UNIVERSITAT POLITÈCNICA DE VALÈNCIA

DEPARTAMENTO DE BIOTECNOLOGÍA

Análisis genético de la percepción del ácido salicílico en *Arabidopsis thaliana*. Caracterización de *NRB4*.

TESIS DOCTORAL

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Valencia, julio de 2012

Dr. D. Pablo Tornero Feliciano, Científico Titular del Consejo Superior de Investigaciones Científicas (CSIC),

CERTIFICA:

Que la presente memoria titulada "Análisis genético de la percepción del ácido salicílico en *Arabidopsis thaliana*. Caracterización de *NRB4*" ha sido realizada por Juan Vicente Canet Pérez bajo mi dirección, en el Instituto de Biología Molecular y Celular de Plantas (U.P.V – C.S.I.C), y constituye su Memoria de Tesis para optar al grado de Doctor por la Universidad Politécnica de Valencia.

Para que así conste a todos los efectos oportunos, firma el presente certificado en Valencia, a catorce de junio de dos mil doce.

Fdo. Pablo Tornero Feliciano

Agradecimientos / Agraïments

En el transcurso de todo un doctorado son muchos los compañeros que, por su colaboración, sus consejos o sus risas (también muy necesarias), deben recibir mi más sincero agradecimiento. La ciencia, al igual que la mayoría de las ocupaciones, requiere de la estrecha colaboración entre colegas, ya sea pasándonos un protocolo que nos ahorre horas de trabajo en "puestas a punto", prestándonos el producto que necesitamos con urgencia (y que aún no le ha llegado a Juni), acompañándonos en nuestras idas y venidas al invernadero, o simplemente tomándonos un café juntos. Por tanto, sois muchos los que merecéis ser nombrados. Aunque, como seguro que de alguno me olvidaré… mis disculpas.

En primer lugar quiero agradecerle a Pablo Tornero la confianza depositada en mí durante estos años. Gracias por haber puesto en mis manos este proyecto que tantos buenos frutos nos ha dado. El BTH será un acrónimo que nunca olvidaré (dicho esto en el mejor de los sentidos). Qué puedo decir de Albor (yo sí que diré cosas, doctor Dobón): has sido muchísimo más que un compañero de laboratorio. Siempre dispuesto a ayudarme y a ejercer de "hermano mayor". Mil gracias. A Brande, le agradezco aquella primera oportunidad de "introducirme" en el mundo científico aunque fuese con un "trabajo aburrido y repetitivo, como un robot" (y a Cristina, por lo del CAP…). I a tu, Lorena, quin *savoir faire*! Gràcies per tants bons moments. Y al resto de mis excompañeros de laboratorio, cuyo trabajo también ha contribuído a que este momento haya llegado (Fede, Álex, Jana, Amparo y Arturo). Y también a Alberto y Vanesa: vuestras estancias fueron muy positivas.

También quiero agradecer a Pablo Vera y a todos los componentes de su laboratorio por todas las críticas constructivas que han hecho a mi trabajo. Por esos seminarios que tanto nos ayudan a enfrentarnos al miedo escénico. Por tantos protocolos, *tupperwares* recalentados, cafés, confidencias y risas. ¡Ay, si la mesa redonda hablara! Jose, gracias por haberme ayudado siempre, por haber respondido siempre con amabilidad a mis dudas. Mariajo, gràcies per haver-me introduït al món del llevat i per tantes altres coses (a dinaaaaar!). Javi, gràcies per la teua ajuda amb el Confocal. Mami, otro hijo tuyo que se hace mayor. A todos los que estáis ahora y a todos los que estuvieron (Vicente, Ana, David, Loli, Begoña, Lourdes, Cristina M, Cristina C, Lorena, Imane, Silvia, Astrid, Bea, Marisa, Sabina…): muchas gracias.

I també les gràcies a tots els meus companys del màster. Quines vesprades tan "entretingudes" que hem passat! Sort que teníem els descansets per injectar-nos cafè a la vena… En especial a Raquel, Regina, Vicent, Zambrano, Miriam i Eva. Vicent, a tu per partida doble, que més d'un favor m'has fet!

A muchos otros compis ibemeceperos, también mi agradecimiento: Leti, Fernando, Ana Cristina, Manu, Sandra, Berta y tantos otros. En general, gracias a todos los que saludáis por los pasillos (algo que debería ser más la norma que la excepción…).

También destacar a los servicios comunes que más he "aprovechado" (esterilizado, secuenciación y microscopía). En especial a Marisol, gracias por tu amabilidad y conocimientos con el Confocal. También quiero reconocer su labor a todos los trabajadores del invernadero, en el que tantas horas he pasado.

Per últim, vull agrair a tota la meua família el recolzament i ànims durant tots aquests anys. En especial, a Mil, culpable principal que aquest dia s'haja fet realitat. Eternament agraït. Als meus pares, per fer possible el que semblava impossible en aquells anys tan durs. El que heu aconseguit amb els vostres quatre fills és un autèntic miracle. Germans i cunyats: moltes gràcies!

Resumen

Las plantas han desarrollado un complejo sistema de defensa frente a sus patógenos. Simplificando enormemente esta complejidad, se distinguen dos rutas de señalización principales: una encaminada a patógenos biotrofos y otra a necrotrofos. La síntesis y percepción del ácido salicílico (SA) es clave para las defensas de la planta, siendo la vía mayoritaria frente a biotrofos. No obstante, aún se desconocen importantes pasos de la ruta del SA, sobre todo los relacionados con su percepción. De hecho, solo se ha descrito un mutante insensible al SA (*npr1*) y no se ha podido demostrar que sea su receptor. La presente Tesis Doctoral parte de la hipótesis de que existen otros componentes genéticos que participan en la percepción de esta hormona aún no descritos.

Se plantea un modelo biológico alternativo a los utilizados previamente en la literatura para estudiar la percepción del SA en *Arabidopsis thaliana*. Dicho modelo se basa en la reducción del peso fresco de la planta provocado por la inducción de resistencia. Debido a la fitotoxicidad del SA cuando se aplica en dosis elevadas, se utiliza el benzotiadiazol (BTH), uno de sus análogos químicos. El BTH incrementa la resistencia de la planta frente al patógeno biotrofo *Pseudomonas syringae*, pero también provoca una considerable pérdida de su biomasa.

Este modelo es aplicado en un rastreo genético a gran escala y el principal grupo de complementación obtenido es *npr1*. Los 43 nuevos alelos encontrados son insensibles al BTH y al SA, hecho que confirma la idoneidad del modelo planteado para detectar mutantes en la percepción del SA. Existe un sesgo en la distribución de las mutaciones presentes en los alelos *npr1*. Este sesgo no corresponde a ninguno de los dominios descritos de la proteína, sino a una zona conservada en los parálogos de *NPR1*. Además, no se han obtenido alelos *npr1* nulos a partir del rastreo debido (como se ha confirmado tras su obtención a partir de bancos de semillas) a su fenotipo intermedio frente al BTH. Este fenotipo se explicaría por la existencia de una redundancia parcial en la percepción del SA, aunque con una fuerte preferencia por *NPR1*. Los cinco parálogos de *NPR1* (*NPR2*, *NPR3*, *NPR4*, *BOP1* y *BOP2*) presentan una función secundaria en la percepción del SA que puede ser detectada en un fondo genético nulo para *NPR1*.

NPR1 también ha sido implicado en la inducción de resistencia tras la aplicación de metil jasmonato (MeJA), pese a que esta hormona controla principalmente la resistencia frente a necrotrofos. Sin embargo, estos estudios se habían basado en los pocos alelos *npr1* descritos hasta la fecha. El análisis de los 43 alelos del rastreo y de los dos alelos nulos permite concluir que *NPR1* no es necesario para la resistencia inducida por MeJA. En cambio, los parálogos *BOP1* y *BOP2* son relevantes en este proceso defensivo. Los *TGAs*, una familia multigénica de factores de transcripción cuya intervención en la ruta de señalización del SA ha sido demostrada, también participan en la resistencia inducida por MeJA.

El otro grupo de complementación caracterizado es *NRB4* (*NON-RECOGNITION-OF-BTH-4*), con tres alelos que presentan mutaciones puntuales. *NRB4* es el segundo gen necesario para la percepción del SA. Los tres alelos *nrb4* del rastreo comparten con *npr1* todos los fenotipos estudiados relacionados con defensa. En cambio, los alelos *nrb4* nulos presentan una pérdida total de la respuesta defensiva mediada por el SA, siendo incluso más extremos que *npr1* en algunos fenotipos. Además, presentan un patrón de crecimiento alterado y son estériles. No se ha detectado interacción alguna entre ambas proteínas y se ha determinado que NRB4 actúa en la ruta después de NPR1. De hecho, *NRB4* forma parte del complejo *Mediator* y es el ortólogo de *MED15* en Arabidopsis. Este complejo es necesario para la regulación general de la transcripción. Pese a su función como conjunto, para muchas de las subunidades se han descrito funciones específicas en procesos fisiológicos concretos, como el relacionado con el SA en *NRB4/MED15*.

Los resultados obtenidos indican que *NRB4* es esencial para la planta. La explicación más sencilla es que dicha subunidad presente otras funciones, además de las relacionadas con el SA, que al ser suprimidas dan lugar a una planta inviable. No obstante, el análisis de los transcriptomas no apunta a la supresión de ninguna otra señalización o función. Además, los alelos nulos presentan fenotipos más severos en defensa, pero ningún otro fenotipo destacable salvo el evidente en desarrollo. Estos resultados y el hecho de que el SA también tenga un importante papel en el crecimiento y desarrollo de la planta sugieren que la relevancia de *NRB4* se debe exclusivamente a su función en la percepción del SA, lo que implicaría que el SA es esencial en el correcto desarrollo de la planta.

Resum

Las plantes han desenvolupat un complex sistema de defensa front a patògens. Simplificant enormement aquesta complexitat, poden distingir-se dues rutes de senyalització principals: una encaminada als patògens biòtrofs i l'altra, als necròtrofs. La síntesi i percepció de l'àcid salicílic (SA) és clau per les defenses de la planta, éssent la via majoritària front a biòtrofs. Tanmateix, encara són desconeguts pasos importants de la ruta del SA, sobretot els que estan relacionats amb la seua percepció. De fet, només s'ha descrit un mutant insensible al SA (*npr1*) i no s'ha pogut demostrar que en siga el receptor. Aquesta Tesi Doctoral arranca de la hipòtesi que existeixen altres components genètics que participen a la percepció d'aquesta hormona, encara no descrits.

Es planteja un model biològic alternatiu als que s'han utilitzat prèviament a la literatura per l'estudi de la percepció del SA en *Arabidopsis thaliana*. L'esmentat model és basat en la reducció del pes fresc de la planta provocada per la inducció de resistència. Degut a la fitotoxicitat del SA quan és aplicat a dosis elevades, s'utilitza el benzotiadiazol (BTH), un dels seus anàlegs químics. El BTH incrementa la resistència de la planta front al patògen biòtrof *Pseudomonas syringae*, però també provoca una pèrdua considerable de la seua biomassa.

Aquest model és aplicat a un rastreig genètic a gran escala i el principal grup de complementació obtingut és *npr1*. Els 43 nous al·lels trobats són insensibles al BTH i al SA, fet que confirma la idoneïtat del model plantejat per detectar mutants en la percepció del SA. Existeix un esbiaixament en la distribució de les mutacions presents als al·lels *npr1*. Aquest esbiaixament no correspon a cap dels dominis descrits de la proteïna, sinó a una zona conservada als paràlegs de *NPR1*. A més a més, no s'han obtingut al·lels *npr1* nuls a partir del rastreig degut (tal i com s'ha confirmat després de la seua obtenció a partir de bancs de llavors) al seu fenotip intermedi front al BTH. Aquest fenotip s'explicaria per l'existència d'una redundància parcial a la percepció del SA, tot i que amb una forta preferència per *NPR1*. Els cinc paràlegs de *NPR1* (*NPR2*, *NPR3*, *NPR4*, *BOP1* y *BOP2*) presenten una funció secundària en la percepció del SA que pot ser detectada en un fons genètic nul per a *NPR1*.

NPR1 també ha estat implicat en la inducció de resistència després de l'aplicació de metil jasmonat (MeJA), malgrat que aquest hormona controla principalment la resistencia front a necròtrofs. Tanmateix, aquests estudis havien estat basats en els pocs al·lels *npr1* descrits fins el moment. L'anàlisi dels 43 al·lels del rastreig i dels dos al·lels nuls permet concloure que *NPR1* no és necessari per la resistència induïda per MeJA. En canvi, els paràlegs *BOP1* i *BOP2* són relevants en aquest procés defensiu. Els *TGAs*, una família multigènica de factors de transcripció la intervenció dels quals en la ruta de senyalització del SA ha estat demostrada, també participen en la resistència induïda per MeJA.

L'altre grup de complementació caracteritzat és *NRB4* (*NON-RECOGNITION-OF-BTH-4*), amb tres al·lels que presenten mutacions puntuals. *NRB4* és el segon gen necessari per la percepció del SA. Els tres al·lels *nrb4* del rastreig comparteixen amb *npr1* tots els fenotips estudiats relacionats amb defensa. Per contra, els al·lels *nrb4* nuls presenten una pèrdua total de la resposta defensiva mediada pel SA, éssent fins i tot més extrems que *npr1* en alguns fenotips. A més a més, presenten un patró de creixement alterat i són estèrils. No s'ha detectat cap interacció entre ambdues proteïnes i s'ha determinat que NRB4 actua en la ruta després de NPR1. De fet, *NRB4* forma part del complex *Mediator* i és l'ortòleg de *MED15* en Arabidopsis. Aquest complex és necessari per la regulació general de la transcripció. Malgrat la seua funció com a conjunt, per moltes de les subunitats han estat descrites funcions específiques de processos fisiològics concrets, com el relacionat amb el SA en *NRB4/MED15*.

Els resultats obtinguts indiquen que *NRB4* és essencial per la planta. L'explicació més senzilla és que l'esmentada subunitat presente altres funcions a més de les relacionades amb SA que, en ser suprimides, donen lloc a una planta inviable. Tanmateix, l'anàlisi dels transcriptomes no apunta la supressió de cap altra senyalització o funció. A més a més, els al·lels nuls presenten fenotips més severs en defensa però cap altre fenotip destacable tret d'aquell evident en desenvolupament. Aquests resultats i el fet que el SA també tinga un paper important en el creixement i desenvolupament de la planta suggereixen que la rellevància de *NRB4* és deguda exclusivament a la seua funció en la percepció del SA, cosa que implicaria que el SA és essencial en el correcte desenvolupament de la planta.

Abstract

Plants have developed a complex defense system against pathogens. Generally speaking, two major signaling pathways can be distinguished: the one related to biotrophs, and another one related to necrotrophs. The synthesis and perception of salicylic acid (SA) are of capital importance to plant defense, since it constitutes the major response against biotrophs. However, several important steps in the SA pathway are still unknown, mostly in relation to its perception. As a matter of fact, only one mutant has been described to be insensitive to SA (*npr1*) and it has not proved to be its receptor. This Dissertation takes as point of departure the hypothesis that other genetic components exist and play some role in the perception of this hormone.

A biological model is proposed as an alternative to the ones previously used in the literature to study SA perception in *Arabidopsis thaliana*. This model is based on the decrease that the plant's fresh weight experiences as a result of the resistance being induced. Due to the phytotoxicity of SA when it is applied at high doses, benzotiadiazole (BTH) is used instead, which is one of its chemical analogues. BTH increases the plant's resistance to the biotrophic pathogen *Pseudomonas syringae* and produces a considerable loss of biomass.

This model is applied in a high-throughput screening. The major complementation group obtained is *npr1*. The 43 new alleles found are insensitive to both BTH and SA, thus confirming the suitability of this model to detect mutants in SA perception. There is a bias in the distribution of the mutants present in *npr1* alleles. Such bias does not correspond to any of the dominions of the protein already described, but to a zone conserved in *NPR1* paralogs. Besides, no nul *npr1* allele has been obtained from the screening, due to its intermediate phenotype (confirmed by getting the lines from seed banks). Such phenotype would be explained by the existence of a partial redundancy in SA perception, although with a marked preference for *NPR1*. The five *NPR1* paralogs (*NPR2*, *NPR3*, *NPR4*, *BOP1* and *BOP2*) present a secondary function in SA perception that can be detected in a null genetic background for *NPR1*.

NPR1 has also been identified to be involved in resistance induction after applying methyl jasmonate (MeJA), even though this hormone primarily controls resistance to necrotrophs. However, such studies had been based on

the few *npr1* alleles described until then. The analysis of the 43 alleles from the screening and the two null alleles allows us to conclude that *NPR1* is not required for resistance induced by MeJA. Furthermore, the paralogs *BOP1* and *BOP2* are relevant in this defensive process. *TGAs*, a multigenic family of transcription factors whose role in the SA signaling pathway has been reported, also participate in resistance induced by MeJA.

The other complementation group characterized is *NRB4* (*NON-RECOGNITION-OF-BTH-4*), with three alleles which present point mutations. *NRB4* is the second gene required for SA perception. The three *nrb4* alleles of the screening share with *npr1* all the phenotypes related to defense that have been studied. On the other hand, *nrb4* null alleles show a total loss of their defensive SA-mediated response, these being even more extreme than *npr1* in some phenotypes. Besides, their growing habit is altered and they are sterile. No interaction between both proteins has been detected, and it has been determined that NRB4 acts in the pathway after NPR1. As a matter of fact, *NRB4* is part of the *Mediator* complex and it is the *MED15* ortholog in Arabidopsis. This complex is required for the general regulation of transcription. In spite of its function as a whole, specific functions have been described for several of its subunits in some concrete physiological processes, like the one related to SA in *NRB4/MED15*.

The results obtained show that *NRB4* is essential for the plant. The simplest explanation is that this subunit may have other functions in addition to the ones related to SA, which when suppressed make the plan unviable. However, the analysis of the transcriptomes does not point out to the suppression of any other signaling or function. Furthermore, the null alleles show phenotypes which are more severe in defense but no other relevant phenotype unless the one which is obvious in development. These results together with the fact that SA has also been described as relevant in plant growth and development suggest that *NRB4* being relevant is exclusively due to its role in SA perception, and this would imply that SA is essential for a correct plant development.

Lista de abreviaturas

4-HBA	*4-Hydroxibenzoate*
AA	Aminoácidos
ABA	Ácido abscísico
ACC	Ácido 1-amonociclopropano-1-carboxílico
amiRNA	*Artificial microRNA*
ARR2	Proteína RESPONSE REGULATOR 2
AtMES	Proteína METHYL ESTERASE
AtMIN7	Proteína HOPM INTERACTOR 7
Avr	Factores de avirulencia
AXR3	Gen *AUXIN RESISTANT 3*
AZI1	Gen *AZELAIC ACID INDUCED 1*
BAK1	Proteína BRI1 ASSOCIATED RECEPTOR KINASE
BOP1	Gen *BLADE ON PETIOLE 1*
BOP2	Gen *BLADE ON PETIOLE 2*
BR	Brasinosteroides
BRI1	Proteína BRASSINOSTEROID INSENSITIVE 1
BSMT1	Proteína BENZOIC ACID/SALICYLIC ACID CARBOXYL METHYLTRANSFERASE 1
BTB/POZ	Dominio *Broad-Complex, Tramtrack, and Bric-à-brac/Pox virus, Zinc finger*
BTH	Benzotiadiazol
CBP60g	Proteína CALMODULIN-BINDING PROTEIN 60-LIKEg
CC-NB-LRR	Proteínas NB-LRR con el dominio *Coiled Coil*
CDPKs	Familia de proteínas *Calcium Dependent Protein Kinase*
CK	Citoquininas
coi1	Mutante *coronatine insensitive 1*
COR	Coronatina
CSP	Familia de proteínas *Cold Shock Proteins*
CUL3	Proteína CULLIN 3
DAMPs	Patrones moleculares asociados a herida
DEX	Dexametasona
DMSO	Dimetilsulfóxido
DNA	Ácido desoxirribonucleico
EDS5	Gen *ENHANCED DISEASE SUSCEPTIBILITY 5*
EFR	Proteína EF-TU RECEPTOR
EF-Tu	Proteína ELONGATION FACTOR Tu
EIL1	Proteína EIN3-LIKE 1
EIN3	Proteína ETHYLENE INSENSITIVE 3
EMS	Etil metano sulfonato
eps1	Mutante *enhanced Pseudomonas susceptibility 1*

ET	Etileno
ETI	Inmunidad activada por efectores
ETI	Inmunidad activada por efectores
etr1	Mutante *ethylene response 1*
ETS	Susceptibilidad activada por efectores
flg22	Péptido de 22 aminoácidos de FLS2
FLS2	Proteína FLAGELLIN SENSITIVE 2
GAs	Giberelinas
GAL4 AD	Dominio de activación de GAL4
GAL4 BD	Dominio de unión a DNA de GAL4
GFP	*Green fluorescent protein*
GH3	Familia de proteínas *Gretchen Hagen*
GTFs	Familia de proteínas *General Transcription Factors*
HBD	Dominio de unión a hormonas esteroides procedente del receptor de glucocorticoides de ratas
HR	Respuesta hipersensible
hrp	Mutante *hypersensitive response and pathogenicity*
IAA	Ácido indolacético
IC	Isocorismato
ICS	Proteína ISOCHORISMATE SYNTHASE
IgG	Inmunoglobulina G
INA	*2,6-dichloroisonicotinic acid*
IPL	Proteína ISOCHORISMATE PYRUVATE LYASE
ISR	Resistencia sistémica inducida
JA	Ácido jasmónico
jar1	Mutante *jasmonate resistant 1*
jin1	Mutante *jasmonate insensitive 1*
KNAT6	Proteína KNOTTED1-LIKE HOMEOBOX GENE 6
LRR	Región rica en leucina
LysM	Motivos de lisina
MAMPs	Patrones moleculares asociados a microorganismos
MAPKs	Familia de proteínas *Mitogen Activated Protein Kinases*
MATE	Familia de proteínas *Multidrug And Toxic compound Extrusion*
MeJA	Metil jasmonato
MeSA	Metil salicilato
MeSAG	Derivados glucosilados del MeSA
MIR	Resistencia inducida por MeJA
MS	Medio de cultivo Murashige y Skoog
NahG	Gen bacteriano *Salicylate hydroxylase*
NB-LRR	Familia de proteínas *Nucleotide Binding-Leucine Rich Repeat*
NDR1	Proteína NON RACE-SPECIFIC DISEASE RESISTANCE 1
NIMIN1	Proteína NIM1-INTERACTING 1

NLS	Dominio de localización nuclear
NPR1	Gen *NON-EXPRESSER OF PATHOGENESIS-RELATED GENES 1*
NPR2	Gen *NPR1-LIKE PROTEIN 2*
NPR3	Gen *NPR1-LIKE PROTEIN 3*
NPR4	Gen *NPR1-LIKE PROTEIN 4*
NRB4/MED15	*NON-RECOGNITION OF BTH 4/MEDIATOR 15*
NtSABP2	Proteína SALICYLIC ACID BINDING PROTEIN 2 de tabaco
NtSAMT1	Proteína SAM TRANSPORTER 1 de tabaco
opr3	Mutante *oxophytodienoate reductase 3*
PAD4	Proteína PHYTOALEXIN DEFICIENT 4
PAL	Proteína PHENYLALANINE AMMONIA LYASE
PAMPs	Patrones moleculares asociados a patógenos
PAN	Proteína PERIANTHIA
PBS1	Proteína AVRPPHB SUSCEPTIBLE 1
PBS3	Gen *AVRPPHB SUSCEPTIBLE 3*
PCR	Reacción en cadena de la polimerasa
PFT1/MED25	Gen *PHYTOCHROME AND FLOWERING TIME 1/MEDIATOR 25*
PFW	*Percentage of Plant Fresh Weight*
PR	Genes *PATHOGENESIS RELATED*
PRRs	Receptores de reconocimiento de patrones
***Psm* CR299**	*Pseudomonas syringae patovar maculicola* CR299
PTI	Inmunidad activada por PAMPs
Pto	*Pseudomonas syringae patovar tomato* DC3000
QTL	*Quantitative Trait Locus*
R	Genes *RESISTANCE*
RbohD	Proteína RESPIRATORY BURST OXIDASE HOMOLOGUE D
RILs	*Recombinant Inbreed Lines*
RLK	Familia de proteínas *Receptor Like Kinase*
RLP	Familia de proteínas *Receptor Like Protein*
RNA	Ácido ribonucleico
ROS	Especies reactivas de oxígeno
RPM1	Proteína RESISTANCE TO P. SYRINGAE PV. MACULICOLA 1
RPS2	Proteína RESISTANT TO P. SYRINGAE 2
RPS5	Proteína RESISTANT TO P. SYRINGAE 5
RT-PCR	Transcripción reversa seguida de PCR
RT-qPCR	Transcripción reversa seguida de PCR cuantitativa
SA	Ácido salicílico
SA-Asp	Saliciloil-L-aspartato

SAG	SA 2-*O*-β-D-glucósido
SAG101	Proteína SENESCENCE ASSOCIATED GENE 101
SAR	Resistencia sistémica adquirida
SARD1	Proteína SAR-DEFICIENT 1
SEM	Microscopio electrónico de barrido
SFR6/MED16	Gen *STRUBBELIG-RECEPTOR FAMILY 6/MEDIATOR 16*
SGE	Éster de la saliciloil glucosa
SGT1	Proteína SALICYLIC ACID GLUCOSYLTRANSFERASE 1
SNI1	Gen *SUPPRESSOR OF npr1-1 INDUCIBLE 1*
ssi2	Mutante *suppressor of SA insensitivity 2*
SWP/MED14	*STRUWWELPETER/MEDIATOR 14*
TAL	Familia de efectores *Transcription Activator Like*
t-CA	Trans-ácido cinámico
TetraFA	2,2,2,2'-tetrafluoroacetophenone
TF	Factor de transcripción
TGAs	Familia de factores de transcripción con el motivo TGACG
TIR-NB-LRR	Proteínas NB-LRR con el dominio *Toll Interleukin 1 Receptor*
TMV	Virus del mosaico del tabaco
TRX-H5	Proteína THIOREDOXIN-H5
TTSS	Sistema de secreción tipo III
t-zea	Trans-zeatina
WRKYs	Familia de factores de transcripción con el motivo Trp-Arg-Lys-Tyr
NILs	*Near isogenic lines*
STAIRs	*Stepped Aligned Inbred Recombinant Strains*
LOD	*Logarithm of the odds*
SAQ	QTL identificado tras los tratamientos con SA
FW	*Fresh weight*
35S	Promotor constitutivo derivado del virus del mosaico de la coliflor
UGT74F1	Proteína UDP-GLYCOSYLTRASFERASE 74 F1
***sid2*-1**	Mutante *SA induction-deficient2*
***eds16*-1**	Mutante *enhanced disease susceptibility 16*
2,4-D	Ácido 2,4-diclorofenoxiacético

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"*Ramón… ¿por qué morir?*"

Alejandro Amenabar. *Mar adentro.*

"*We're off to see the Wizard, The Wonderful Wizard of Oz.*"

Victor Fleming. *The Wizard of Oz.*

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1. Introducción general

Los seres vivos han de ser capaces de adaptarse a los cambios continuos que tienen lugar en el ambiente que los rodea con el fin de asegurar su supervivencia. Estos cambios ambientales a los que han de enfrentarse pueden clasificarse en función de su origen en dos grandes grupos: bióticos (derivados de las interacciones con individuos de la misma o de diferentes especies) y abióticos (aquellos que constituyen las características fisico-químicas del ecosistema). El éxito evolutivo de una especie dependerá del resultado favorable de estos enfrentamientos.

Las plantas también han tenido que adaptarse a estos cambios ambientales, siendo la relación con los patógenos que las rodean un tipo de estrés biótico clave al que deben hacer frente. Dado que son organismos sésiles, no pueden depender de la huída y, además, tampoco pueden generar anticuerpos. Sin embargo, el reino vegetal ha desarrollado diversos mecanismos de defensa, con procesos enormemente eficaces y complejos, que permiten a la planta responder en cada situación de la forma más adecuada. Por ello, la resistencia frente a un patógeno es más la norma que la excepción (Agrios 2005).

En las últimas tres décadas, gran parte de la investigación realizada con plantas se ha centrado en una pequeña planta herbácea de la familia *Brassicaceae*, carente de interés comercial, denominada *Arabidopsis thaliana* (L.) *Heynh* (Arabidopsis) (Somerville y Koornneef 2002). Se trata de una especie utilizada como sistema modelo en estudios genéticos, bioquímicos y fisiológicos. Esto es debido a una serie de características que la hacen idónea para su investigación en laboratorio. Entre estas características destacan su ciclo de vida corto (6-8 semanas), su elevada producción de semillas, que permanecen viables durante muchos años, su facilidad de cultivo en invernaderos o en cámaras de cultivo, (ya que requieren poco espacio para su cultivo) y el relativamente reducido tamaño de su genoma. Además, éste puede ser modificado por transgénesis de forma rápida y sencilla. Arabidopsis también presenta los tipos de respuesta defensiva más importantes descritos en otras plantas (Glazebrook et al. 1997), por lo que es una buena herramienta para el estudio de la interacción planta-patógeno.

2. El reconocimiento planta-patógeno

Las plantas pueden ser atacadas por patógenos con diferentes estilos de vida. Estos han sido agrupados en dos categorías principales, aunque también existen otras intermedias. Los patógenos biotrofos son aquellos especializados en alimentarse y crecer en el interior de tejidos vivos. El ácido salicílico (SA, de *Salicylic Acid*) es la principal hormona implicada en las respuestas defensivas frente a patógenos biotrofos (por su importancia en esta Tesis, se dedicará un apartado posterior a dicha hormona). Los patógenos necrotrofos actúan provocando la muerte de la célula hospedadora, normalmente mediante la producción de toxinas, para poder nutrirse a partir del tejido muerto. Estas diferencias provocan que los biotrofos establezcan una relación mucho más íntima con sus hospedadores. De hecho, muchos de ellos han desarrollado la capacidad de vivir en el espacio intercelular del mesófilo, un ambiente muy rico en nutrientes. En el caso de aquellos que son patógenos obligados, esta relación es tan extrema que no pueden ser cultivados en un medio de cultivo artificial. Los necrotrofos, al contrario que los biotrofos, si pueden vivir como saprófitos fuera de sus hospedadores, alimentándose de materia orgánica muerta (Agrios 2005; de Wit 2007).

Para defenderse de sus patógenos, las plantas han desarrollado un sofisticado sistema inmunitario (utilizándose este concepto en un sentido amplio) para defenderse de una gran variedad de patógenos. A diferencia de los animales, no presentan unas células inmunológicas especializadas. En contraposición, todas las células vegetales parecen tener una habilidad innata para reconocer a los patógenos y activar una respuesta defensiva adecuada (Kim et al. 2008). Sin embargo, para que dicha respuesta activa sea requerida, los patógenos primero han de traspasar las defensas constitutivas de la planta. Se trata de un conjunto de barreras físicas y químicas que dificultan la entrada del patógeno. Representan una resistencia inespecífica, puesto que es efectiva frente a un gran número de patógenos (Heath 2000a). Un claro ejemplo es la cutícula que, sin embargo, los patógenos pueden penetrar a través de heridas (previas o generadas por el propio patógeno) o de aberturas naturales tales como los estomas. Entonces, el siguiente obstáculo a superar será la compleja pared celular vegetal (Hückelhoven 2007). Si los patógenos son capaces de superar estas barreras constitutivas, la planta activará una serie de mecanismos

inducibles que actúan tanto a nivel local como sistémico (Shah et al. 1999). Dentro de esta respuesta inmune inducida, es posible diferenciar dos niveles.

La primera respuesta activa de la planta se produce gracias a un conjunto de receptores de reconocimiento de patrones (PRRs, de *Pattern Recognition Receptors*) situados en la superficie de la célula vegetal. Estos receptores pueden detectar la presencia de unos patrones moleculares asociados a patógenos (PAMPs, de *Pathogen Associated Molecular Pattern*). Como consecuencia de esto se induce la denominada inmunidad activada por PAMPs (PTI, de *PAMP Triggered Immunity*) (Jones y Dangl 2006). Sin embargo, los microorganismos han desarrollado estrategias para superar esta respuesta defensiva, tanto mediante interferencias en el reconocimiento de los PAMPs como, sobre todo, a través de la secreción de proteínas efectoras en el interior del citoplasma de la célula vegetal. Esta respuesta de los patógenos recibe el nombre de susceptibilidad activada por efectores (ETS, de *Effector Triggered Susceptibility*) (Jones y Dangl 2006).

Como segunda respuesta activa, las plantas han desarrollado mecanismos más especializados para detectar a los microorganismos invasores que se engloban en la denominada inmunidad activada por efectores (ETI, de *Effector Triggered Immunity*). Por este mecanismo, la planta es capaz de reconocer de forma directa o indirecta muchos de estos efectores gracias a las proteínas codificadas por genes *R* (de *Resistance*), principalmente a las tipo NB-LRR (de *Nucleotide Binding–Leucine Rich Repeat*), (Jones y Dangl 2006; Boller y He 2009). Cabe destacar que esta respuesta mediada por las NB-LRR es efectiva frente a patógenos biotrofos o hemibiotrofos, pero no frente a necrotrofos (Glazebrook 2005).

Evidentemente, con la ETI las plantas no han conseguido una resistencia plena. La selección natural dirige a los patógenos hacia nuevas vías que les permitan suprimir la ETI, bien modificando o eliminando los efectores desencadenantes o bien generando otros nuevos (de Wit 2007). Al mismo tiempo, esto dirigirá a las plantas a reconocer estos nuevos efectores. De esta forma, el "tira y afloja" entre patógeno y hospedador se mantendrá mientras ambas especies coevolucionen.

El modelo de zig-zag propuesto por Jones y Dangl en 2006 (Figura 1) es un buen resumen de las diferentes etapas existentes en la interacción planta-patógeno. Al mismo tiempo, puede ser visto como una aproximación válida al

proceso coevolutivo existente entre una planta y un patógeno dados. A continuación, se abordarán con mayor detalle las tres primeras etapas descritas en dicho modelo.

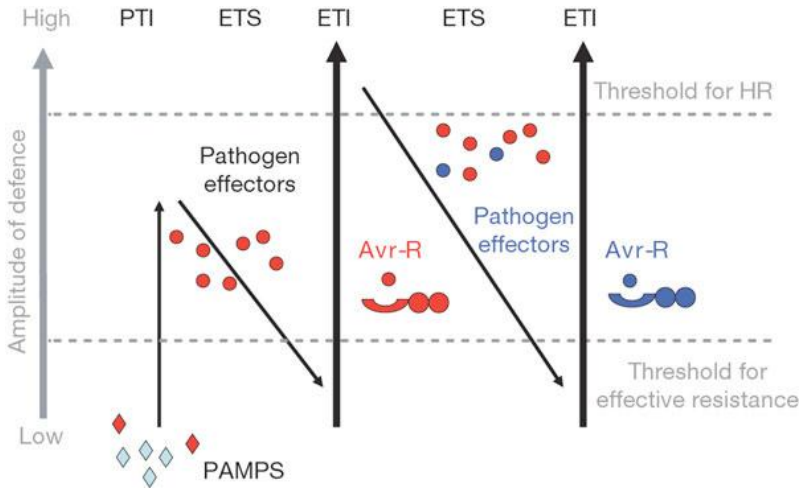

Figura 1. Modelo de zig-zag (tomado de Jones y Dangl 2006). Esquema que representa las diferentes etapas que tienen lugar en la interacción planta-patógeno. Etapa 1: Las plantas detectan el ataque de los patógenos a través de los receptores PRRs que reconocen a un conjunto de patrones moleculares asociados a patógenos (PAMPs) y se desencadena la inmunidad activada por PAMPs (PTI). Etapa 2: Los patógenos evaden la PTI mediante la secreción de efectores, generando la susceptibilidad activada por efectores (ETS). Etapa 3: Las proteínas R de las plantas son capaces de reconocer determinados efectores de los patógenos (en rojo) y activar la inmunidad activada por efectores (ETI). La ETI es más rápida y contundente que la PTI, llegando a superar el umbral de inducción de la muerte celular programada (HR). Etapa 4: Los patógenos pierden o modifican algunos de los efectores previamente reconocidos (en rojo) o bien generan otros nuevos (en azul). Como consecuencia, logran suprimir la ETI y provocar enfermedad. En etapas sucesivas, la selección favorecerá la aparición de nuevas proteínas R que permitan a la planta reconocer estos nuevos efectores.

2.1 Inmunidad activada por PAMPs (PTI)

La mayoría de los patógenos necesitan acceder al interior de la planta, bien por heridas o bien por aberturas naturales, para que tenga lugar el proceso de infección. El siguiente paso consiste en penetrar la pared celular vegetal. Es en este punto cuando el patógeno queda expuesto al reconocimiento por parte de los receptores PRRs (Chisholm et al. 2006). Estos receptores están presentes tanto en plantas como en animales y tienen la capacidad de reconocer una serie de huellas o patrones moleculares que identifican a todo tipo de patógenos y que no están presentes en el propio hospedador. Esto les permite identificar la presencia de un agente externo (Boller y Felix 2009).

Estos patrones moleculares reciben el nombre genérico de MAMPs (*Microbe Associated Molecular Pattern*). Los MAMPs incluyen a los ya mencionados PAMPs y a otros no necesariamente asociados a microorganismos patogénicos. Por tanto, si bien ambos acrónimos se utilizan prácticamente como sinónimos, el término MAMPs es más acertado por ser menos restrictivo, aunque el de PAMPs es más comúnmente utilizado. Son moléculas muy conservadas, propias de un conjunto amplio de microorganismos y esenciales para su ciclo vital (Ausubel 2005; Segonzac y Zipfel 2011). Los MAMPs pueden tener naturalezas muy distintas. Los más comunes en los hongos son la quitina y el ergosterol, componentes principales de su pared celular. En las bacterias, destacan los lipopolisacáridos, en las Gram negativas; o los peptidoglucanos, en las Gram-positivas. Pero no todos los MAMPs forman parte de estructuras extracelulares. Las proteínas bacterianas CSP (de *Cold Shock Proteins*) o el factor de elongación Tu (EF-Tu, ELONGATION FACTOR Tu) son dos ejemplos de elementos intracelulares que actúan como MAMPs (He et al. 2007; Schwessinger y Zipfel 2008).

Existe otro grupo de moléculas capaces de ser reconocidas por los PRRs y disparar la PTI. Durante el proceso infectivo, el patógeno genera una serie de heridas en los tejidos de la planta que liberan al apoplasto un conjunto de moléculas (p. ej. fragmentos de pared celular). Estas moléculas reciben el nombre de patrones moleculares asociados a herida (DAMPs, de *Damage Associated Molecular Pattern*) y pueden ser percibidas como señales de peligro por los PRRs. En el pasado, estos compuestos han sido denominados "elicitores" endógenos (Lotze et al. 2007).

Los pocos receptores PRR identificados en plantas son proteínas transmembrana que pueden clasificarse en dos familias según presenten o no un dominio serín/treonín (Ser-Thr) quinasa en su región citoplasmática. Así, en las proteínas de la familia RLK (de *Receptor Like Kinase*) este dominio sí está presente y en las de la familia RLP (de *Receptor Like Protein*) no. Estas proteínas también presentan un dominio extracelular que es el responsable del reconocimiento y unión del MAMP o DAMP. Este dominio puede contener una región rica en leucina (LRR, de *Leucine Rich Region*), hecho que sucede en la mayoría de los PRRs descritos, o bien motivos de lisina (LysM, de *Lysine Motifs*). Los PRRs presentan una gran afinidad y especificidad por un MAMP concreto pudiendo llegar a detectarlo a concentraciones por debajo de nanomolar (Chisholm et al. 2006; Boller y He 2009; Segonzac y Zipfel 2011).

El ejemplo de PTI mejor estudiado en plantas es el que tiene lugar tras la percepción de la flagelina bacteriana por parte del receptor FLS2 (FLAGELLIN SENSITIVE 2) de Arabidopsis. Esta proteína es uno de los componentes principales del flagelo bacteriano y es la responsable de su motilidad, característica fundamental para su patogenicidad en plantas. Se ha comprobado que el péptido flg22, correspondiente a una región de 22 aminoácidos (AA) muy conservada de su dominio N-terminal, es el causante del reconocimiento por FLS2 y de la consiguiente activación de defensas. Esto se ha demostrado al poder reproducir esta activación con la mera aplicación de dicho péptido generado artificialmente. Sin embargo, el sitio exacto de unión a flg22 en FLS2 es aún desconocido (Felix et al. 1999; Zipfel 2008).

El receptor FLS2 pertenece a la familia LRR-RLK, es decir, presenta un dominio extracelular rico en repeticiones de leucina, un dominio transmembrana y un dominio citoplásmico capaz de unir a proteín-quinasas mediante una unión Ser-Thr. Por tanto, presenta todos los dominios necesarios para traducir este reconocimiento extracelular en una cascada de señalización intracelular. Sin embargo, se ha descrito que tanto FLS2 como otras LRR-RLKs descritas (p. ej. EFR, EF-TU RECEPTOR, que reconoce el péptido conservado elf18 del EF-Tu bacteriano), necesitan de la acción de otras proteínas para poder generar una función completa (Zipfel 2009). Es el caso de la LRR-RLK BAK1 (BRI1 ASSOCIATED RECEPTOR KINASE). BAK1 fue identificado como el principal regulador positivo del receptor BRI1 (BRASSINOSTEROID INSENSITIVE 1) (Kim y Wang 2010). BAK1

interacciona, entre otras, con FLS2 y EFR, de manera dependiente de ligando. Tanto la heteromerización como la fosforilación del complejo FLS2-BAK1 tienen lugar de forma casi instantánea (menos de 15 segundos) tras la detección de flg22. Se puede concluir que BAK1 actúa como un adaptador de diferentes señales defensivas mediadas por LRR-RLKs (Segonzac y Zipfel 2011). Se han descrito homólogos funcionales a FLS2 en *Solanum lycopersicum* (tomate), *Oryza sativa* (arroz) y *Nicotiana benthamiana*. Esto indica que el reconocimiento de esta proteína bacteriana por FLS2 se ha conservado a lo largo del proceso evolutivo (Zipfel 2009).

Como resultado del reconocimiento de flg22, o de otros MAMPs, se inician rápidamente múltiples eventos de fosforilación y se activan varias cascadas de señalización mediadas por MAPKs (de *Mitogen Activated Protein Kinases*). Como consecuencia, se limita el crecimiento del patógeno mediante la inducción de la expresión de genes de defensa, en muchos casos a través de la activación previa de los factores de transcripción (TFs, de *Transcription Factors*) tipo WRKY (Asai et al. 2002; Segonzac y Zipfel 2011).

Se ha podido validar la importancia de la PTI. Por una parte, las plantas que son defectivas en alguno de los PRR identificados presentan una detectable susceptibilidad a bacterias. Es el caso del mutante *fls2* de Arabidopsis, que presenta una mayor susceptibilidad a la bacteria biotrofa *Pseudomonas syringae patovar tomato* DC3000 (*Pto*, una cepa virulenta que ha sido ampliamente utilizada en los experimentos presentados en capítulos posteriores). Por otra parte, para los patógenos es totalmente imprescindible suprimir o evitar la PTI para poder continuar con la infección (Zipfel 2004; Segonzac y Zipfel 2011). Otra prueba en este sentido es la identificación de *FLS2* como carácter genético cuantitativo (QTL, de *Quantitative Trait Locus*) principal contribuyente de la resistencia basal presente en Arabidopsis en su interacción con *Pseudomonas syringae pv. phaseolicola* 1448A. Se trata en este caso de un patógeno que no es capaz de provocar infección en la planta silvestre (Forsyth et al. 2010).

2.2 La supresión de la PTI: los efectores patogénicos

Los verdaderos patógenos de plantas son aquellos capaces de suprimir o evadir su resistencia basal o PTI. Para ello han desarrollado un elevado número de factores de virulencia que les permiten manipular las funciones celulares del

hospedador en su propio beneficio. Dentro de este conjunto de mecanismos de virulencia destaca la secreción de unas moléculas denominadas efectores. Estos efectores no están presentes en los microorganismos en general, sino que son característicos de un grupo de patógenos concreto. En muchos casos poseen actividades enzimáticas que les permiten modificar determinadas proteínas del hospedador con el objetivo de aumentar la virulencia del patógeno y evadir su detección, promoviendo así la ETS (Jones y Dangl 2006; Speth et al. 2007).

Las bacterias patógenas de plantas y de animales presentan al menos cuatro sistemas diferentes de secreción de efectores, siendo el denominado sistema de secreción tipo III (TTSS, de *Type III Secretion System*) el más relevante para la virulencia. El TTSS permite a las bacterias inyectar un número considerable de efectores en el interior de la célula hospedadora, con valores de entre 15 y 30 efectores distintos. Estos efectores contribuyen a la virulencia del patógeno bien mimetizando o bien inhibiendo funciones celulares concretas. La importancia del TTSS puede observarse en cepas patogénicas de *P. syringae* mutadas en dicho sistema de secreción para que no sea funcional. Estas bacterias no pueden suprimir la PTI y pasan a ser no patogénicas (Jones y Dangl 2006; de Wit 2007).

Existen varios ejemplos documentados de efectores bacterianos que actúan directamente sobre los complejos PRRs inhibiendo la PTI. Es el caso de los efectores AvrPto y AvrPtoB de *Pto*, que son capaces de interaccionar físicamente con el dominio quinasa de FLS2, EFR y BAK1. Respecto a AvrPto, aún existe controversia sobre cual es su verdadera diana. Esto se debe a que, si bien puede interaccionar *in vivo* con las tres LRR-RLKs mencionadas, solamente es capaz de inhibir *in vitro* las actividades de autofosforilación de FLS2 y EFR. Sin embargo, interacciona preferentemente con BAK1, interfiriendo así en la formación del complejo FLS2-BAK1 (Shan et al. 2008; Xiang et al. 2008). AvrPtoB, al igual que otros efectores tipo III, es una proteína con dos dominios. Su extremo N-terminal es el responsable de la interacción con FLS2, mientras que en el extremo C-terminal presenta un dominio E3-ubiquitín ligasa que es responsable de inducir la degradación de FLS2. Sin embargo, mutaciones que eliminan esta actividad ligasa no son capaces de alterar la supresión de las respuestas defensivas inducidas tras la detección de flg22. Esto sugiere que el extremo N-terminal es suficiente para interaccionar con FLS2 y suprimir dichas respuestas. No obstante, dicha actividad sí es necesaria para suprimir las

respuestas defensivas mediadas por otras PRRs. Por tanto, actúa como un mecanismo redundante necesario para alcanzar una virulencia completa. AvrPtoB también puede interaccionar con BAK1 (no degradarlo), e inducir la vía de señalización del ácido abscísico (ABA, de *Abscisic Acid*), favoreciendo la patogenicidad (de Torres-Zabala et al. 2007; Zipfel 2009).

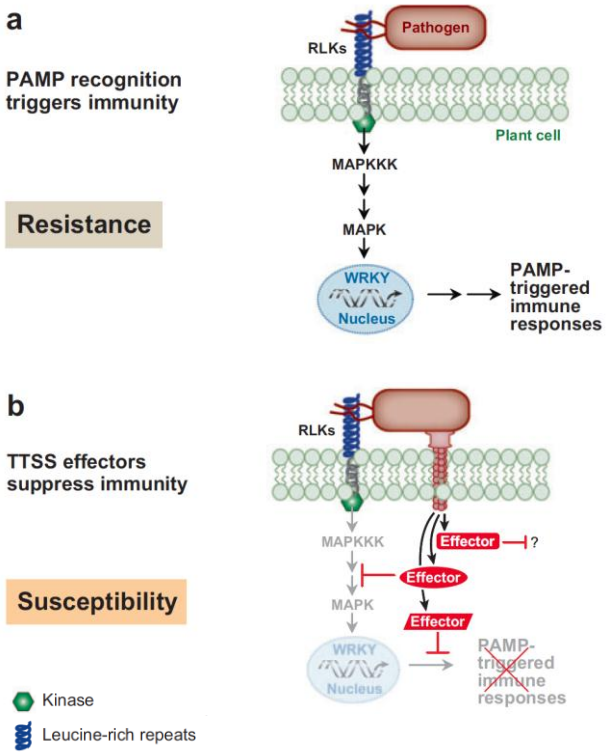

Figura 2. Modelo para la inducción y supresión de la PTI en plantas por un patógeno bacteriano (tomado de Bent y Mackey 2007). a La planta reconoce determinados patrones moleculares asociados a patógenos mediante receptores extracelulares con actividad quinasa. Esto dispara la inmunidad basal de la planta principalmente a través de cascadas de señalización mediadas por MAPKs y modificación transcripcional mediada por factores de transcripción WRKY. **b** Las bacterias patógenas utilizan el sistema de secreción tipo III para introducir múltiples efectores en el interior celular, donde actúan sobre diferentes dianas con el objetivo de suprimir la PTI y permitir la acumulación de bacterias en el apoplasto vegetal. Este modelo es extrapolable a otros patógenos.

Otro efector de *P. syringae* que es capaz de comprometer la PTI es HopAI1. Interacciona directamente con dos de las MAPKs que participan en la cascada de señalización iniciada tras la percepción de los MAMPs (MPK3 y MPK6). Presenta una actividad fosfotreonina-liasa que elimina el grupo fosfato de ambas proteín-quinasas y las inactiva (Li et al. 2007; Zhang et al. 2007a). También se han identificado efectores que pueden alterar el tráfico vesicular intracelular para suprimir la inmunidad del hospedador. El efector HopM1 de *P. syringae* que interacciona físicamente con una de las proteínas de Arabidopsis encargadas de regular este proceso (AtMIN7, HOPM INTERACTOR 7) y media su degradación vía proteasoma (Nomura et al. 2006). Los efectores TAL (de *Transcription Activator Like*) son aquellos capaces de introducir cambios significativos en la expresión de muchos genes del hospedador. Como ejemplo, los efectores AvrBs3 y AvrXa27 de *Xanthomonas spp.* presentan un dominio de localización nuclear (NLS de *Nuclear Localization Signals*) que les permite entrar en el núcleo de la célula hospedadora y alterar la transcripción de sus genes diana (Speth et al. 2007).

Otra estrategia utilizada por las bacterias para evadir la PTI es alterar en la medida de sus posibilidades los MAMPs detectados por las defensas de la planta. Es el caso de la flagelina de *Xanthomonas campestris pv. Campestris*, que no puede ser reconocida por la FLS2 de Arabidopsis (Schwessinger y Zipfel 2008).

Respecto a los efectores producidos por patógenos eucariotas de plantas (hongos y oomicetos), sus funciones y mecanismos son peor conocidos que los de bacterias. Estos efectores pueden actuar tanto en la matriz extracelular como en el interior de la célula hospedante. Muchos hongos biotrofos presentan la capacidad de generar una estructura de infección especializada denominada haustorio, que se expande por el tejido de la planta a través del espacio intercelular o apoplasto sin introducirse en las células vegetales. Los efectores fúngicos saldrían al apoplasto a través del haustorio y, si bien es sabido que algunos de ellos pueden introducirse en la célula hospedadora, se desconoce el mecanismo (Chisholm et al. 2006; Jones y Dangl 2006).

La quitina, componente principal de la pared celular de los hongos, es reconocida por las defensas de la planta para disparar la PTI. Además, las plantas han desarrollado quitinasas que liberan pequeños polímeros activos de la pared celular de los hongos que actúan como amplificadores de la señal de peligro. El efector Avr4 de *Cladosporium fulvum* presenta un dominio de unión a

quitina que actúa protegiendo su pared celular de las quitinasas, disminuyendo así parte de la inducción de defensas de la planta (Chisholm et al. 2006).

El principal mecanismo de defensa de las plantas frente a infecciones virales es el conocido como silenciamiento del RNA viral. La mayoría de los virus patógenos de plantas presentan un genoma de RNA y durante su replicación forman moléculas intermediarias de doble cadena. Dichas moléculas son las principales activadoras de este silenciamiento. En contrapartida, los virus han desarrollado efectores que tienen como diana los componentes principales de la maquinaria de silenciamiento de la planta y que actúan como supresores de dicho mecanismo (Speth et al. 2007). Los efectores virales también modifican el tráfico inter e intracelular en las células hospedadoras. Muchos de ellos presentan proteínas de movimiento que facilitan su paso a través de los plasmodesmos que intercomunican las células vegetales. Otros efectores virales actúan directamente sobre complejos PRRs. Es el caso de la proteína de transporte nuclear de los geminivirus que es capaz de interaccionar e inhibir la actividad quinasa de tres LRR-RLKs de Arabidopsis necesarias para la respuesta defensiva antiviral (Fontes et al. 2004; Speth et al. 2007).

2.3 Inmunidad activada por efectores (ETI)

Los procesos defensivos que se desencadenan en la planta con la ETI son muy similares a los activados por la PTI. Por ello, hay autores que consideran que esta diferenciación no es necesaria. Esto viene avalado por el hecho de que los mecanismos descritos como propios de cada tipo de defensa han coexistido desde el principio de la evolución de las plantas con semillas. Además, existe un elevado grado de solapamiento entre los datos de expresión de genes obtenidos en plantas independientemente del tipo de inmunidad activada. Las plantas tampoco parecen discriminar entre el tipo de patógeno causante de la activación. Así, plantean un modelo por el que la planta ejecuta el mismo tipo de programa defensivo cuando percibe cualquiera de las posibles señales de peligro (MAMPs, DAMPs o efectores), si bien existen diferencias cinéticas y cuantitativas en dicha inducción. A grandes rasgos, la ETI suele ser más rápida, eficaz y prolongada que la PTI (Boller y Felix 2009). No obstante, para una mejor comprensión de estos mecanismos defensivos, en el presente trabajo se ha mantenido la separación entre ambas etapas.

En este punto, y debido a su importancia histórica, es conveniente introducir la hipótesis de la resistencia gen a gen expuesta por Flor en 1971. Según esta teoría, las plantas poseen un conjunto de genes *R* cuyas proteínas resultantes actúan como receptores capaces de detectar una serie de proteínas Avr del patógeno (o factores de avirulencia). De acuerdo con esta hipótesis, si existía una interacción entre la proteína R de la planta y la proteína Avr del patógeno, la planta desencadenaría una respuesta inmunitaria que la haría resistente al patógeno (interacción incompatible). Si no existía tal interacción, la planta sería susceptible (interacción compatible). Resultados experimentales posteriores condujeron a una modificación de este modelo y el planteamiento de la hipótesis del gen guardián, según la cual no es necesaria una interacción física entre la proteína R y el efector del patógeno. Esta hipótesis propone que en la mayoría de los casos se produce un reconocimiento indirecto de los efectores a través de la monitorización del estado de diferentes componentes de la célula vegetal. Por tanto, estas proteínas R actuarían como sensores que dan la señal de alarma cuando detectan la más mínima perturbación provocada por los efectores (van der Biezen y Jones 1998; Jones y Takemoto 2004).

La ETI también suele conllevar la aparición de una respuesta hipersensible (HR, de *Hypersensitive Response*). La HR es una respuesta defensiva robusta, asociada principalmente a la resistencia mediada por genes *R*, que incluye una muerte programada de células de la planta en el lugar de infección (Heath 2000b). La detección de patógenos a través de las proteínas R es muy específica, mientras que la HR que induce la planta no lo es, siendo efectiva para combatir a diferentes patógenos (de Wit 2007). Sin embargo, también se han descrito MAMPs capaces de inducir esta HR (Naito et al. 2008). La identificación de los mutantes *hrp* (*hypersensitive response and pathogenicity*) en bacterias fitopatógenas corrobora la estrecha relación entre la virulencia o avirulencia de un patógeno y la actividad de sus efectores. Esto se debe a que las bacterias *hrp* pierden la habilidad tanto de inducir HR en hospedadores resistentes como de producir patogénesis en hospedadores susceptibles (Lindgren et al. 1986; Bent y Mackey 2007).

Como ya se ha mencionado, la clase mayoritaria de genes *R* codifica proteínas receptoras del tipo NB-LRR. Su nombre hace referencia a sus dos dominios característicos (un sitio conservado de unión a nucleótido en la zona central y una región rica en repeticiones de leucina en el extremo C-terminal,

respectivamente) (Dangl y Jones 2001). Las proteínas NB-LRR son citoplasmáticas y también pueden presentar dominios funcionales en su extremo N-terminal. De hecho, se pueden subdividir en dos categorías en función de estos dominios: las TIR-NB-LRR (de *Toll Interleukin 1 Receptor*) y las CC-NB-LRR (de *Coiled Coil*). El dominio LRR estaría implicado en el reconocimiento de efectores o de las perturbaciones que estos generan (en muchos casos junto con los dominios en N-terminal). En cambio, el NB ejercería como dominio de activación mediante la sustitución de un nucleótido difosfato (ADP) por otro trifosfato (ATP) (Chisholm et al. 2006; Takken et al. 2006; Collier y Moffett 2009). Durante el trabajo experimental de esta Tesis se han utilizado como herramientas dos cepas de *Pto* con los efectores AvrRpm1 (Ritter y Dangl 1996) y AvrRpt2 (Debener et al. 1991) con el objetivo de estudiar el comportamiento de determinados genotipos en la resistencia mediada por estas interacciones incompatibles. En Arabidopsis, estos efectores son reconocidos de forma indirecta por las proteínas RPM1 (RESISTANCE TO P. SYRINGAE PV. MACULICOLA 1) y RPS2 (RESISTANT TO P. SYRINGAE 2), respectivamente. Así pues, los mutantes *rpm1* (Grant et al. 1995) y *rps2* (Mindrinos et al. 1994) no son capaces de reconocer la presencia de uno de estos efectores y son más susceptibles a la cepa que lo posee. Un ejemplo de reconocimiento indirecto de un efector a través de su actividad enzimática es el que se da entre la proteína de tipo NB-LRR RPS5 (RESISTANT TO P. SYRINGAE 5) de Arabidopsis y el efector AvrPphB de *P. syringae*. Este efector es una proteasa que, tras ser introducida por el TTSS, tiene como diana a la proteína PBS1 (AVRPPHB SUSCEPTIBLE 1). El efector rompe esta proteín-quinasa del hospedador y RPS5 puede detectar dicha rotura y lanzar la respuesta defensiva (Shao et al. 2003).

Una diferencia sustancial entre los MAMPs y los efectores radica en que los primeros han sido estables evolutivamente, puesto que forman parte de elementos imprescindibles para la viabilidad del patógeno. En cambio, los efectores sí pueden variar considerablemente, o incluso desaparecer en determinadas cepas del patógeno, porque no son necesarios para el crecimiento y desarrollo del microorganismo fuera del hospedador. La posible modificación de estos efectores está influenciada por cómo son percibidos por las proteínas R. En el caso de que exista una interacción física, es posible que se seleccionen mutaciones que introduzcan cambios en su secuencia que imposibiliten tal interacción, siempre y cuando no se altere su actividad en la virulencia. Este

proceso evolutivo es mucho más complicado en los efectores reconocidos de forma indirecta (Bent y Mackey 2007).

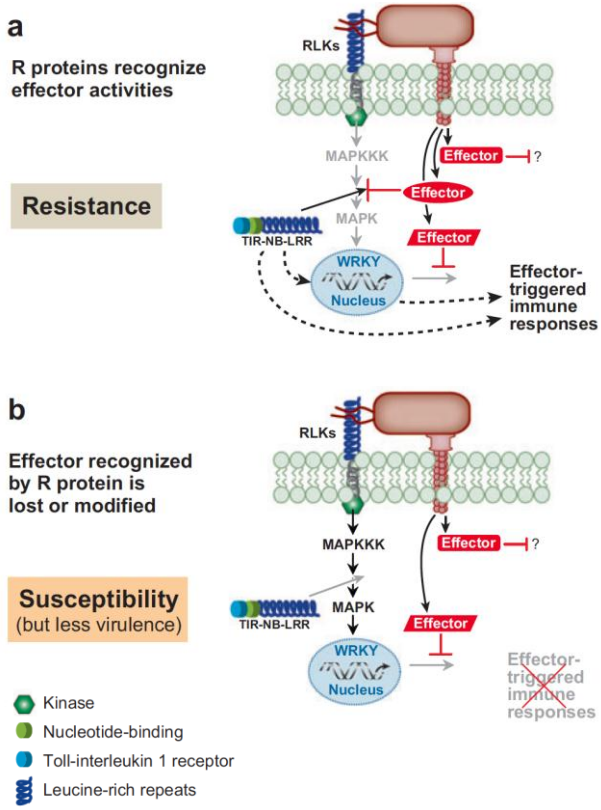

Figura 3. Modelo para la inducción y supresión de la ETI en plantas por un patógeno bacteriano (tomado de Bent y Mackey 2007). a Las proteínas de resistencia de las plantas (caso de las TIR-NB-LRR) reconocen directa o indirectamente la presencia de los efectores bacterianos restaurando la resistencia mediante la más contundente ETI. **b** Los patógenos evaden la resistencia basada en genes *R* mediante la modificación o eliminación de los efectores que la inducen. La virulencia o grado de patogenicidad consecuente es menor respecto al ocurrido tras la supresión de la PTI. Este modelo es extrapolable a otros patógenos.

3. Las respuestas defensivas

La detección de la presencia del patógeno, independientemente de que esta se haya producido vía PRRs o vía genes *R*, conduce a unas respuestas defensivas que, dada su similitud, pueden ser analizadas en conjunto (Lopez et al. 2008). Uno de los efectos más inmediatos es la alteración del flujo de iones a través de la membrana plasmática. Destaca el rápido incremento de la concentración citoplasmática de Ca^{2+} debido a la mayor entrada de este ion desde el apoplasto. El Ca^{2+} actúa como segundo mensajero, tanto promoviendo la apertura de otros canales iónicos en la membrana como activando a las quinasas dependientes del calcio (CDPKs, de *Calcium Dependent Protein Kinase*). También se produce una alcalinización del espacio extracelular debido a la salida de iones K^+ (Boller y Felix 2009; Segonzac y Zipfel 2011).

Otro efecto casi inmediato es la producción de especies reactivas de oxígeno (ROS, de *Reactive Oxygen Species*). En Arabidopsis, la producción de ROS requiere de la activación de una oxidasa (RbohD, RESPIRATORY BURST OXIDASE HOMOLOGUE D) que es parcialmente dependiente de la concentración de Ca^{2+} citoplasmática (Ogasawara et al. 2008). Las ROS pueden actuar directamente como antibióticos sobre los patógenos. También pueden reforzar la pared celular, favoreciendo su resistencia a las enzimas líticas de los patógenos. En el interior celular, actúan como mensajeros secundarios pudiendo inducir directa o indirectamente la expresión de genes de defensa. No obstante, si la infección persiste se producirá una mayor producción de ROS relacionada con la HR. En este proceso, conocido como explosión oxidativa, también es relevante la producción de óxido nítrico (Boller y Felix 2009).

Como se ha mencionado anteriormente, la percepción del patógeno provoca la activación de varias cascadas de señalización mediadas por MAPKs cuyo resultado final es inducir la expresión de los genes de defensa. En Arabidopsis se ha planteado que las CDPKs actúen sinérgica e independientemente de las MAPKs para inducir esta activación. Para conseguirlo, estas cascadas de señalización conducen a la fosforilación de un elevado número de TFs. Son estos los que regulan finalmente la expresión de dichos genes. Destacan por su relevancia los TFs tipo WRKYs y la familia de los TGAs (de Wit 2007; Boudsocq et al. 2010).

Como resultado de la expresión de estos genes tiene lugar una gran acumulación de proteínas. Entre ellas sobresale el heterogéneo grupo de proteínas asociadas a defensa, codificadas en los genes *PR* (*PATHOGENESIS RELATED*). Fueron identificadas durante el estudio de la HR desencadenada en *Nicotiana tabacum* (tabaco) por la interacción con el virus del mosaico del tabaco (TMV, de *Tobacco Mosaic Virus*), por dos grupos independientes (Gianinazzi et al. 1970; van Loon y van Kammen 1970). Las proteínas PR se definen como aquellas que no son detectables, o solo lo son a niveles basales, en los tejidos sanos y para las que se ha demostrado una acumulación en condiciones patológicas en dos o más tipos de interacciones planta-patógeno (van Loon et al. 2006; Sels et al. 2008). Esta definición podría incluir a proteínas presentes constitutivamente pero cuya expresión aumenta durante las infecciones. Para evitarlo se ha propuesto el concepto más restrictivo de proteínas PR inducibles (van Loon et al. 2006). El término condiciones patológicas de la definición se utiliza en un sentido amplio. De esta forma, incluye tanto el ataque directo de un patógeno como la aplicación de productos que mimeticen dicho ataque (caso de las fitohormonas defensivas) o la producción de heridas. Se habla de proteínas asociadas o relacionadas con defensa porque el hecho de que se induzcan durante las respuestas defensivas no implica que presenten un papel funcional en defensa (van Loon et al. 2006; Sels et al. 2008).

Las proteínas PR descritas han sido clasificadas en 17 familias, numeradas en función del orden de descubrimiento. Para algunas de estas familias es posible destacar una propiedad que indicaría cuál es su papel general en la respuesta de la planta al ataque del patógeno. Es el caso de la actividad β-1,3-glucanasa en PR-2 (Kauffmann et al. 1987) y de la actividad quitinasa de PR-3 (Legrand et al. 1987), PR-4, PR-8 y PR-11, cuyos sustratos diana en ambos casos son componentes de la pared celular de los hongos (van Loon et al. 2006). La actividad ribonucleasa detectada en PR-10 sería relevante para la defensa frente a virus (Park et al. 2004). Otro ejemplo es la actividad inhibidor de proteinasa en la familia PR-6. Esta función puede limitar, entre otras, la actividad de las proteasas digestivas secretadas por los insectos herbívoros (Tamhane et al. 2005) o la finalización del ciclo de replicación en los virus (Gutierrez-Campos et al. 1999). En el caso de las defensinas, PR-12, y de las tioninas, PR-13, se ha descrito para ambas familias una actividad general antibacteriana y antifúngica, que actuaría sobre la permeabilización de las membranas celulares (Sels et al.

2008). La actividad peroxidasa presente en PR-9 podría contribuir al fortalecimiento de la pared celular mediante la lignificación (Passardi et al. 2004). La presencia y funcionalidad de las citadas actividades puede variar enormemente entre los diferentes miembros de cada una de las familias. También existen muchas PRs para las que aún no se ha descrito ninguna función biológica directa relacionada con las defensas de la planta. Este es el caso del grupo más abundante, la familia PR-1. Se trata de una familia altamente conservada y que ha sido identificada en todas las especies de plantas estudiadas Además, estas proteínas han sido utilizadas (también en la parte experimental de esta Tesis) como marcadores válidos de la inducción de una respuesta defensiva adecuada tras el ataque del patógeno (van Loon et al. 2006). La expresión de este conjunto de genes también es inducida por la acumulación del SA (véase el apartado correspondiente a esta hormona).

Por tanto, las funciones de los genes de defensa incluyen, entre otras, la producción de agentes antimicrobianos o de proteínas capaces de catalizar dicha producción, el reforzamiento de la pared celular mediante la deposición de calosa en los puntos de infección y una mayor lignificación o la aparición de la HR en el lugar de inicio de la infección limitando su avance. Pero, sobre todo, regulan la biosíntesis y señalización de aquellas fitohormonas con mayor implicación en la defensa (Dangl y Jones 2001; de Wit 2007). Es el caso del SA, el ácido jasmónico (JA, de *Jasmonic Acid*) y el etileno (ET, de *Ethylene*). A grandes rasgos, es posible hablar de dos rutas principales de señalización defensiva. La vía dependiente del SA está más relacionada con la inducción de resistencia frente a patógenos biotrofos y virus. En cambio, la vía dependiente del JA y del ET combate principalmente a los patógenos necrotrofos e insectos. Por tanto, la activación de las rutas de señalización defensivas depende del tipo de vida del patógeno y de su modo de infección (Glazebrook 2005; Lopez et al. 2008). Además, existe una inhibición cruzada entre estas dos vías de señalización. De esta forma, una elevada resistencia a biotrofos está correlacionada con un incremento de la susceptibilidad a necrotrofos. Asimismo, una elevada resistencia a necrotrofos condiciona una mayor susceptibilidad a biotrofos (Robert-Seilaniantz et al. 2011). Este antagonismo ha sido aprovechado por algunos patógenos para superar las defensas de la planta. Algunas cepas de *P. syringae* producen el efector coronatina (COR, de *Coronatine*), muy similar a la hormona JA. Su secreción consigue simular varias de las respuestas activadas por esta hormona. De hecho, se ha demostrado que

la COR de *Pto* contribuye a su virulencia en Arabidopsis mediante la supresión indirecta de los mecanismos defensivos mediados por SA, más relevantes para combatir a este patógeno (Brooks et al. 2005).

En los últimos años, existe un número creciente de trabajos que implican a otras hormonas, además de las tres ya mencionadas, en las respuestas de la planta frente al ataque del patógeno; bien favoreciendo la resistencia o bien la susceptibilidad. Es el caso del ABA (Ton et al. 2009), las citoquininas (CK, de *Cytokinins*; Choi et al. 2010), las giberelinas (GAs, de *Gibberellins*; Navarro et al. 2008), las auxinas (Wang et al. 2007a) o los brasinosteroides (BR, de *Brassinosteroids*; Nakashita et al. 2003). Dicho ataque provoca una modificación de la abundancia relativa de todas estas hormonas (Lopez et al. 2008). Esta nueva homeostasis hormonal conduce a la expresión de los genes defensivos adecuados que permitan a la planta presentar una respuesta defensiva eficiente. No obstante, los patógenos provocan una alteración de esta homeostasis como parte de su estrategia de virulencia, llegando incluso a producir fitohormonas o compuestos que las mimetizan. De hecho, ha quedado probada la producción de CK, ABA, auxinas, JA y ET en diferentes especies de bacterias y hongos. Además, los patógenos también pueden inducir la producción de hormonas en el hospedador (Lopez et al. 2008; Robert-Seilaniantz et al. 2011).

En los siguientes apartados se abordará el papel que juegan estas hormonas en las defensas de la planta. No obstante, el grueso de la información se centrará en el SA, puesto que el estudio de la percepción de dicha hormona es el eje central del presente trabajo. Previamente, se introducirá el concepto de señalización sistémica defensiva y algunos de los avances realizados recientemente en esta materia.

3.1 Señalización sistémica defensiva

Desde hace años se ha constatado que las plantas son capaces de transmitir información a hojas distales sobre ataques en hojas locales (Ross 1961). Esta comunicación a grandes distancias en el interior de la planta requiere tanto de la existencia de una señal sistémica móvil como de un buen conducto que permita una eficiente translocación de la misma (Shah 2009). El tejido vascular de la planta representa una perfecta vía de transporte y distribución de estas señales sistémicas. También se ha comprobado que determinados compuestos volátiles

pueden contribuir a esta señalización sistémica sin necesidad de utilizar el tejido vascular. Dichos compuestos volátiles pueden ser translocados por vía aérea y actuar sobre la propia planta o sobre plantas vecinas. También pueden ser emitidos por plantas atacadas por ciertos herbívoros, induciendo una defensa indirecta mediante la atracción de los insectos predadores de estos herbívoros (Frost et al. 2008; Heil y Ton 2008).

Para que un determinado metabolito sea considerado como una señal sistémica de las defensas de la planta, ha de poder translocarse desde el tejido atacado hasta las zonas distales antes de que se induzcan en ellas las respuestas defensivas. Además, ha de ser el responsable de influir en la activación de las mismas. En este contexto cabe destacar el concepto de resistencia sistémica adquirida (SAR, de *Systemic Acquired Resistance*), como un buen ejemplo de mecanismo defensivo inducible que es activado en los tejidos distales de la planta en respuesta a una infección local por un patógeno. La SAR proporciona a la planta una resistencia acrecentada frente al posible ataque posterior por parte de una amplia gama de patógenos (Vlot et al. 2008a; Shah 2009). La resistencia sistémica inducida (ISR, de *Induced Systemic Resistance*) es otro tipo de señalización sistémica defensiva que tiene lugar en la planta tras su colonización por parte de microorganismos beneficiosos. Un ejemplo típico de ISR es la colonización de las raíces de la planta por parte de rizobacterias no patogénicas, hecho que proporciona a dicha planta una mayor resistencia frente a infecciones posteriores por parte de diversos patógenos (van Loon 2007). Durante los últimos años se han hecho bastantes esfuerzos para identificar posibles metabolitos que funcionen como señales sistémicas defensivas, especialmente con aquellos involucrados en la SAR (Shah 2009).

El SA es considerado como una señal sistémica asociada con la SAR desde hace muchos años. Sin embargo, experimentos con injertos en los que se emplearon plantas de tabaco que expresaban el gen bacteriano *NahG* descartaron que el SA sea la única molécula translocada responsable de la inducción de resistencia en los tejidos sistémicos (Vernooij et al. 1994). *NahG* es un gen de *Pseudomonas putida* que codifica una salicilato hidroxilasa que degrada el SA a catecol. Asimismo, estos y otros trabajos han determinado que el SA sí es necesario para el establecimiento y mantenimiento de la SAR, aunque no suficiente (Lawton et al. 1995; Truman et al. 2007). Trabajos posteriores, basados también en la interacción entre plantas de tabaco y el TMV, plantean que uno de los

derivados del SA sería la señal sistémica responsable de la SAR. El metil salicilato (MeSA, de *Methyl Salicylate*) es producido en tabaco por la NtSAMT1 (SAM TRANSPORTER 1) en la primera hoja inoculada con TMV, a partir del SA acumulado. Posteriormente, el MeSA es translocado a las hojas distales vía floema. Allí es hidrolizado por la NtSABP2 (SALICYLIC ACID BINDING PROTEIN 2) obteniéndose SA, responsable último del inicio de la inducción defensiva en las hojas distales (Park et al. 2007a; Park et al. 2009). El 2,2,2,2'-tetrafluoroacetophenone (tetraFA) es un compuesto sintético que actúa como inhibidor competitivo de la actividad esterasa de la NtSABP2. Su uso demostró que para la inducción de SAR en tabaco es necesaria la actividad de la NtSABP2 solo en hojas distales y no en la primera hoja inoculada. La aplicación de dicho compuesto en hojas distales de Arabidopsis también atenúa la SAR, sugiriendo un papel clave para el MeSA en Arabidopsis (Park et al. 2009). Sin embargo, existe controversia sobre esta conclusión.

En Arabidopsis, BSMT1 (BENZOIC ACID/SALICYLIC ACID CARBOXYL METHYLTRANSFERASE 1) es la principal enzima responsable de la síntesis de MeSA. Las plantas *bsmt1* no son capaces de acumular niveles significativos de MeSA ni en hojas inoculadas ni en hojas distales. Sin embargo, estas plantas mutantes sí son capaces de inducir SAR. Además, estos mismos autores demuestran que, si bien se producen elevados niveles de MeSA en las plantas silvestres en respuesta a la inoculación del patógeno, la gran mayoría (97%) es emitido a la atmósfera (Attaran et al. 2009). Cabe destacar que el MeSA es un compuesto volátil. A su vez, estos resultados son contrarios a otros obtenidos también en Arabidopsis. En ellos se observa una supresión de la SAR tanto por la falta de acumulación de MeSA debida a una mutación en *BSMT1* como por un elevado ratio MeSA:SA debido a una sobreexpresión de *BSMT1* (Liu et al. 2009). Por tanto, tenemos dos estudios independientes con mutantes de *BSMT1* cuyos resultados de inducción de SAR son contradictorios. Un trabajo posterior ha intentado clarificar estas discrepancias, concluyendo que se deben a diferencias en las condiciones de luz utilizadas. Si las plantas, tras recibir la primera inoculación reciben poca exposición a la luz, ven comprometida la activación de SAR por la inhibición de la síntesis de MeSA. En cambio, si a la infección primaria le siguen 3,5 o más horas de exposición a la luz, la SAR puede desarrollarse en ausencia de MeSA. Sin embargo, el MeSA sí es necesario para un desarrollo óptimo de esta respuesta sistémica (Liu et al. 2011).

El papel clave del MeSA en la SAR también se ha demostrado en *Solanum tuberosum* (patata). Esto se debe a que la SAR puede ser suprimida en patata tanto por tratamientos con tetraFA como por el silenciamiento de la expresión de *StMES1*(*METHYL ESTERASE*), un ortólogo de NtSABP2 (Manosalva et al. 2010). En Arabidopsis se han identificado 18 posibles ortólogos (AtMES), de los que al menos 5 pueden hidrolizar el MeSA (Vlot et al. 2008b).

El JA y alguno de sus derivados (en conjunto denominados "jasmonatos") también han sido implicados con la SAR en Arabidopsis. En concreto, se ha publicado que, tras la inoculación con una cepa avirulenta de *P. syringae*, el contenido de JA aumenta rápidamente en los exudados de los peciolos de dichas hojas. Además, se produce una rápida inducción de la expresión de genes de respuesta al JA en hojas distales, sugiriéndose que el JA puede ser el factor translocado por el tejido vascular necesario para la SAR. La no activación de SAR en mutantes deficientes (*opr3*, *oxophytodienoate reductase 3*) o insensibles (*jin1*, *jasmonate insensitive 1*) al JA avala esta hipótesis (Truman et al. 2007). Estos resultados entran en contradicción con los obtenidos en otros estudios. En ellos se demuestra que estos mutantes (*opr3* y *jin1*) y otros también implicados en la vía del JA (*coi1*, *coronatine insensitive 1*; *jar1*, *jasmonate resistant 1*) sí son competentes para SAR (Cui et al. 2005; Mishina y Zeier 2007; Attaran et al. 2009). Estas discrepancias pueden deberse en parte a la dosis de patógeno inoculado. Según esta interpretación, los jasmonatos jugarían un papel condicional en este proceso. Si se aplica una dosis baja de patógeno, no se induce una HR y sí son necesarios para activar la SAR. En cambio, con una dosis mayor sí tiene lugar la HR y no son necesarios (Shah 2009).

Otro compuesto postulado como relevante para la SAR es el ácido azelaico. Fue identificado debido a sus elevados niveles en los exudados de los peciolos de hojas de Arabidopsis inoculadas con cepas de *P. syringae* capaces de disparar SAR. Además, el marcaje de esta molécula ha permitido comprobar que puede moverse sistémicamente. Su aplicación directa puede promover la resistencia en hojas distales en plantas silvestres. Esto no sucede en aquellos mutantes de la vía del SA que son defectivos en SAR. Todo ello indica que la inducción de resistencia por el ácido azelaico requiere de la vía de señalización del SA, es decir, que induce la acumulación de esta hormona defensiva (Jung et al. 2009). Por otra parte, el gen *AZI1* (*AZELAIC ACID INDUCED 1*), que se expresa a niveles elevados en plantas tratadas con el ácido azelaico, es necesario para la

sensibilización defensiva mediada por esta molécula. Esto se observa por la pérdida de la SAR inducida bien por el patógeno o bien por esta molécula en el mutante *azi1*. Por tanto, el ácido azelaico y la proteína AZI1 se postulan como relevantes para la inducción de resistencias sistémicas en plantas (Jung et al. 2009). Existen más ejemplos de compuestos que han sido postulados como posibles señales sistémicas relevantes para la inducción de defensas. Es el caso del terpenoide dehydroabietinal, el aldehído (E)-2-hexenal, el alcohol (Z)-3-hexenol, diferentes azúcares y algunos péptidos pequeños.

En resumen, a pesar de los últimos avances realizados, aún quedan bastantes preguntas por resolver en este campo. No obstante, se puede concluir que una comunicación eficiente entre aquellos tejidos que han sido atacados por un patógeno y el resto de la planta es fundamental para una activación adecuada de sus defensas que contribuya a evitar la difusión sistémica del patógeno (Shah 2009).

3.2 Ácido salicílico (SA)

El SA pertenece a la amplia gama de compuestos fenólicos producidos por las plantas. Tradicionalmente, estos compuestos se consideraron como metabolitos secundarios con poca importancia para la planta o incluso como meros residuos. Pero este concepto cambió drásticamente al descubrirse que sí cumplen funciones importantes (Revisado en Dempsey et al. 2011). En el caso concreto del SA se ha descrito que presenta un papel relevante en la regulación de procesos relacionados con el crecimiento y desarrollo de la planta (germinación de semillas, crecimiento vegetativo, fotosíntesis, respiración, termogénesis, formación de flores, producción de semillas, senescencia y muerte celular no asociada con la HR). También puede influir en otros muchos procesos tales como el establecimiento de la plántula, el cierre de los estomas, la nodulación en legumbres o las respuestas frente a estreses abióticos (sequía, frío, calor, metales pesados, estrés osmótico, etc.). En muchos de estos procesos, el efecto del SA es indirecto mediante la alteración de las vías de señalización de otras hormonas vegetales (Vlot et al. 2009; Rivas-San Vicente y Plasencia 2011).

Además de los ya citados, el SA juega un papel clave como molécula señalizadora en la respuesta inmunitaria de la planta, sobre todo frente a

patógenos biotrofos. Participa en los procesos activados tanto en la PTI como en algunos tipos de ETI y es esencial para la SAR. La infección por el patógeno conduce a una acumulación de SA tanto en las hojas infectadas como en las distales, donde tiene lugar la SAR. Esta acumulación está correlacionada con el aumento de la expresión de los genes *PR*. La inducción de algunas de estas proteínas se ha utilizado como marcador de la ruta de señalización de la hormona y del establecimiento de SAR. De hecho, en la parte experimental de esta tesis se utiliza la detección de PR-1 como un indicador del buen funcionamiento de la vía. Además, la aplicación exógena de SA o de alguno de sus análogos funcionales, como la aspirina, el INA (*2,6-dichloroisonicotinic acid*) (Ward et al. 1991) y el BTH (*benzothiadiazole S-methyl ester*) (Lawton et al. 1996), activan la expresión de genes *PR* y la resistencia frente a una amplia gama de patógenos. Como veremos a continuación, mutaciones en genes implicados en la biosíntesis y/o acumulación del SA aumentan la susceptibilidad de la planta, pudiéndose restaurar la resistencia con SA exógeno (An y Mou 2011). Por tanto, se ha podido concluir que plantas con bajos niveles de SA, caso de las transgénicas *NahG* ya mencionadas (Friedrich et al. 1995), son más susceptibles a patógenos compatibles. En cambio, las plantas con elevados niveles de SA, como las transgénicas *c-SAS* y *p-SAS* de Arabidopsis (Mauch et al. 2001), son más resistentes (Nawrath et al. 2005). Además, este papel clave del SA como hormona defensiva ha sido confirmado tanto en dicotiledóneas como en algunas monocotiledóneas (Vlot et al. 2009).

Biosíntesis del SA

Existen dos rutas diferentes de biosíntesis del SA en plantas, aunque ambas parten del mismo metabolito, el corismato. La primera ruta, se inicia a partir de un derivado del corismato, la L-fenilalanina (Phe, de *L-Phenylalanine*). Posteriormente, la fenilalanina amonio liasa (PAL, PHENYLALANINE AMMONIA LYASE) convierte la Phe en trans-ácido cinámico (t-CA, de *trans-cinnamic acid*). El t-CA es un precursor de la biosíntesis de otros compuestos fenólicos (p. ej. lignina o flavonoides). Finalmente, la biosíntesis del SA a partir del t-CA se realiza mediante la participación de dos posibles intermediarios finales: el ácido orto-cumárico o el ácido benzoico. En conjunto, esta primera se denomina ruta PAL y tiene lugar en el citoplasma (Dempsey et al. 2011; Rivas-San Vicente y Plasencia 2011). Varios géneros bacterianos pueden sintetizar SA generando como intermediario el isocorismato (IC, de

Isochorismate), y en las plantas el corismato se sintetiza en los plastidios. También es conocido que muchas de las rutas metabólicas localizadas en estos orgánulos han derivado de procariotas endosimbiontes. Estas observaciones fomentaron la búsqueda de una segunda ruta de biosíntesis en plantas. Finalmente, se confirmó que el SA puede también obtenerse a partir del corismato por una ruta más corta en la que se utiliza como intermediario el IC. En bacterias, las enzimas ICS (ISOCHORISMATE SYNTHASE) e IPL (ISOCHORISMATE PYRUVATE LYASE) son las que catalizan esta biosíntesis. En plantas, esta segunda vía se conoce como ruta IC y tiene lugar en el cloroplasto (Vlot et al. 2009; Dempsey et al. 2011; Rivas-San Vicente y Plasencia 2011).

La mayoría del SA producido en respuesta a un patógeno es obtenido por la ruta IC en Arabidopsis (Wildermuth et al. 2001), en tomate (Uppalapati et al. 2007) y en *N. benthamiana* (Catinot et al. 2008). En el caso de Arabidopsis se han identificado de forma tentativa dos genes *ICS*, *ICS1* e *ICS2*. Sin embargo, parece que ICS1 es la responsable del 90% de la producción de SA inducida por el patógeno en esta especie (Wildermuth et al. 2001). Tal conclusión se obtiene a partir de los estudios realizados con dos mutantes defectivos en ICS1(también conocida como SID2 o EDS16): *sid2*-1 (*SA induction-deficient 2*) (Nawrath y Metraux 1999) y *eds16*-1/*sid2*-2 (*enhanced disease susceptibility 16*) (Dewdney et al. 2000). Los fenotipos de estos mutantes confirman el papel fundamental de la ICS1, y por ende del SA, en la PTI, la ETI y la SAR (mayor susceptibilidad a patógenos virulentos, menor resistencia a patógenos avirulentos, reducción de la inducción de genes de defensa y no inducción de SAR en hojas distales). La aplicación de SA complementa estos fenotipos de mayor susceptibilidad (Wildermuth et al. 2001; Tsuda et al. 2008). No obstante, el hecho de que el doble mutante *ics1 ics2* presente una pequeña cantidad de SA (~5% del nivel total de SA obtenido en plantas silvestres tratadas) sugiere que la ruta IC no es la única fuente de esta hormona en Arabidopsis. Cabe destacar que este doble mutante casi carente de SA presenta una pigmentación amarillenta, un menor tamaño y solamente puede sobrevivir mediante el cultivo *in vitro* (Garcion et al. 2008). La ruta PAL sería la responsable del SA residual presente en *ics1 ics2*. De hecho, trabajos realizados con el cuádruple mutante *pal1 pal2 pal3 pal4* muestran una reducción del 50 al 75% en la producción de SA (Huang et al. 2010). La aparente contradicción entre los datos obtenidos con mutantes que bloquean cada una de las dos vías puede explicarse por el hecho de que ambas parten del

corismato. Por otra parte, aún no ha sido identificado ningún gen que codifique una IPL en plantas (An y Mou 2011; Dempsey et al. 2011).

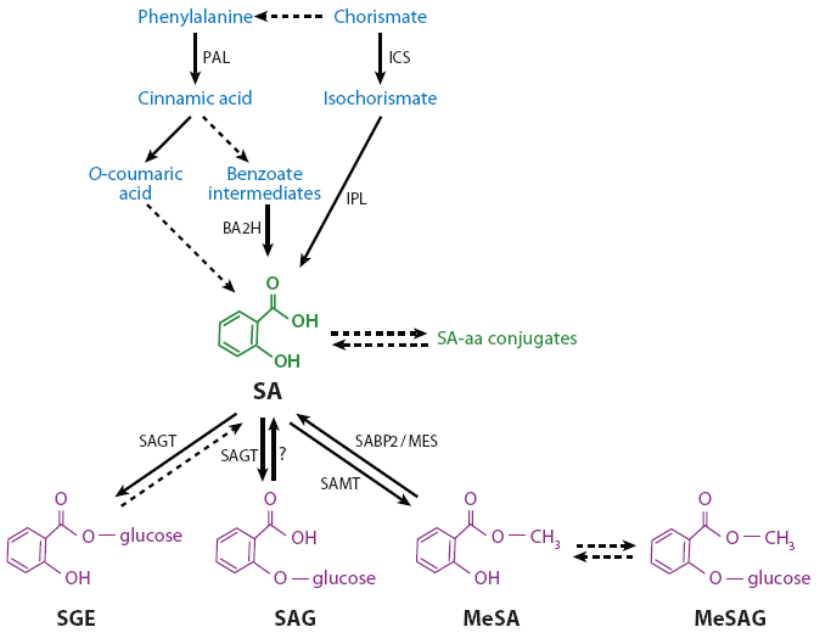

Figura 4. Biosíntesis y modificaciones del SA (tomado de Vlot et al. 2009). Esquema simplificado de las dos rutas de biosíntesis del SA: la ruta de la fenilalanina amonio liasa y la ruta del isocorismato. Se muestran también algunas de las modificaciones químicas del SA que tienen lugar tras su biosíntesis.

Modificaciones del SA

Una vez el SA se ha sintetizado, puede experimentar varias modificaciones químicas como son la glucosilación, la metilación o la conjugación con AA. Estas modificaciones alteran la actividad, acumulación, función y/o movilidad del SA (Dempsey et al. 2011). De hecho, la mayor parte del SA producido en la planta es glucosilado y/o metilado.

La glucosilación inactiva al SA pero permite su almacenamiento vacuolar en cantidades relativamente grandes gracias a su toxicidad reducida respecto a la molécula original. En Arabidopsis se han descrito dos enzimas que pueden

glucosilar al SA: UGT74F1 (UDP-GLYCOSYLTRASFERASE 74 F1) y UGT74F2/SGT1 (SALICYLIC ACID GLUCOSYLTRANSFERASE 1) (Dean y Delaney 2008). Ambas pueden catalizar la conjugación de glucosa en el grupo hidroxilo del SA dando lugar a la formación del SA 2-*O*-β-D-glucósido (SAG, de *SA Glucoside*). Además, la SGT1 también cataliza esta reacción sobre el grupo carboxilo del SA, formándose el éster de la saliciloil glucosa (SGE, de *Salicyloyl Glucose Ester*) (Dempsey et al. 2011). Estas reacciones tienen lugar en el citosol y son inducibles por la aplicación de SA o por la infección de un patógeno tanto en Arabidopsis (Song 2006) como en tabaco (Lee y Raskin 1999). El SAG, el principal compuesto conjugado producido, es transportado activamente del citosol a la vacuola. Allí es almacenado, pudiendo ser requerido para convertirse nuevamente en SA libre (Rivas-San Vicente y Plasencia 2011).

La metilación también inactiva al SA, pero al mismo tiempo aumenta su permeabilidad y volatilidad. Esto permite un transporte más efectivo de esta señal defensiva a largas distancias. Además de lo comentado previamente respecto a SAR, se ha implicado al MeSA en procesos como el transporte de SA entre plantas, la atracción de predadores o de polinizadores y la regulación cruzada existente entre esta vía de señalización y la dependiente del JA (Dempsey et al. 2011). A partir del MeSA también pueden formarse derivados glucosilados (MeSAG), igualmente inactivos. Su posible función sería actuar como forma de almacenamiento de MeSA no volátil, aunque parece que no se acumulan en la vacuola (Dean et al. 2005; Dempsey et al. 2011).

Poco se sabe aún sobre el papel que desempeña la conjugación de AA con el SA. En Arabidopsis se ha descrito la familia de enzimas GH3 (de *Gretchen Hagen*) como responsable de la conjugación de AA, sobre todo con la auxina ácido indolacético (IAA, de *Indole-3-acetic acid*). De todas ellas, solamente GH3.5 es activa con el SA, aunque tiene una mayor afinidad por el IAA. El principal conjugado SA-AA formado en plantas es el Saliciloil-L-aspartato (SA-Asp). El SA-Asp es también una forma inactiva del SA que podría estar implicada en su catabolismo (Dempsey et al. 2011). La sobreexpresión de *GH3.5* provoca un aumento de los niveles de SA-Asp tras la infección del patógeno. También induce la resistencia a patógenos y la expresión de genes de defensa. En el mutante nulo *gh3.5*-2 no se detectaron alteraciones en los niveles de SA, SAG o SA-Asp. Esto indica que otras proteínas GH3 también deben catalizar la formación de SA-Asp. Este mutante presenta fenotipos asociados a auxinas y

una reducción del nivel de IAA-Asp. Además, la sobreexpresión de *GH3.5* provoca un aumento de los niveles de IAA-Asp considerablemente superior al registrado para SA-Asp. En conjunto, todos estos hallazgos confirman el papel principal de esta enzima sobre el IAA. Por tanto, el papel de *GH3.5* en la resistencia a patógenos se debería a su influencia en la regulación cruzada existente entre las hormonas IAA y SA (Park et al. 2007b; Zhang et al. 2007b).

Regulación de la acumulación de SA

En primer lugar se abordará la descripción de aquellas proteínas reguladoras que disparan y amplifican la señal de acumulación del SA. Los primeros análisis genéticos indicaron que, tras la detección del patógeno por Arabidopsis, las proteínas R tipo CC-NB-LRR activarían la señalización defensiva vía NDR1 (NON RACE-SPECIFIC DISEASE RESISTANCE 1). En cambio, las tipo TIR-NB-LRR lo harían vía EDS1 (Aarts et al. 1998). Posteriormente, se identificaron dos interactores de EDS1: PAD4 (PHYTOALEXIN DEFICIENT 4) (Feys et al. 2001) y SAG101 (SENESCENCE ASSOCIATED GENE 101) (Feys et al. 2005). Más adelante se ha comprobado que EDS1 también participa en las defensas mediadas por las CC-NB-LRR (Venugopal et al. 2009). Además de en la ETI, estas cuatro proteínas (NDR1, EDS1, PAD4 y SAG101) han sido implicadas en la PTI (Dempsey et al. 2011). La acumulación de SA inducida por el patógeno es dependiente de una o varias de estas proteínas. Tratamientos con SA restablecen la expresión de genes de defensa en los mutantes *eds1* y *pad4*. Por tanto, dichas proteínas intervienen por encima del SA en esta cascada de señalización. Además, dichos tratamientos inducen la expresión de *EDS1* y *PAD4* en plantas silvestres. Esto sugiere la existencia de un bucle de retroalimentación positiva que amplifica esta señalización defensiva. Este bucle estaría regulado por los diferentes complejos formados por EDS1 (Vlot et al. 2009; Dempsey et al. 2011). EDS1 está localizado en el núcleo y el citoplasma, siendo necesario en ambos compartimentos para una función defensiva completa. Puede formar homodímeros en el citosol, heterodímeros nucleares con SAG101 y heterodímeros con PAD4 en ambas localizaciones. EDS1 es necesario para la acumulación de sus dos interactores, si bien los tres genes actúan cooperativamente en la señalización defensiva. Además, las funciones de ambos interactores son parcialmente redundantes, aunque sí parecen actuar en rutas separadas para transducir la señalización de EDS1 (Feys et al. 2005; García et al. 2010). En el caso de *NDR1*, su

sobreexpresión conlleva un aumento de la resistencia de Arabidopsis a cepas virulentas de *P. syringae*. Además, puede complementar los fenotipos de *ndr1*, que se deben a la menor capacidad de estas plantas de acumular SA tras la infección. Por tanto, NDR1 también actúa en la ruta por encima del SA (Shapiro y Zhang 2001; Coppinger et al. 2004).

Otro gen descrito como relevante para la acumulación de SA durante la respuesta defensiva es *EDS5/SID1*. La proteína EDS5 presenta una gran similitud con la familia de transportadores MATE (*Multidrug And Toxic compound Extrusion*) de animales, que está implicada en el transporte de pequeñas moléculas orgánicas, compuestos fenólicos incluidos, a través de membranas (Nawrath et al. 2002; Kuroda y Tsuchiya 2009). Además, se ha determinado que EDS5 se localiza en el cloroplasto (Ishihara et al. 2008). Todo ello, unido a que la mayor parte del SA se sintetiza en el cloroplasto, sugiere que EDS5 regula la acumulación de la hormona mediante el control del transporte de determinadas moléculas a través de la membrana del orgánulo. Esta molécula podría ser el propio SA, que ha de ser traslocado al citoplasma para realizar parte de sus funciones. Además, la salida del SA del cloroplasto ayudaría a prevenir una posible inhibición de su síntesis debida a una elevada acumulación en el cloroplasto (Dempsey et al. 2011).

Otro gen de Arabidopsis identificado mediante rastreos genéticos de mutantes que presentaban una menor resistencia a enfermedad es *PBS3* (Warren et al. 1999). Mutantes nulos en este gen (también llamado *WIN3*, *GDG1* o *GH3.12*) presentan una reducción en la acumulación de SA tras la infección, básicamente debida al descenso de los niveles de SAG, de la expresión de *PR-1* y de la resistencia a *P. syringae* (Jagadeeswaran et al. 2007; Lee et al. 2007; Nobuta et al. 2007). Sin embargo, en estas mismas condiciones inductivas, estos mutantes no presentaban alterada la expresión de *ICS1* y sí acumulaban una mayor cantidad de SA-Asp. *PBS3* codifica GH3.12, otro miembro de la familia de enzimas GH3, que no utiliza ni el SA ni el IAA como sustratos sino otros como el 4-HBA (*4-Hydroxibenzoate*). Estos y otros hallazgos sugieren que PBS3 potencia la acumulación de SA mediante la supresión de su catabolismo, posiblemente mediante la inhibición de GH3.5 y/o de otras GH3 aún no descritas que inactiven al SA conjugándolo con Asp (Okrent et al. 2009; Dempsey et al. 2011).

El mutante *eps1* (*enhanced Pseudomonas susceptibility 1*) de Arabidopsis también fue identificado debido a su mayor susceptibilidad a cepas virulentas y avirulentas de *P. syringae*. Tras la infección, *eps1* acumula una menor cantidad de SA y no es capaz de inducir la expresión de genes *PR*. Dado que tratamientos con SA restauran dicha inducción, EPS1 estaría implicada en la regulación de la biosíntesis de dicha hormona. La expresión de *EPS1* aumenta tras la infección con *P. syringae* y esta inducción es dependiente de la ruta de señalización del JA porque está bloqueada en *coi1*. De hecho, la expresión de *EPS1* es inducida por tratamientos con metil jasmonato (MeJA, de *Methyl Jasmonate*), pero no con SA. Estos y otros resultados obtenidos con mutantes *eps1* indican que este gen podría actuar en la regulación cruzada entre las vías del SA y del JA, con ciertas diferencias en función del ecotipo. *EPS1* codifica una posible aciltransferasa tipo BAHD, aunque no ha sido aún confirmada su actividad transferasa (Zheng et al. 2009).

Se han descrito varios TFs que pueden influir en la expresión de *ICS1* y por tanto en la acumulación de SA. Los TFs CBP60g (CALMODULIN-BINDING PROTEIN 60-LIKEg) y SARD1 (SAR-DEFICIENT 1) funcionan como activadores de la expresión de *ICS1*. El doble mutante *cbp60g sard1* tiene comprometidas la PTI, la ETI y la SAR; todo ello relacionado con la poca o nula acumulación de SA tras la infección. Ambos TFs pueden unirse al promotor de *ICS1* y presentan una redundancia funcional parcial, puesto que los mutantes simples no tienen fenotipos tan drásticos. Entre sus diferencias destaca la mayor implicación de CBP60g en la PTI relacionada con la señalización temprana vía Ca^{2+} (Wang et al. 2009; Zhang et al. 2010a; Wang et al. 2011). Otro TF que actúa como regulador positivo de la expresión de *ICS1* es WRKY28. Recientemente se ha demostrado que WRK28 puede unirse a dos motivos del promotor de *ICS1* y favorecer su expresión (van Verk et al. 2011).

Los TFs EIN3 (ETHYLENE INSENSITIVE 3) y EIL1 (EIN3-LIKE 1) son dos reguladores positivos de las respuestas dependientes de ET. Además, actúan reprimiendo la síntesis de SA y por ende las respuestas defensivas asociadas a la misma. El doble mutante *ein3*-1 *eil1*-1 acumula niveles elevados de SA, expresa constitutivamente *ICS1*, *PR-1* y *PR-2,* y tiene una mayor resistencia a *P. syringae*. Por contra, la sobreexpresión de *EIN3* muestra una marcada susceptibilidad. Estos resultados sugieren que ambos TFs pueden unirse al

promotor de *ICS1* y reprimir su expresión. Esta unión directa se ha demostrado para EIN3 (Chen et al. 2009).

Percepción del SA: dependiente e independiente de *NPR1*

La parte de la vía de señalización del SA que transcurre tras la acumulación de la hormona está regulada principalmente por *NPR1* (*NON-EXPRESSER OF PATHOGENESIS-RELATED GENES 1*), también conocido como *NIM1* o *SAI1*. Por tanto, NPR1 es un regulador clave de múltiples respuestas defensivas, incluyendo SAR (Dong 2004; Pieterse y van Loon 2004). Su importancia en la percepción del SA se demuestra en que ha sido encontrado en al menos cuatro rastreos genéticos diferentes (Cao et al. 1994; Delaney et al. 1995; Glazebrook et al. 1996; Shah et al. 1997). De hecho, hasta la fecha, es el único gen descrito que si es mutado convierte a la planta en insensible a la hormona. Así pues, fenotipos como la no inducción de determinados genes *PR* tras una infección patogénica o la incapacidad de disparar una SAR efectiva, presentes en el mutante *npr1*, no pueden ser superados con tratamientos con SA, BTH o INA.

El mecanismo por el que se piensa que la planta percibe el SA está relacionado con el estado redox celular. El modelo propuesto en la literatura indica que cuando la planta no se enfrenta a un estrés biótico, NPR1 se encuentra principalmente en el citosol en su forma inactiva, formando oligómeros asociados mediante puentes disulfuro. La acumulación de SA que tiene lugar durante la respuesta defensiva genera un cambio en el estado redox de la célula. Este cambio provoca la reducción de dos residuos de cisteína (Cys82 y Cys216) por parte de las enzimas TRX-H5 (THIOREDOXIN-H5) y/o TRX-H3. De esta forma, los oligómeros inactivos de NPR1 se convierten en monómeros que han de atravesar la membrana nuclear para ser activos. Una vez en el núcleo, estos monómeros interaccionan con varios TFs tipo TGA, que a su vez son los responsables de la inducción de genes de defensa mediados por SA. Mutaciones en Cys82 o Cys216 en ausencia de patógeno, elevan el nivel de NPR1 monomérico, de localización nuclear, así como la expresión de los genes mediados por NPR1. Sin embargo, estos mutantes no son capaces de inducir una SAR dependiente de SA plena debido a la rápida degradación de NPR1. Esto sugiere que la reoligomerización de la proteína es necesaria para la existencia de una respuesta defensiva mediada por SA completa. Este proceso

tiene lugar con la S-nitrosilación de la Cys156, una prueba más de la estrecha relación entre el SA y el estado redox de la célula (Mou et al. 2003; Tada et al. 2008). NPR1 contiene un dominio BTB/POZ (*Broad-Complex, Tramtrack, and Bric-à-brac/Pox virus, Zinc finger*) y un motivo de repeticiones Ankyrin, que ha sido relacionado con procesos de interacciones entre proteínas (Cao et al. 1997). Además, proteínas con el dominio BTB pueden interaccionar con las proteínas CULLIN, como CUL3, dando lugar a complejos con actividad E3-ubiquitin ligasa que actúan degradando determinados sustratos (Pintard et al. 2004). Las propias proteínas con el BTB pueden ejercer de sustrato. En el caso de NPR1, se ha confirmado que es degradado, preferentemente en su forma nuclear y monomérica, vía proteasoma. Esta degradación ocurre en ausencia de patógeno para así amortiguar la expresión basal de los genes de defensa. Sorprendentemente, también ocurre tras la inducción defensiva dependiente del SA. Tras la entrada de los monómeros de NPR1 al núcleo, dos residuos de serina (Ser11 y Ser15) son fosforilados. Esta modificación facilita su interacción con CUL3 y por ende su degradación (Spoel et al. 2009). De hecho, al bloquear la degradación de NPR1 mediante mutantes defectivos en la actividad CUL3 se compromete la correcta inducción de la SAR. Por tanto, la degradación y reciclaje de NPR1 cumple una doble función en la regulación de la expresión de los genes diana de la respuesta defensiva mediada por SA: por un lado, bloquea el inicio de su transcripción allí donde no es necesaria; por otra parte, permite una completa activación de su transcripción en las células atacadas (Spoel et al. 2009).

NPR1 no presenta un dominio de unión a DNA. Por tanto, la señalización mediada por NPR1 requiere de la participación de otras proteínas. NPR1 interacciona en planta con al menos siete TGAs (incluídas en la clase bZIP de TFs) de Arabidopsis (Kesarwani et al. 2007). Los TGAs pueden interaccionar directamente con la región promotora de *PR-1*, uniéndose a la conocida como secuencia de activación, y promoviendo su expresión (Lebel et al. 1998). Los análisis con mutantes han mostrado una redundancia funcional de los genes *TGA* en la vía de señalización del SA (Zhang et al. 2003). NPR1 también interacciona con otras 3 proteínas, NIMIN1, 2 y 3 (NIM1-INTERACTING 1), y los tres genes se inducen transitoriamente tras un tratamiento exógeno con SA. Sin embargo, NIMIN1 es un regulador negativo de la señalización vía SA/NPR1. De hecho, su sobreexpresión compromete respuestas defensivas como la ETI y la SAR. Además, también puede formar complejos con algunas

TGAs, NPR1 y el promotor *PR-1*, modulando así la activación transcripcional dependiente de los TGAs (Weigel et al. 2001; Weigel et al. 2005).

Además de los TGAs, otros TFs de tipo WRKY participan, en este punto de la vía de señalización del SA, de su regulación. La mayoría de estos genes *WRKY* son inducidos por la aplicación exógena de SA o la infección con un patógeno. Esta inducción es principalmente dependiente de NPR1. Algunos de estos WRKY actúan como reguladores positivos de la resistencia mediada por SA (p. ej. WRKY18, 53, 54 y 70). Otros, en cambio, actúan como represores de estas respuestas defensivas (p. ej. WRKY7, 11, 17 y 38). Además, muchos de estos WRKY presentan efectos opuestos en las vías de señalización del SA y del JA. Por tanto, constituyen puntos relevantes en el antagonismo cruzado existente entre ambas rutas (Vlot et al. 2009).

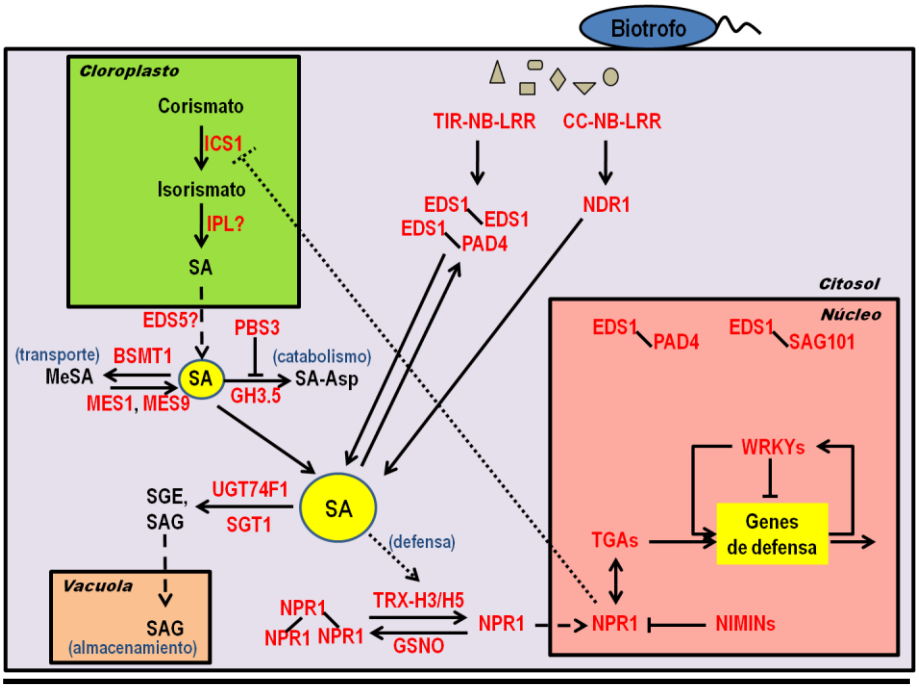

Figura 5. Esquema de la vía de señalización defensiva mediada por SA (adaptado de Vlot et al. 2009 y de Dempsey et al 2011). Modelo simplificado de las reacciones que tienen lugar en la célula vegetal tras la detección de un patógeno biotrofo y que conducen a la activación de los genes de defensa mediados por el SA. Se incluyen también los procesos de biosíntesis y modificaciones químicas de la hormona más relevantes.

NPR1 también regula negativamente la acumulación de SA. Esto sucede mediante la supresión de la expresión de *ICS1*, para lo que es necesaria la localización nuclear de NPR1. Así pues, en el mutante *npr1* existe una mayor expresión de *ICS1* y una mayor acumulación de SA tras la infección (Wildermuth et al. 2001; Zhang et al. 2010b). Si bien se desconoce el mecanismo por el cual dicha supresión tiene lugar, esta sucedería una vez las respuestas defensivas correspondientes fueran activadas (Dempsey et al. 2011).
Existe una ruta de señalización dependiente del SA pero independiente de *NPR1*. Dicha ruta ha sido menos estudiada e invita a pensar en la existencia de proteínas relevantes para la percepción de la hormona aún no descritas (Robert-Seilaniantz et al. 2011). La existencia de esta vía independiente de *NPR1* ha sido demostrada con el estudio de varios mutantes de Arabidopsis que pueden acumular SA e inducir la expresión de genes *PR* incluso en ausencia de un *NPR1* funcional. Dichos mutantes han sido identificados principalmente en rastreos genéticos cuyo objetivo era identificar supresores de mutantes *npr1* (An y Mou 2011). Es el caso del regulador negativo de SAR *SNI1* (*SUPPRESSOR OF npr1-1 INDUCIBLE 1*). El doble mutante *npr1*-1 *sni1* es capaz de restaurar la inducción de genes *PR* por el SA y la resistencia (Li et al. 1999). Otro ejemplo es el mutante *ssi2* (*suppressor of SA insensitivity 2*) que acumula constitutivamente SA y expresa *PR-1*. Las plantas *ssi2* presentan una mayor resistencia a patógenos biotrofos. De nuevo, el doble mutante *npr1*-5 *ssi2* es capaz de revertir los fenotipos de *npr1*-5 (Shah et al. 2001).

A pesar de los esfuerzos realizados, el SA es la única hormona de plantas para la que aún no se ha descrito un receptor (Nawrath et al. 2005). Esto puede deberse a que sea percibido por una familia de receptores redundante. También es posible que no exista un receptor como tal y que sea NPR1 quien perciba las modificaciones activadas por el SA (Robert-Seilaniantz et al. 2011). De hecho, recientemente se ha demostrado que tanto NPR1 como otras proteínas relacionadas con ella son sensibles al SA. Estas proteínas ven alteradas algunas de sus capacidades bioquímicas, tales como la interacción con otras proteínas, en presencia de SA (Maier et al. 2011).

Una vez finalizada la redacción de la presente Tesis (mayo 2012), ha sido publicado un artículo que presenta a los parálogos de *NPR1*, *NPR3* y *NPR4*, como los posibles receptores de la hormona. Estas proteínas, a diferencia de NPR1, sí se pueden unir al SA y actuarían como adaptadores de CUL3,

regulando su actividad de degradación de NPR1 en función del nivel de SA (Fu et al. 2012).

SA y biotecnología

La utilización del SA en investigación y/o biotecnología está limitada por su toxicidad para las plantas. En el caso de Arabidopsis, aquellos tratamientos exógenos que dan lugar a un efecto reproducible en la inducción de las defensas de la planta presentan unas concentraciones de la hormona (de 0,2 a 1 mM) muy cercanas a las que le resultan fitotóxicas (van Leeuwen et al. 2007). Con el objetivo final de una posible aplicación biotecnológica, se han desarrollado diferentes análogos químicos de la hormona en los que se ha intentado eliminar las desventajas del SA. Entre estos análogos destacan los ya mencionados INA (Ward et al. 1991) y BTH (Lawton et al. 1996). Durante el trabajo experimental de esta Tesis se ha utilizado ampliamente el BTH. Tratamientos con concentraciones similares a las utilizadas con el SA no son tóxicas y sí presentan un efecto positivo para la defensa. Además de su baja fitotoxicidad, el BTH presenta otras ventajas con respecto al SA tales como la mayor estabilidad, actividad y movilidad por la planta. El BTH ha sido incluso comercializado, si bien su uso como herramienta biotecnológica en la agricultura ha sido limitado. Esto se debe a una característica que está directamente relacionada con la inducción de defensas en las plantas: la pérdida de biomasa (Heil 2002). Si bien este hecho limita su uso en el campo, puede ser altamente interesante en el laboratorio. Así pues, en la presente Tesis se propone un modelo biológico para estudiar la percepción del SA en Arabidopsis basado en el fenotipo de pérdida de biomasa producido por el BTH (véase artículo 1). Este nuevo modelo es posteriormente llevado a la práctica mediante un rastreo genético (véase artículo 2). Dicho rastreo proporciona un conjunto de nuevas herramientas biológicas cuyo estudio permite generar nuevos conocimientos en este campo (véase artículos 2, 3 y 4).

3.3 Otras hormonas en la respuesta defensiva: su relación con el SA

Ácido jasmónico (JA)

Las rutas de señalización del JA y del SA son antagónicas, como ya se ha comentado previamente, aunque en casos puntuales se ha observado

sinergismo entre ambas vías. Este antagonismo se debería a que controlan la resistencia frente a patógenos con diferentes estrategias infectivas. De esta forma, la planta podría controlar los costes que supone la inducción de respuestas defensivas (Vlot et al. 2009). Si bien este antagonismo es bidireccional, el flujo principal sería el de la represión de la vía del JA. Se ha observado que son más los genes dependientes del JA reprimidos por la vía de señalización del SA que los dependientes del SA reprimidos por la del JA (Glazebrook et al. 2003). Además, experimentos posteriores con Arabidopsis en los que se emplearon patógenos biotrofos, necrotrofos e insectos indicaron que en la planta existe una priorización de la ruta del SA sobre la del JA (Koornneef et al. 2008).

En los apartados anteriores ya se han presentado argumentos en favor de la existencia del antagonismo SA-JA (COR, EPS1, WRKYs). Este efecto también puede observarse en mutantes que afectan a la acumulación del SA, como *eds4* y *pad4*. Estos mutantes presentan una mayor expresión de genes de respuesta a JA tras la aplicación de un inductor (Gupta et al. 2000). NPR1, clave para la percepción del SA, también participa de la inhibición de la vía del JA. Se ha propuesto que en dicho proceso no sería necesaria su localización nuclear, sino que la NPR1 citosólica modularía el antagonismo entre SA y JA (Spoel et al. 2003). Otro actor que participaría en el control de esta regulación cruzada es la glutaredoxina GRX480. Esta proteína fue identificada en un rastreo de interactores de los TGAs. La expresión de *GRX480* está inducida por SA de manera dependiente de *NPR1*. Su sobreexpresión no altera prácticamente la inducción de *PR-1* por SA. En cambio, reprime casi por completo la expresión de genes utilizados como marcadores de la vía del JA tras la aplicación de MeJA. Esta represión es independiente de *NPR1*, pero dependiente de los TGAs (Ndamukong et al. 2007).

Etileno (ET)

El ET es también antagonista de la vía de señalización del SA. Como se ha indicado anteriormente, EIN3 y EIL1, dos reguladores positivos de esta vía, pueden unirse al promotor del gen *ICS1*, reprimiendo la biosíntesis del SA. EIN2 es una proteína clave en la señalización por ET y de su actividad depende la acumulación de EIN3 y EIL1. Estos TFs controlan directamente la expresión de *FLS2*, que codifica una de las LRR-RLKs presentadas en el

apartado de la PTI. Por tanto, esta hormona tiene un papel directo en la regulación de uno de los receptores implicados en la defensa innata. Tanto el mutante *ein2*-1 como el doble *ein3*-1 *eil1*-1 tienen suprimidas las respuestas defensivas mediadas por FLS2. Sin embargo, debido al antagonismo SA-ET, estos mutantes presentan una mayor resistencia a *P. syringae* (Chen et al. 2009; Boutrot et al. 2010)

Ácido abscísico (ABA)

El ABA está implicado principalmente en la tolerancia de las plantas a estreses abióticos. No obstante, también está relacionado con las interacciones planta-patógeno. Se le considera como un regulador negativo de la resistencia a patógenos, aunque puntualmente también puede inducir resistencia (Ton et al. 2009). Con relación al SA, existe una interacción claramente antagonista entre ambas hormonas, que tiene lugar en diferentes puntos de sus rutas de señalización. Tratamientos con ABA pueden suprimir la inducción de SAR mediante la inhibición de la ruta de señalización del SA tanto antes como después de la acumulación de dicha hormona. Esta inducción de SAR también puede ser suprimida mediante la aplicación de estrés abiótico a la planta. Esto confirma que dicha supresión es dependiente del ABA. Por otra parte, la inducción efectiva de SAR afecta negativamente a la expresión de genes tanto relacionados con la biosíntesis como con las respuestas mediadas por ABA (Yasuda et al. 2008).

Citoquininas (CK)

Las CK constituyen un grupo de hormonas implicado en el control del crecimiento (división celular, movilización de nutrientes, longevidad de las hojas...). Tradicionalmente, no habían sido relacionadas con la regulación de las defensas de la planta. Sí se había destacado la existencia de patógenos que secretan análogos de las CK o activan su producción en la planta, desviando así nutrientes hacia los tejidos infectados (Choi et al. 2011). Es el caso de determinados hongos biotrofos que mediante esta estrategia retrasan la senescencia y aumentan el aporte de nutrientes en la región infectada (Walters y McRoberts 2006). Se había asumido que las CK actuarían suprimiendo mecanismos de la defensa basal de las plantas como consecuencia de su efecto en el crecimiento (Robert-Seilaniantz et al. 2007). Sin embargo, recientemente

se ha demostrado que las CK producidas por la planta inducen la resistencia de Arabidopsis a *Pto* (Choi et al. 2010). Estos autores observan una correlación entre el mayor nivel de CK y la mayor biosíntesis de SA y expresión de *PR-1*. También observan una correlación entre los bajos niveles de CK y la mayor susceptibilidad a *Pto*. Además, proponen un posible mecanismo por el que tendría lugar la regulación entre las dos vías. ARR2 (RESPONSE REGULATOR 2) es un TF activado por CK. ARR2 puede interaccionar directamente con TGA3, un TF implicado en la respuesta mediada por SA. La interacción entre ambos TFs sería esencial para la inducción de *PR-1* por CK (Choi et al. 2010).

Giberelinas (GAs)

Las GAs participan en la modulación del crecimiento de la planta. Estas hormonas provocan la degradación de las proteínas DELLA, que son reguladores negativos de la vía, permitiendo así la expresión de los genes de respuesta a GAs (Jiang y Fu 2007). Las proteínas DELLA promueven la resistencia a patógenos necrotrofos y la susceptibilidad a biotrofos mediante la modulación del balance entre las rutas de señalización defensivas dependientes del SA y del JA/ET. Esta conclusión se deduce de los resultados obtenidos con un cuádruple mutante que carece de cuatro de las cinco proteínas DELLA descritas en Arabidopsis. Dicho mutante es susceptible a patógenos necrotrofos pero más resistente a patógenos biotrofos como *Pto* (Navarro et al. 2008). Por tanto, las GAs promueven la resistencia a biotrofos y la susceptibilidad a necrotrofos, puesto que aumentan la acumulación de SA y ROS y disminuyen la señalización vía JA (Robert-Seilaniantz et al. 2011).

Auxinas

Existe una regulación cruzada entre la ruta de señalización del SA y la de las auxinas, principalmente de tipo antagonista. La activación de la vía dependiente de auxinas está correlacionada con una mayor susceptibilidad a patógenos biotrofos debida a la menor biosíntesis de SA. De hecho, tratamientos con auxinas pueden suprimir la expresión de *PR-1* (Park et al. 2007b; Robert-Seilaniantz et al. 2011). La regulación entre ambas hormonas puede tener lugar a través de la modificación de la estabilidad de determinados reguladores negativos de la respuesta mediada por una de estas hormonas. En concreto, se

ha visto que la aplicación exógena de SA estabiliza algunas proteínas AUX-IAA, que son reguladores negativos de los genes de respuesta a auxinas. Es el caso de AXR3 (AUXIN RESISTANT 3) y AXR2. Además, muchos de los genes inducibles por auxinas están reprimidos en hojas distales tras la inducción de SAR (Wang et al. 2007a). Otro punto de regulación entre ambas vías se situaría en la proteína GH3.5. Como ya se ha comentado, esta enzima es capaz de conjugar AA tanto con el IAA como con el SA, provocando la inactivación de dichas hormonas (Park et al. 2007b). Por otra parte, la aplicación exógena de auxinas puede aumentar la susceptibilidad al patógeno (Chen et al. 2007). Diferentes efectores introducidos por *Pto* en las células hospedadoras actúan induciendo la producción de auxinas y de ABA. Puesto que ambas hormonas son antagónicas al SA, dicha producción favorece su patogenicidad (Robert-Seilaniantz et al. 2011).

Brasinosteroides (BR)

Los BR son un grupo de hormonas asociado a la regulación de procesos relacionados con el desarrollo y el crecimiento de la planta. Sin embargo, también han sido implicados en la adaptación a estreses de tipo abiótico y biótico. De hecho, los tratamientos con BR aumentan la resistencia frente a varios patógenos biotrofos (Nakashita et al. 2003). Este efecto tendría lugar por la mayor acumulación de SA y expresión de *PR-1*. No obstante, la inducción de *PR-1* observada es independiente de NPR1. Por tanto, los BR podrían controlar directamente la expresión de *PR-1* (Divi et al. 2010). Otro argumento en favor del papel de estas hormonas en las defensas de la planta es el relacionado con BAK1. Como ya se ha comentado, BAK1 actúa como adaptador de diferentes señales defensivas mediadas por receptores tipo LRR-RLK, siendo relevante para la PTI. Además, también participa de la vía de señalización de BR dado que interacciona con BRI1, el receptor principal de estas hormonas. En ambos casos, BAK1 interviene como un correceptor necesario para la transducción de la señal (Robert-Seilaniantz et al. 2011).

4. El complejo *Mediator*

En la presente Tesis se mostrará un conjunto de fenotipos asociados a mutantes correspondientes a una de las subunidades del *Mediator* cuya función aún no había sido descrita (véase artículo 4). Estos resultados demuestran que la percepción del SA es otra de las funciones relacionadas con este complejo.

El *Mediator* es un complejo multiproteico formado por unas 20-30 subunidades, dependiendo de la especie. Está conservado en todos los eucariotas estudiados, desde levaduras a humanos. Su función es controlar el ensamblaje y la actividad de la maquinaria transcripcional, actuando como un puente de unión entre la misma y el enorme conjunto de TFs presentes en la célula (Kidd et al. 2009). Mediante trabajos *in vitro* con *Saccharomyces cerevisae* (levadura) se demostró que la RNA polimerasa II y otro grupo de proteínas denominadas GTFs (*General Transcription Factors*) son los componentes mínimos necesarios para la transcripción en eucariotas (Flanagan et al. 1990). Sin embargo, la imposibilidad de aumentar la eficiencia de este sistema de transcripción *in vitro* con la adición de activadores transcripcionales purificados sugirió la existencia de un "mediador" entre dichos activadores y la maquinaria transcripcional (Flanagan et al. 1991). Finalmente, el complejo *Mediator* de levadura fue aislado poco después, confirmándose tanto su papel de puente necesario para este proceso como su efecto positivo en la transcripción basal no regulada por activadores ni represores (Kim et al. 1994). Por tanto, se puede concluir que durante la transcripción en eucariotas intervienen al menos la RNA pol. II, los GTFs, un elevado número de activadores y represores y el complejo *Mediator* (Kidd et al. 2011).

El *Mediator* está organizado en cuatro módulos. Tres de ellos, denominados cabeza, intermedio y cola, forman la parte central del complejo. El cuarto es el módulo quinasa, que puede estar o no separado del conjunto. El módulo cola sería el responsable de la interacción con los TFs. En cambio, los módulos cabeza e intermedio se unirían al dominio C-terminal de la RNA pol. II (Malik y Roeder 2005). Respecto al módulo quinasa, solamente está asociado con el resto del *Mediator* cuando éste no se encuentra unido a la RNA pol. II (Samuelsen et al. 2003). Así pues, la función del complejo dependerá de su interacción con el módulo quinasa. En ausencia de dicho módulo, el *Mediator* provoca el reclutamiento de la RNA pol. II y activa la transcripción. En

cambio, su presencia bloquea el ensamblaje de esta polimerasa y reprime la transcripción (Taatjes 2010).

El complejo *Mediator* también ha sido identificado en plantas. Su purificación, a partir de Arabidopsis, no tuvo lugar hasta trece años después de la identificación en levaduras (Backstrom et al. 2007). Este hallazgo mostró la existencia de una baja similitud de secuencia entre las subunidades del *Mediator* de plantas respecto a las ya identificadas en levaduras y metazoos. Una posible explicación de esta baja similitud se debería al elevado número de TFs estimado en el genoma de Arabidopsis (aproximadamente 1700) de los que casi la mitad serían específicos de plantas (Riechmann y Ratcliffe 2000). De hecho, el módulo cola, que interaccionaría directamente con los TFs, es el menos conservado. En total se identificaron 21 subunidades conservadas respecto a los complejos descritos en otros eucariotas, seis subunidades específicas del *Mediator* de plantas y otros seis parálogos de alguna de estas proteínas. En cambio, no se detectaron homólogos a otras ocho subunidades conservadas en levaduras y/o metazoos. Tampoco se detectaron las subunidades presentes en el módulo quinasa, puesto que el complejo estaba unido a la RNA pol II, pero sí se han identificado los homólogos de Arabidopsis correspondientes a las mismas (Backstrom et al. 2007). La posterior utilización de pequeños motivos de secuencia conservados durante la evolución ha permitido una mejor comparación de los componentes descritos del *Mediator*. Con este nuevo análisis, solamente habría dos subunidades conservadas ausentes en Arabidopsis y el número de las específicas de plantas bajaría a cuatro (Bourbon 2008).

Además de su función conjunta como coactivadores transcripcionales, para algunas de las subunidades del *Mediator* se ha descrito un papel específico como reguladores en procesos fisiológicos concretos. Entre ellos, destacan la respuesta a estreses de tipo biótico y abiótico, el control del desarrollo, la floración y la fertilidad, y también funciones nucleares esenciales como la actividad helicasa del DNA o el procesamiento del RNA. De hecho, en una revisión reciente se indica que hasta quince subunidades del *Mediator* de plantas tendrían una función asignada o un fenotipo descrito para sus mutantes (Kidd et al. 2011). La mayoría de estas proteínas presentan una doble nomenclatura debido a que fueron funcionalmente caracterizadas antes de su identificación como miembros de este complejo. Es el caso de uno de los genes del *Mediator*

implicados en la respuesta a estrés biótico: *MED25* (*MEDIATOR 25*)/*PFT1* (*PHYTOCHROME AND FLOWERING TIME 1*). Este gen fue identificado como un regulador del proceso adaptativo que induce a las plantas a evitar la sombra. Esto se lograría promoviendo la floración en función de los cambios en la calidad de la luz (Cerdan y Chory 2003). Posteriormente, se comprobó que el mutante *pft1* presentaba una mayor susceptibilidad a hongos necrotrofos acompañada de una reducción de la expresión de genes de defensa inducidos en respuesta al JA (Kidd et al. 2009). Recientemente, este gen también ha sido relacionado con la tolerancia a estreses de tipo abiótico (Elfving et al. 2011). Por tanto, MED25 es una subunidad muy versátil que puede regular la floración, la señalización hormonal y las respuestas a estreses abióticos y bióticos (Figura 6).

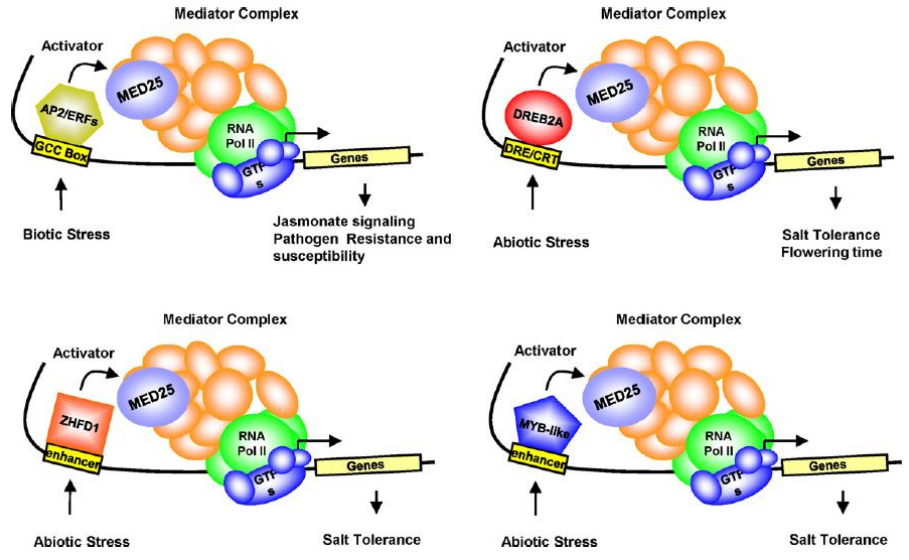

Figura 6. MED25: reguladora de múltiples procesos en Arabidopsis (tomado de Kidd et al. 2011). La interacción de MED25 con diferentes activadores transcripcionales permite a la planta regular diferentes procesos. Es el caso de la tolerancia a salinidad, la floración, la resistencia o susceptibilidad a patógenos y la señalización de la vía del JA.

Cabe mencionar que entre las subunidades del *Mediator* de plantas, sí existe una elevada conservación de la secuencia. Además, en determinados casos se ha podido confirmar una conservación de la función (Kidd et al. 2011). El ortólogo de *MED25/PFT1* en *Triticum aestivum* (trigo), *TaPFT1*, puede complementar los fenotipos de patogenicidad y floración del mutante *pft1* de Arabidopsis (Kidd et al. 2009). El ortólogo de *MED16/SFR6* (*STRUBBELIG-RECEPTOR FAMILY 6*) en arroz, *OsSFR6*, puede restaurar los fenotipos de falta de tolerancia a estrés osmótico y al frío presentes en el mutante *sfr6* de Arabidopsis (Wathugala et al. 2011).

objetivos

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Objetivos

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Este trabajo parte de la hipótesis de que existen otros componentes genéticos en la percepción del SA que aún no han sido descritos. Para poder identificarlos, obtener nuevas herramientas biológicas y ampliar el conocimiento científico en este campo se presentan los siguientes objetivos:

- Plantear un nuevo modelo para el estudio de la percepción del SA que no esté basado en la inoculación de un patógeno y el posterior análisis de su crecimiento. Validar dicho modelo en *Arabidopsis thaliana* mediante la Variación Natural (colecciones nucleares de ecotipos) y la Variación Artificial (colección de mutantes publicados).

- Aplicar este modelo en un rastreo genético que responda a la pregunta de si el gen *NPR1* es el único necesario para la percepción del SA. Estudiar las señales que empiezan tras la aplicación y/o inducción del SA y que desencadenan la activación de las defensas de la planta.

- Analizar el papel de *NPR1* en la resistencia inducida por metil jasmonato. Estudiar la participación de sus parálogos en esta ruta de señalización.

- Caracterizar fenotípicamente a *nrb4*. Localizar la mutación responsable de dichos fenotipos. Estudiar la función de *NRB4* en la percepción del SA y la relación entre esta ruta y el complejo *Mediator*.

Artículos

Artículo 1

Resistance and biomass in Arabidopsis: a new model for Salicylic acid perception

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Este artículo ha sido publicado en la revista:
Plant Biotechnology Journal (2010) 8, 126-141

Summary

Salicylic acid (SA) is an essential hormone for plant defence and development. SA perception is usually measured by counting the number of pathogens that grow *in planta* upon an exogenous application of the hormone. A biological SA perception model based on plant fresh weight reduction caused by disease resistance in *Arabidopsis thaliana* is proposed. This effect is more noticeable when a chemical analogue of SA is used, like Benzothiadiazole (BTH). By spraying BTH several times, a substantial difference in plant biomass is observed when compared to the mock treatment. Such difference is dose-dependent and does not require pathogen inoculation. The model is robust and allows for the comparison of different Arabidopsis ecotypes, recombinant inbreed lines, and mutants. Our results show that two mutants, *non-expresser of pathogenesis-related genes 1* (*npr1*) and *auxin resistant 3* (*axr3*), fail to lose biomass when BTH is applied to them. Further experiments show that *axr3* responds to SA and BTH in terms of defence induction. *NPR1*-related genotypes also confirm the pivotal role of NPR1 in SA perception, and suggest an active program of depletion of resources in the infected tissues.

Keywords: salicylic acid, BTH, NPR1, AXR3, Arabidopsis.

Introduction

Plants have a sophisticated defence system that is triggered or not depending on the nature of the pathogen. Some plant defences are specialised in necrotrophic pathogens (van Kan 2006) while others are effective against biotrophic pathogens (Bent and Mackey 2007). Salicylic acid (SA) is a key molecule in the triggering of plant defences against biotrophs. SA is also relevant for some developmental events (e.g. Vanacker et al. 2001 and Martinez et al. 2004). Despite the importance of this hormone in defence, little is still known about it. In Arabidopsis (*Arabidopsis thaliana*) there have been developed transgenic lines that degrade SA (*NahG*, Friedrich et al. 1995) and also others that produce more SA (*c-SAS* and *p-SAS*, Mauch et al. 2001). Moreover, there are two mutants impaired in SA biosynthesis: *eds5/sid1* (Nawrath et al. 2002) and *sid2/ics1* (Wildermuth et al. 2001). Additionally, there

are other mutants with less SA, e.g. *eds1* and *pad4* (Wiermer et al. 2005). SA biosynthesis is under a positive feedback loop; SA triggers the expression of *EDS1* and *PAD4*, and these genes are required for the expression of the SA biosynthetic genes. The metabolism of SA is also under control (Shah 2003). Most of the SA present in the plant is conjugated with glucose, forming a pool of temporary inactive SA that can be slowly released in an active form (Nawrath et al. 2005).

The scientific community has made an important effort to find the SA receptor. *NPR1* is the only known gene that, when mutated, renders plants insensitive to SA, and yet it is not clear if it is the receptor itself. It has been found in at least four different screenings (Cao et al. 1994; Delaney et al. 1995; Glazebrook et al. 1996; Shah et al. 1997), which indicates the essential role it plays in SA perception. NPR1 has been shown to accumulate in the cytosol and migrate to the nucleus upon SA perception. In the proposed model, SA triggers the expression of a thioredoxin that acts over NPR1 oligomers, rendering monomers that migrate to the nucleus (Tada et al. 2008). NPR1 is degraded by the proteosome in the nucleus, a process that is required for the activation of defence when SA is present (Spoel et al. 2009).

In parallel with the search for mutants, other biochemical approaches aimed at searching for proteins with SA binding have been adopted. Although some of the candidates have a strong affinity (Kumar and Klessig 2003), it is likely that none of them is a conventional SA receptor, if such a thing exists.

An intriguing feature of plant defence is the resulting loss of fitness (Heil 2002). It may seem intuitive that, upon a pathogen insult, the plant produces toxic compounds that negatively affect the plant, but other mechanisms can be proposed. For example, the plant may prioritise defence vs. development, redirecting all available resources to stop invasion. A third option is a "scorched earth defence", i.e. the tissue where the pathogen is perceived is deprived of the elements that the microorganism requires (including oxygen, water, solutes, etc). SA negatively regulates the effect of auxins, and this hormone is a good candidate to be the vehicle used to reduce plant development when a strong defence is triggered (Wang et al. 2007a).

In order to find the genes required for SA perception and its consequences, we have had to screen and accurately measure different Arabidopsis genotypes. The exponential growth of the pathogens used (Katagiri et al. 2002) has proved to be a handicap. The relationship between plant defence and development is also affected by the presence of the pathogen in the system. Therefore, we have developed a biological model for the perception of SA in Arabidopsis not based on pathogen inoculation but on the application of benzothiadiazole (BTH). BTH is a biotechnological development of the research in plant defence (Lawton et al. 1996), a chemical analogous to SA that triggers plant defence and biomass loss in a consistent and dose-dependent manner. With this system, we can analyse the natural and artificial variation of Arabidopsis in response to SA. Small differences were found in both cases. Arabidopsis ecotypes have shown no extreme behaviour, and only two mutants have been selected, *axr3-1* (A semidominant allele, Ouellet et al. 2001) and *npr1*. Complementary experiments have proved that *axr3* can perceive SA, confirming the unique role of *NPR1* and related genes. We also have found that the presence of *sni1* in the plant (Li et al. 1999) implies that *NPR1* is relevant for a programmed down-regulation of plant metabolism which could affect the pathogen.

Results

An experimental model for SA perception

We are interested in unveiling the signal transduction that starts with salicylic acid (SA) application to Arabidopsis and results in the triggering of the plant defences. The amounts of SA that trigger plant resistance are close to phytotoxicity, and this is why BTH (Lawton et al. 1996) is commonly used in research. BTH is a chemical analogous to SA with no phytotoxicity. It is commercialised under different names (Actigard® and BION® among others, www.syngenta.com). The standard way of measuring SA perception is by means of a western blot of a defence marker (e.g.. PR2, Cao et al. 1997), or by monitoring the growth of a inoculated pathogen (e.g. *Pseudomonas syringae pv tomato* DC3000 (*Pto*), Katagiri et al. 2002). Figure 1a shows the result of *Pto* inoculation. *Pto* is able to grow several orders of magnitude more in mock-treated plants than in BTH 350 μM treated plants. Although the procedure of inoculating and measuring *Pto* is straightforward (Tornero and Dangl 2001), it is

subject to important variations; small changes in the initial conditions can lead to a lack of reproducibility. Besides, factors that affect pathogen growth also affect *Pto* measurement. During the experiments, we noticed that BTH treated plants have less biomass than the mock-treated ones (Figure 1b). This fact has been described in (Heil 2002) and it is indicated in the label of the commercial product (www.syngenta.com). We repeated the experiments without pathogen inoculation and obtained the same results (data not shown); a single 350 μM application of BTH can reduce Arabidopsis biomass. Note that this effect is not exclusive of BTH. It has been described the relationship between the cost of fitness and resistance in absence of pathogen; for instance in the R-gene-mediated resistance (Tian et al. 2003) or constitutive defence mutant (Heidel et al. 2004). Conversely, plants deficient in SA level increase yield production (Abreu and Munne-Bosch 2009), providing that no pathogen is present.

As a single treatment lacked reproducibility and there was statistical overlapping between mock vs. BTH treated plants, we tried different applications and treatments. Briefly, we applied BTH by imbibing, drenching, spraying, and in vitro culture (data not shown). The optimal method consists in spraying BTH four times for two weeks (see Experimental procedures) and recording plant weight when the plants are three weeks old. No special limit was observed after four treatments; up to eight treatments were applied during four weeks with no evident toxicity (data not shown). Increasing the number of treatments improves the difference between mock vs. BTH treated plants when the plants have enough room to grow. When the treatment finished, Col-0 plants outgrew the treatment and were able to set seeds.

The results are more clearly shown when the ratio between BTH and mock treated plants is used (Figure 1c). Different BTH concentrations were used to find the optimal option, starting with 350 μM (used in Figure 1a) and diluting by a factor of 10. BTH concentrations higher than 3.5 μM still produced a measurable effect on Arabidopsis biomass, whereas lower BTH concentrations failed to differentiate mock and BTH treated plants. Therefore, 350 μM is the standard BTH concentration or "High BTH", and 350 nM the subclinical BTH concentration or "Low BTH".

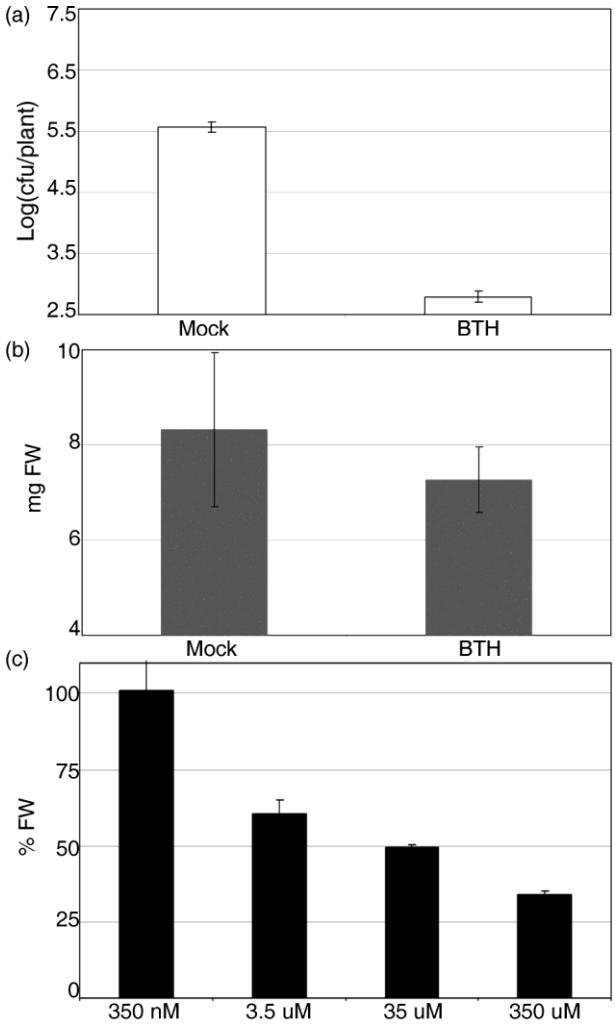

Figure 1. BTH increases disease resistance and decreases biomass accumulation. (a), Arabidopsis plants were pretreated with either mock or benzothiadiazole (BTH) 350 µM and then inoculated with *Pseudomonas syringae pv tomato* isolate DC3000 (*Pto*) one day later. Three days later, the bacteria (measured as Logarithm of colony forming units per plant) were measured. (b), Plant weight of (a) before bacterial extraction, in mg of fresh weight. (c), The same effect after considerable optimisation that includes four treatments (see Experimental procedures). All panels show the average and standard deviations, and at least three independent experiments were performed with similar results.

Phenotypes of the model

The differences in plant biomass caused by High BTH treatments are numerically significant, corresponding to the strong phenotype of Figure 2a. Figure 2a shows Col-0 treated with mock (left) and High BTH (right) the difference in plant size is worth noting, while the number of leaves is similar. Therefore, a visual inspection can discern in most cases whether a genotype responds or not to BTH. This is a simple way to evaluate SA perception and characterise the response to BTH in the Col-0 ecotype. There were no observable macroscopic plant lesions, so we looked for microscopic lesions. Trypan blue staining pinpoints cell death and membrane damage (and fungi if present, Keogh et al. 1980). Figure 2b, c and d show the Trypan Blue staining of cotyledons from plants treated with mock, Low BTH, and High BTH treatments respectively. While subclinical BTH concentrations produced no measurable effects, standard amounts of BTH triggered Program Cell Death in a small number of cells. Callose depositions are a hallmark of defence induction, and are easily seen with aniline blue under ultraviolet light (Conrath et al. 1989). Therefore, cotyledons of mock, Low BTH, and High BTH treated plants were stained with aniline blue (Figure 2e, f, and g, respectively. Figure 2h, i and j show the same cotyledons exposed to visible light). The result is that mock and subclinical BTH concentrations do not produce callose deposition. Standard BTH concentrations, on the other hand, produce abundant callose depositions, of several sizes and distributions. A 3.3' diaminobenzidine stain showed no difference in Reactive Oxygen Species (ROS) at the time of the sampling (data not shown).

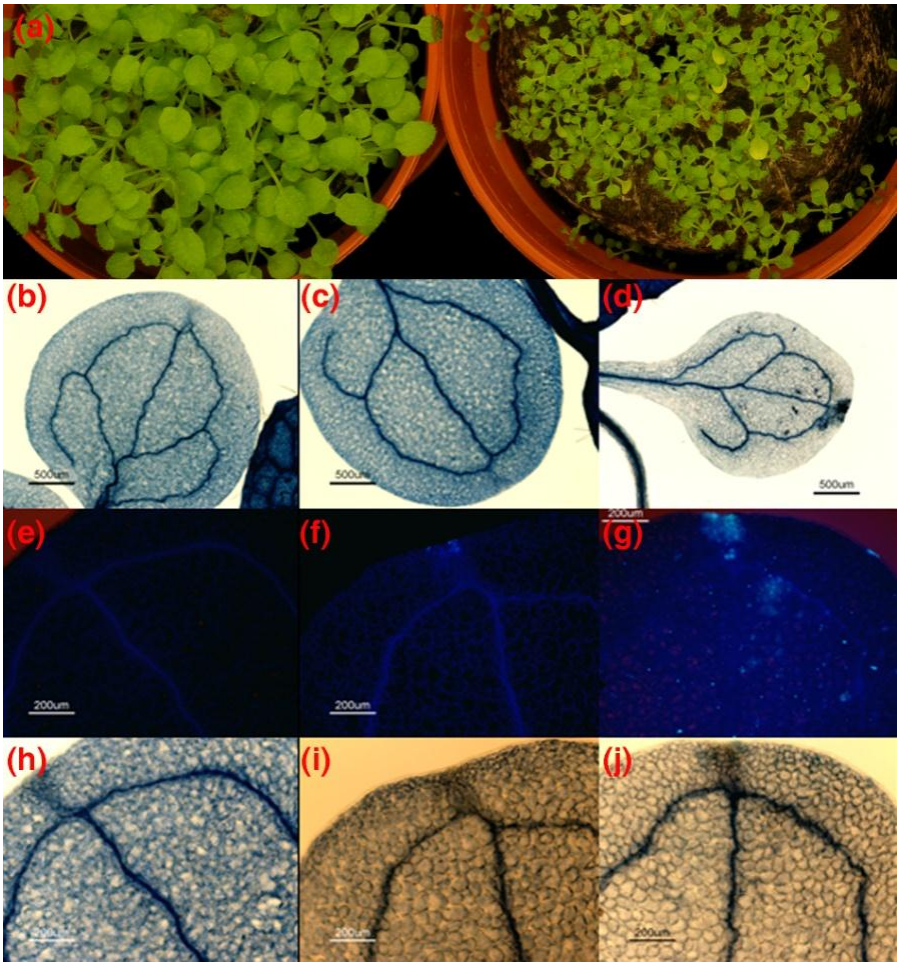

Figure 2. The continuous triggering of plant resistance produces a distinctive macroscopic and microscopic phenotype. (a), Macroscopic phenotype of plants either treated with mock (left) or with BTH 350 µM (right) at the same time as Figure 1c. BTH-treated plants have the same number of leaves as mock-treated plants, and are able to survive and set seeds. (b), (c) and (d) correspond to Trypan blue stains, unveiling cell death and membrane damage. (e), (f) and (g) show Aniline blue stains under ultraviolet light, which detects callose depositions. (h), (i), and (j) are the same micrographs under visible light. (b), (e) and (h) are from representative plants treated with mock, (c), (f) and (i) are from BTH 350 nM treated plants, and (d), (g), and (j) are from BTH 350 µM treated plants. Only BTH 350 µM produces microscopic cell death in few and isolated cells (dark blue staining outside the veins in (d)), and triggers plant defence, as observed in the callose depositions (fluorescent in (g)). Three independent experiments were performed with similar results.

Salicylic acid is a hormone with a fine-regulated homeostasis. Thus, there is evidence of a positive feedback loop in SA synthesis and of negative regulation upon SA perception (Shah 2003). There are two possible explanations for BTH effects on biomass: BTH could trigger the SA positive feedback loop increasing thus SA concentration, or it could be the responsible of the effect by itself. To check these hypotheses, the amounts of SA (free and total) in mock, Low BTH, and High BTH treated plants were measured three days after the last treatment (Figure 3a, the order of magnitude of all these values are in agreement with reported concentrations (Defraia et al. 2008)). Low BTH treatment does not change dramatically the amount of total and free SA, while High BTH treatment produces a decrease in free SA and a strong decrease in the total amount of SA. The subclinical amounts of BTH do not induce the expression of the marker PR1 (Figure 3b), a standard stress marker (Uknes et al. 1992), nor enough resistance to be detected in *Pto* growth curves (data not shown). Standard BTH concentrations induced a strong PR1 expression, even if the western blot was repeated with only mock and subclinical BTH treatments to avoid a possible signal masking due to the strong High BTH signal (data not shown).

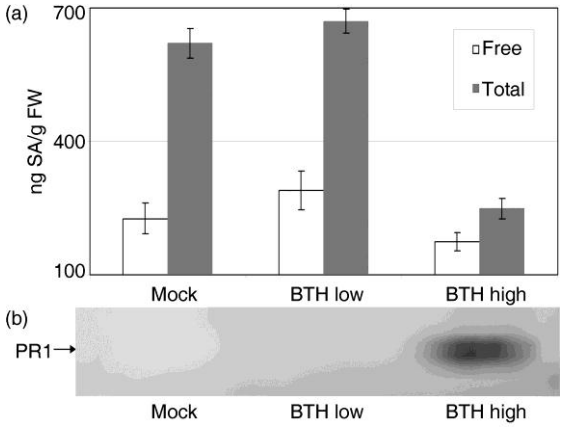

Figure 3. SA accumulation and defence induction upon BTH application. (a) Quantification of SA upon mock, BTH 350 nM, and BTH 350 µM treatments as described in Figure 1c. Both free and total SA (i.e. glucosylated derivates released after hydrolysis plus the free SA) were measured, showing the average and standard deviations of three samples. (b) Western blot for PR1. This defence marker was immunodetected in samples from the same experiments as in (a). The arrow points to the expected size of PR1 (14 kDa). Three independent experiments were performed with similar results.

Natural variation and SA perception

Once the right conditions were set, we intended to assess whether Col-0 was the best ecotype to work with. Figure 4 shows the analysis of two sets of ecotypes and Col-0. Figure 4a corresponds to a nuclear core collection of 48 ecotypes (McKhann et al. 2004), while Figures 4b and c show a set of 96 ecotypes (Nordborg et al. 2005). Col-0 is a valid representative of the ecotypes tested; in the three panels it ranked in the middle of the ecotypes (between 40th and 56th percentiles) when ordered by percentage of plant fresh weight (PFW). Some ecotypes like Col-0, Ws-0, L*a*e*r*-0 and No-0 were repeated with different stocks (e.g., Col-3, Col-4, Col-5, etc), because they are the background of mutations or are used for mapping. None of them behaved in a different way (data not shown).

Another option in Natural Variation is to search for quantitative trait loci (QTLs) in mapping populations. This can be done even if the parentals have a similar behaviour, a phenomenon called transgression (Koornneef et al. 2006). We analysed seven recombinant inbreed lines (RILs) available at the beginning of this research searching for transgression (see Experimental procedures). The RILs were: Col-0 x Nd-1 (Deslandes et al. 1998), Col-gl1 x Kas-1 (Wilson et al. 2001), Cvi-1 x L*a*e*r*-2 (Alonso-Blanco et al. 1998a), L*a*e*r*-0 x Sha-0 (Clerkx et al. 2004), Bay-0 x Sha-0 (Loudet et al. 2002), Col-4 x L*a*e*r*-0 (Lister and Dean 1993), and L*a*e*r*-0 x No-0 (Magliano et al. 2005) (Figure 5 and data not shown). There are three QTLs detected only in the mock-treated plant's fresh weight (Colgl1 x Kas-1, L*a*e*r*-0 x Sha-0 and L*a*e*r*-0 x No-0, Figure 5b, d and e, respectively). There is, however, no significant QTL specific of the response to BTH in terms of fresh weight.

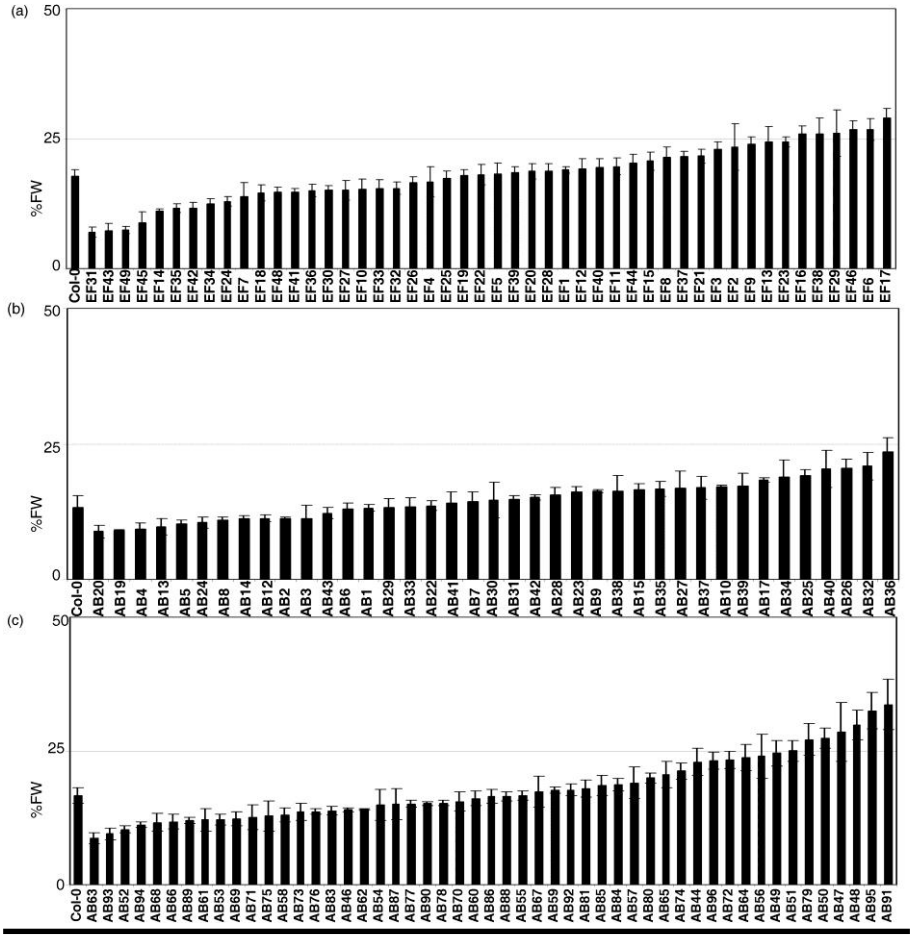

Figure 4. Arabidopsis ecotypes tested show a similar phenotype. Two collections of ecotypes were tested as described in Figure 1c. (a) Col-0 and the McKhann collection (McKhann et al. 2004) ranked for its percentage of fresh weight. (b) and (c) Col-0 and the Nordborg collection (Nordborg et al. 2005) were measured in two separate lots. The full names of the ecotypes shown, as well as other ecotypes tested, are listed in Table S1. None of the ecotypes tested shows an extreme behaviour under these conditions, and Col-0 ranked between 40th and 56th percentile in the three panels. Two independent experiments were performed with similar results.

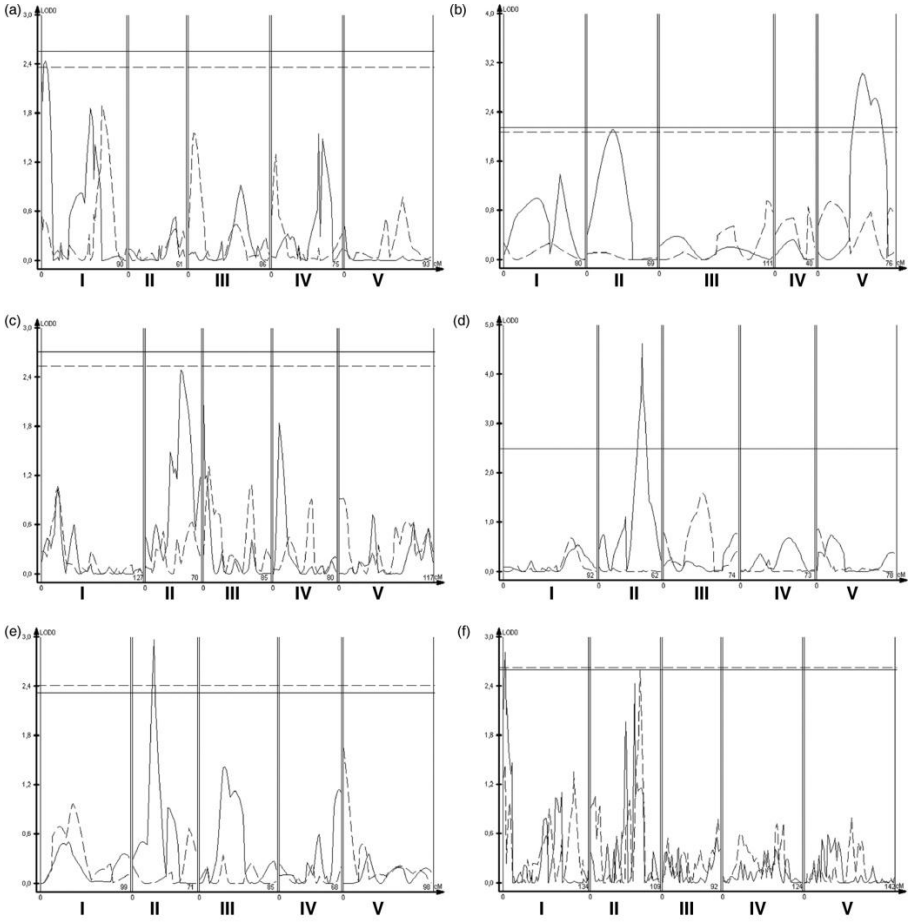

Figure 5. There are no significant QTLs in the tested populations specific to SA perception. Plants were treated with either mock or BTH 350 µM as described in Figure 1c. The output showed is the likelihood of a QTL (in logarithm of odds; LOD, in the Y axis) in a particular region of the genome (X axis). The horizontal line shows the threshold of significance. The continuous lines show the QTLs for mock, and the dotted line the QTLs for BTH treatment. The populations analysed were (a) Col-0 x Nd-1, (b) Col-gl1 x Kas-1, (c) Cvi-1 x La*er*-2, (d) La*er*-0 x Sha-0, (e) La*er*-0 x No-0, (f) Col-4 x La*er*-0.

Most signal transductions do not affect SA perception

The next step was to analyse the wealth of information generated in the form of mutants. SA biosynthesis is regulated by a positive feedback loop, so the mutations related to SA were the first objective.. Thus, we assayed the mutant that failed to perceive SA; *npr1*, mutants of SA biosynthesis; *eds5* and *sid2*, transgenic lines with altered SA content (*NahG* less SA, and *c-SAS* more SA); and mutants with a down regulation of SA biosynthesis, *eds1* and *pad4* (Figure 6a). Only *npr1* failed to respond to SA. This clear result prompted us to keep *npr1* as a negative control, and to extend the list of mutants in defence (Figure 6a and b). Then, we tested mutants in basal resistance (either more resistant or more susceptible), Systemic Acquired Resistance (SAR), specific resistance, and non-host resistance (see Table S1). None of the tested mutants in defence, except *npr1*, differed from the wild type (wt) in their response to BTH.

SA signal transduction has been reported to crosstalk with several signal transductions (Lopez et al. 2008), being Jasmonic Acid, Ethylene, Abscisic Acid, Auxins, Light and ROS the most commonly cited. Therefore, the response to BTH of a representative set of mutants in each of these pathways was measured. For the Auxin pathway, nineteen mutants were tested (Figure 6c), and only *axr3* did not respond to BTH in a consistent manner. Note that the allele used in this work is *axr3-1*, a semidominant mutation that enhances the stability of the protein (Ouellet et al. 2001). Mutants in other pathways, like Light (Figure 6c), Abscisic Acid (Figure 6d), Ethylene (Figure 6d), ROS (Figure 6d) or Jasmonic Acid and/or response to necrotrophs (Figure 6e), had a response to BTH similar to that of wt. A complete list of the mutants tested is provided in Table S1.

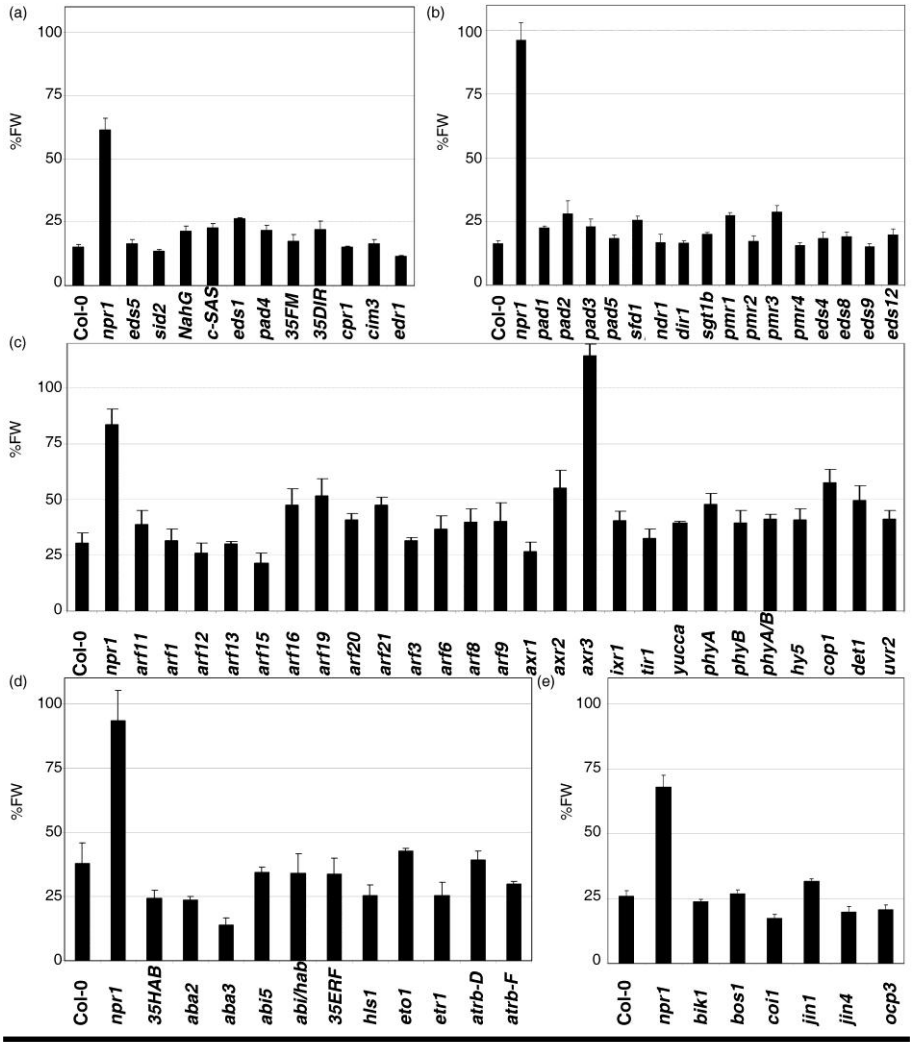

Figure 6. Analysis of a collection of mutants points out only to two candidates for SA perception. All the mutants were tested as described in Figure 1c. The complete list is shown in Table S1. (a) and (b) show defence mutants, (c) corresponds to mutations in Auxin and Light signalling, (d) mutations in Abscisic Acid, Ethylene and Reactive Oxygen Species, and (e) mutations in Jasmonic Acid and/or response to necrotrophs. *35FM* stands for *35S:FMO1, 35DIR* is *35S:DIR1, phyA/B* is the double *phyA phyB, 35HAB* is *35S:HAB1, abi/hab* is *abi1-2 hab1-1, 35ERF* is *35S:ERF1, atrb-D* is *atrboh-D,* and *atrb-F* is *atrboh-F*. Three independent experiments were performed with similar results.

axr3 and *npr1* show a distinct response to SA

The conclusion of Figure 6 and other data not presented is that from a total of 98 mutants tested, only two did not respond to BTH; *npr1* and *axr3*. *NPR1* is a gene clearly involved in SA perception, but the result of *axr3* was unexpected. While it was tempting to discard *axr3* due to the small size of this mutant, other small mutants like *cpr1* (Bowling et al. 1994), showed percentages of fresh weight in the same order of magnitude as the wt (Figure 6a). Therefore, a detailed characterisation of *axr3* in terms of response to SA and BTH was performed. Figure 7a shows *Pto* growth in Col-0, *npr1* and *axr3* pretreated with mock or High BTH. BTH is clearly able to trigger defence in *axr3*, as opposed to the effect caused in *npr1*. The levels of the PR1 protein were determined by western blot (Figure 7b) in plants either treated with mock or BTH 350 μM and proved to be basically the same. While *npr1* fails to induce this defence marker upon High BTH, *axr3* is able to increase the expression of this defence protein. Note that in *axr3* plants there is a small but detectable amount of PR1 even in the mock treated ones.

An interesting feature of plants mutated in *npr1* is that they fail to regulate the levels of SA (Cao et al. 1997). When growing *npr1* in MS plates supplemented with SA 500 μM, the cotyledons are bleached and the plant is unable to grow (Figure 7c). The easiest interpretation is that *npr1* fails to perceive SA, and therefore it is unable to trigger SA degradation and SA accumulation has deleterious effects. Col-0 and *axr3* plants, on the other hand, grow in plates containing SA 500 μM (Figure 7c).

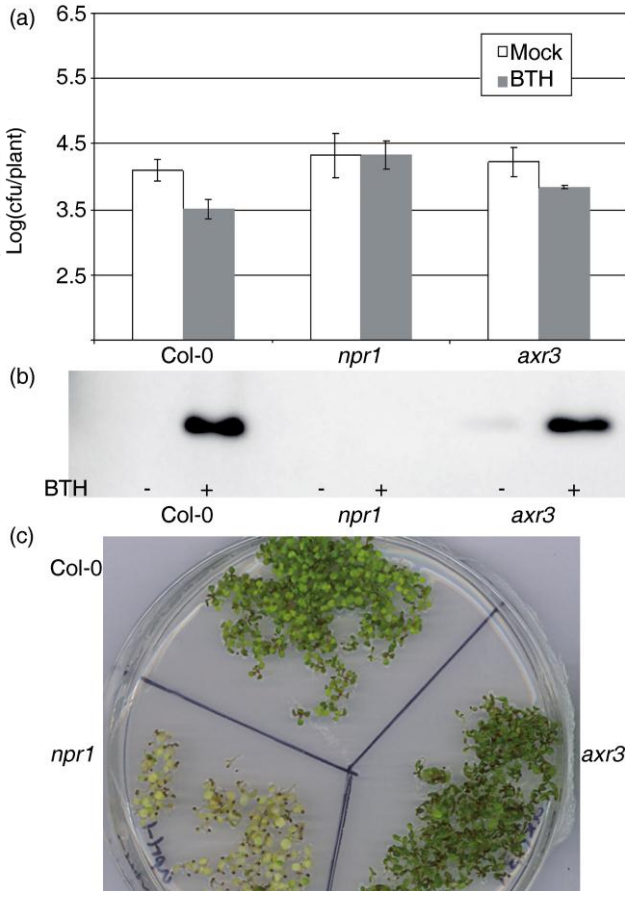

Figure 7. *axr3* can sense SA and BTH. (a) *Pto* growth in Col-0, *npr1* and *axr3* treated either with mock or BTH 350 µM, as described in Figure 1a. The resistance triggered by BTH is significant (Student´s T test, $p<0.05$) in both Col-0 and *axr3*, not in *npr1*. (b) Western blot for PR1 of the same experiment as (a), but prior to bacterial inoculation. The arrow points to the expected size of PR1 (14 kDa). (c) Phenotype of the same genotypes in MS plates supplemented with SA 500 µM. *npr1* plants do not perceive SA and therefore can not avoid accumulation to toxic levels. Col-0 and *axr3* can perceive SA and are able to grow in this medium. Three independent experiments were performed with similar results.

npr1-related genes and SA perception

Then we focused on *npr1* and related genes. The previous experiments were repeated with *npr1-1*, but there are eleven alleles of *npr1* (Cao et al. 1994; Delaney et al. 1995; Glazebrook et al. 1996; Shah et al. 1997). We assayed four of them (Figure 8), and -with some variation- all the alleles tested show no response to BTH in terms of PFW. There are no mutants with an increasing sensitivity to SA; therefore the next best candidates are the transgenics that overexpress *NPR1*. *35S:NPR1* is an overexpression of *NPR1*, and the plants can more strongly perceive BTH, as reported in the literature (Cao et al. 1998). *35S:NPR1:HBD* is a version of *NPR1* fused to the glucocorticoid receptor in a *npr1* background (Wang et al. 2005). The result is a protein not subjected to the nuclear vs. cytoplasm traffic, vital to its function in SA perception (Dong 2004). Figure 8 shows that the mere presence of NPR1 in the cytosol is not enough to trigger response to BTH and the nuclear localisation is required.

There are five genes in Arabidopsis with a high homology to *NPR1* (Liu et al. 2005). *NPR3* and *NPR4* have been reported to play a key role in plant defence (Zhang et al. 2006) and mutations in *BOP1* and *BOP2* affect the identity of the floral organs and the shape of the leaves (Ha et al. 2007; McKim et al. 2008). Plants from these two double mutants respond to BTH in the same way as in wt (Figure 8).

Regarding its biochemistry, NPR1 has been shown to interact in yeast two-hybrid with two sets of proteins, TGAs (Zhang et al. 1999) and NIMINs (Weigel et al. 2001), and in vivo with some of them. T-DNA insertions in *TGA1* and *TGA7* show small but consistent differences between these mutants and wt in their response to BTH (Figure 8). This small effect is more noticeable when a triple mutant *tga6 tga2 tga5* is used (Zhang et al. 2003), and the plants show an intermediate macroscopic phenotype (data not shown).

The *npr1* phenotypes are quite straightforward, which has led to a number of suppressor screenings. One of these suppressors is *sni1* (Li et al. 1999), and the double *sni1 npr1* regains the ability to activate defences upon BTH application. Interestingly, the double *sni1 npr1* does not behave as a suppressor in our system (Figure 8). We also tested T3 seeds from insertions in the homologue *NPR2*, the interactors *NIMIN1, NIMIN2, NIMIN3, TGA3* and *TGA4* and the

suppressors *SSI2* (Shah et al. 2001) *SON1* (Kim and Delaney 2002), and *SNI1* but no *npr1*-like phenotype was observed in the segregating families (data not shown).

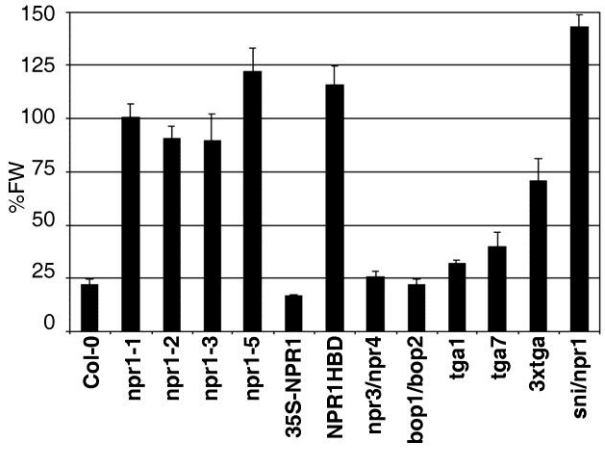

Figure 8. Behaviour of genotypes related to NPR1 in SA perception. Alleles of *npr1*, loss of function of related genes and transgenic plants that overexpress the protein were assayed as described in Figure 1c. *3xtga* stands for the triple mutant *tga6 tga2 tga5* and *NPR1HBD* for the transgenic *35S:NPR1:HBD*. Three independent experiments were performed with similar results.

Discussion

SA perception and plant fitness

Salicylic acid is a necessary hormone in plants for full resistance against biotrophic pathogens such as *Pto*. While the amount of SA can be measured in the laboratory (Defraia et al. 2008), for the quantification of SA perception we usually rely on the growth of the pathogen we are interested to start with. This is a potential circular problem, since we use a tool to answer a question that affects the tool.

Another potential problem is the nature of the pathogen. Pathogens like *Pto* grow exponentially, and small differences in the input lead to considerable differences in the output. For example, note the difference between the growths of *Pto* in Figure 1a vs. Figure 7a. There are alternatives, like immunodetection of defence markers (Uknes et al. 1992; Figures 2 and 7), or measurement of phytoalexin accumulation (Glazebrook and Ausubel 1994). These alternatives can produce quantitative data, but are not suitable for high throughput assays.

One side effect of several resistances is their negative effect on plant fitness (Heil 2002). In general, the more resistant an individual is, the less fit it is to compete when the pathogen is not present. There are several hypotheses to explain this fact. The first one is that, since the plant produces molecules that eventually stop the growth of the pathogen, it is plausible that the same molecules affect the plant. Other alternative is an economic consideration; the triggering of defence genes involves the use of resources that have to be obtained from normal plant growth. A somewhat related argument is the "scorched earth" defence, where the plant tries to stop the infection by starving the pathogen.

Resistance and fresh weight are inversely correlated

In the case of BTH, a single application can produce measurable effects in terms of plant fresh weight (Figure 1b). This subtle effect (measured 4 days after a single BTH treatment) was optimised for measurement and screen. While different ways of applying BTH produce visible differences, the best condition for our goals is to spray the plants with BTH four times on separate dates (see Experimental procedures). This procedure provides us with an accurate quantification of genotypes such as the mutants and ecotypes described above (Figure 1c). But most importantly, it gives us a tool to do a screening (Figure S1). There is a correlation between size and fresh weight that can be used to search for new mutants in a high-throughput fashion, and the next generation can be retested with two simple measurements (fresh weight of mock vs BTH treated plants). In principle, this model is analogous to screen for mutants in auxin perception with plates of 2,4-D (Maher and Martindale 1980). 2,4-D is more stable and has a stronger effect on the plant than the endogenous auxin, like BTH vs. SA. The main differences are that BTH does not work in

plates, and it is not lethal. But in both cases we can recover mutations impaired in the perception of the hormone by using an analogue and a set of extreme conditions (Mockaitis and Estelle 2008).

In order to use the biological model, several steps must be taken. First we need to characterise plant response in terms of macroscopic, microscopic and molecular phenotypes, to be sure that the observed effects on fresh weight correspond to the activation of plant defences. Second, the choice of the ecotype to be used, because Col-0 may not be the best background. And third, there is the question of genetic specificity; the biological model proposed should not mislabel mutants that affect the growth of the pathogen as a mutant in SA perception (e.g. *cpr1,* Figure 6a), and it should correctly label *npr1* as defective in SA perception.

The response to BTH in terms of PFW is dose-dependent (Figure 1c). The highest BTH concentration tested is 350 μM, a concentration frequently used in Arabidopsis (Lawton et al. 1996). To put it in context, this corresponds to approximately nine times the recommended dose for *Pto* infection in tomato (www.epa.gov), but it is between two and six times lower than SA concentrations used in Arabidopsis (1mM, e.g. Cao et al. 1994; 2 mM, e.g. Aviv et al. 2002). The loss of fresh weight can be detected as low as 3.5 μM, but not at 350 nM. Low BTH is unable to trigger cell death or callose deposition (Figure 2c and f). High BTH, on the other hand, is able to cause cell death in a small number of cells (Figure 2d), as it is also reported in the literature for SAR (Alvarez et al. 1998) and labelled as micro-HRs. Although a plausible hypothesis was that these micro-HR sites are similar, we did not observe any oxidative burst (data not shown). It is therefore possible that the micro-HRs are different, and while in SAR they are caused by oxidative burst, the cell death shown in Figure 2d is caused by other effector. Another alternative is that in our model a transient oxidative burst occurs immediately after the treatments, but it disappears when the tissue is stained (3 days after the last treatment). In any case, the small number of cell deaths observed does not account for the difference in PFW, and it seems an effect rather than a cause of resistance. While it has been reported that BTH by itself does not strongly trigger callose depositions, a second mock treatment (water infiltration) after BTH had the ability to do so (Kohler et al. 2002). Consistently with this result (we sprayed the plant several times), there is a strong callose staining with High BTH.

The next step was to analyse the molecular events that occur in this system. The amount of SA is under the control of feedback loops, positive in SA biosynthesis and negative in SA accumulation (Shah 2003). Therefore, it was relevant to measure the amount of SA in this system. High BTH produced a strong reduction in the amount of total SA (Figure 3a). There is a small reduction in the amount of free SA, but it is clear that the plant responded to High BTH with a reduction of the conjugated form of SA (mainly glucoside) (Nawrath et al. 2005). Therefore, this constitutes additional evidence in favour of a negative feedback loop that regulates the accumulation of SA. The other piece of evidence is the amount of SA in *npr1* (Cao et al. 1997). This mutant has more SA than the wt, both in mock and pathogen-inoculated plants. Another form of this phenotype is the low tolerance of *npr1* plants to SA in vitro (Figure 7c). It cannot detect SA, and therefore it cannot avoid SA accumulation and toxicity. The same mechanism impaired in *npr1*, is triggered continuously in the plants treated with High BTH in Figure 2, in an effort to maintain the homeostasis of the SA levels. The result is that the levels of total SA are depleted. It has been reported that a single application of BTH increase SA content (von Rad et al. 2005). We speculate that this discrepancy is due -as it happens with the callose staining and with the fresh weight- to the repetition of treatments (four in our case vs. one), rather than the difference of age (8 days in the first treatment vs. 4-6 weeks) or growth conditions.

The detection of the defence marker PR1 (Figure 3b) and *Pto* growth (Figure 1a and data not shown) confirms that fresh weight loss and disease defence are closely correlated, as low concentrations that do not produce fresh weight loss, do not trigger defence. Correspondingly, high concentrations are able to produce both phenotypes.

SA perception in natural variation

Before starting the search for new mutants, the best genotype has to be chosen. Col-0 is the ecotype most widely used for mutant screening (www.arabidopsis.org), but it could be an extreme ecotype in response to BTH. Figure 4 shows that Col-0 is a representative Arabidopsis ecotype, because it ranks between the 40th and 56th percentile among the collections tested. Another reason for these experiments was to search for natural variation, but there is no extreme ecotype in the response to BTH.

We also searched for transgression in seven RILs (Figure 5 and data not shown), but found none. The three QTLs found are only relevant to the differences in growth when a mock treatment is applied, but there is no difference in the response to BTH. This does not mean that there are not variations in the SA response (van Leeuwen et al. 2007), but that none was both significant and specific to SA perception with the populations and system under study.

SA perception in defence and signalling mutants

From the comprehensive list of mutants tested, there is no evidence of desensitisation. That is, mutants that have more SA than their corresponding wt (*e.g. c-SAS* and *cpr1*) are still able to respond to exogenous BTH applications (Figure 6). A direct consequence is that we can assay genotypes that are more resistant to bacteria and unequivocally discriminate if it is due to an enhanced SA perception. So far we have found no evidence for such genotype, with the exception of *35S:NPR1* (see below). Regarding the different kind of defences, mutants in SAR, basal, specific (or gene-for-gene) and non-host resistance were tested and found not to be different from the wt, with the exception of *npr1*, as discussed below.

The more we study plant biology, the clearer it becomes that everything is interconnected. If two decades ago plant defence and development could be seen as two separate programs, evidence in the last years reveals a much more intricate signal network with complex interactions. Thus, there are reports on the interactions between SA and Auxins, Light perception, Ethylene, Jasmonic Acid, Abscisic Acid and ROS, among others (reviewed by Lopez et al. 2008). Mutations in pathways different from Auxin do not have a measurable impact on SA perception when measured as described. Regarding Auxins, only *axr3* does not respond to BTH in weight, and there is no visible difference between mock and BTH treated plants (data not shown). *AXR3* belongs to the family of IAAs, genes that are rapidly induced with auxins, and behave as activators or repressors of the auxin response (Reed 2001). The allele of *axr3* used is a semidominant mutant that stabilises the protein, causing an increase in auxin perception and phenocopying the overexpression of the wt protein (Ouellet et al. 2001). It is tempting to speculate that AXR3 is the link between defence and development in Arabidopsis.

In favour of this hypothesis, there are solid evidences of the interaction between SA and Auxins (Wang et al. 2007a), and the overexpression of *AXR3* reproduces the *axr3* phenotype (Reed 2001). Thus, the phenotype that responds to BTH could be explained by an increase in the amount of the AXR3 protein. However, this hypothesis has serious drawbacks. *AXR3* is slightly repressed under pathogenic conditions (www.genevestigator.com), which does not fit with a prominent role in the response to BTH. Mechanistically, exogenous Auxin applications reduces SA perception (Wang et al. 2007a). But *axr3* has Auxin hypersensitivity, so instead of sensing more SA, it should perceive less SA, which contradicts the model. A closer examination proves that *axr3* is indeed able to perceive SA and BTH, as measured by *Pto* growth, western blot of PR1, and tolerance to SA in plates (Figure 7). This perception is slightly attenuated (Figure 7a and 7b), as expected by the interaction between Auxins and SA.

The second hypothesis is that the small size of the plant does not allow it to lose weight, as it is already at minimal levels. The average weight of *axr3* in mock is less than Col-0 with BTH in Figure 6c, while in other replicates both weights were similar (data not shown). The difference with respect to the first hypothesis is that the small fresh weight is not related to a defence mechanism. The results of our experiments support this second hypothesis.

NPR1- related genotypes mark the relationship between plant defence and development

NPR1 is a gene necessary for SA perception (Figure 6), among other roles in plant defence (Pieterse and van Loon 2004) and development (Vanacker et al. 2001). The extreme *npr1* phenotype in response to BTH (Figure 6) is not allele specific, because the available alleles behave in the same way. It is worth mentioning that the *npr1-3* allele is still functional for the so-called Induced Systemic Resistance (Pieterse and van Loon 2004). In fact, the overexpression of *NPR1* fused to the glucocorticoid receptor (*35S:NPR1:HBD* in Figure 8) reproduces the same phenomenon, i.e. a functional NPR1 protein that is unable to migrate to the nucleus. Therefore, the response to BTH is dependent on the NPR1 protein acting in the nucleus. The overexpression of *NPR1* increases sensitivity to SA and its analogues in terms of pathogen growth and defence

markers (Cao et al. 1998; Friedrich et al. 2001), and we can reproducibly detect this enhanced SA perception (Figure 8).

In the Arabidopsis genome there are five genes with high homology to *NPR1* (Liu et al. 2005). Certain functional redundancy could exist in the genes of this family; therefore we assayed loss of function mutations in these genes. Fortunately, there are two double mutants available, *npr3 npr4* and *bop1 bop2*, and none of them is consistently different from wt. In the case of *NPR2*, T3 seeds from a T-DNA insertion (Table S1) were found to be like wt (data not shown). Therefore, there is no measurable functional redundancy, at least in a *NPR1* wt background.

NPR1 interacts with proteins from two families of genes, *TGAs* (Zhang et al. 1999) and *NIMINs* (Weigel et al. 2001). *TGAs* are a subclass of the family of *bZIP* transcription factors (Jakoby et al. 2002) that physically interact with promoters of PR genes (Johnson et al. 2003). The transcriptional activation of these PR genes is dependent on the interaction of the TGAs with NPR1 (Després et al. 2000). There are reports about functional redundancy in this family (Jakoby et al. 2002).

In our model, T-DNA insertions in *tga1* and *tga7* have a small but measurable phenotype (Figure 8), but the best indication of the significant role of this gene family in SA perception is the phenotype of the triple mutant *tga6 tga2 tga5* (Zhang et al. 2003; Figure 8). In this case the phenotype is visible to the naked eye (data not shown). T3 seeds from T-DNA insertions in *TGA3* and *TGA4* (Table S1) were phenotypically similar to wt (data not shown).

NIMINs are a family of three small genes, and their proteins interact in vitro with NPR1. Mechanistically, *NIMIN* genes would act as repressors of SA signalling (Weigel et al. 2001). T3 seeds from T-DNA insertions in *NIMIN1, NIMIN2* and *NIMIN3* (Table S1) were found to behave like wt (data not shown).

NPR1 is the only gene necessary for SA perception, and several suppressor screenings have been carried out to identify other players (Li et al. 1999). T3 seeds from T-DNA insertions in *SSI2*, and *SNI1* behave like wt (data not shown). Interestingly, the double *sni1 npr1* does not behave as a suppressor in

our system (Figure 8). *SNI1* encodes a nuclear protein rich in leucine and it is assumed to be a negative SAR regulator (Li et al. 1999). We were able to confirm the suppression of the *npr1* phenotype by *sni1* in *Pto* growth curves (data not shown), but not in weight.

An obvious hypothesis is that the signal that goes from SA to NPR1 is genetically divided into two; one is repressed by *SNI1* and activates defence genes (e.g. *PR1*), causing the measurable reduction of the infection. The other one is *SNI1* independent, and reduces the growth of the plant. This branching could be achieved through different signal thresholds, since *sni1* induces defence at lower concentrations of SA analogues (Li et al. 1999) both in wt and in *npr1*. In any case, the evidence that a genotype produces defence (PR gene expression included) with no loss of fresh weight contradicts the first two hypotheses presented to explain the interaction between plant defence and development ("defence is toxic", and "defence is expensive"). Thus, the third hypothesis ("scorched earth defence") is favoured by the results presented here. In other words, the plant has two programs: active synthesis of defences and active depletion of nutrients.

Experimental procedures

Inoculation and plant treatment

For all the experiments, *Arabidopsis thaliana* was sown in small pots, kept at 4 °C for 3 days and then transferred to growing conditions under a short-day regime (8 hours of light (150 μmol m-2 s-1) at 21 °C, 16 hours of dark at 19 °C). The treatments, inoculations, and sampling started 30 minutes after the initiation of the artificial day to ensure reproducibility. *Pseudomonas syringae pv. tomato* DC3000 (*Pto*) containing pVSP61 (empty vector) were maintained as described (Ritter and Dangl, 1996). The bacteria were grown, inoculated and measured as described (Tornero and Dangl 2001) with minor changes. Trypan Blue and Aniline Blue staining were performed as described (Tornero et al. 2002b); (Conrath et al. 1989), respectively). For all the experiments, three independent treatments were performed (three independent sets of plants sown and treated on different dates), only two in the case of the large collection of ecotypes.

BTH and fresh weight

Benzothiadiazole (BTH, CGA 245704), in the form of commercial product (Bion® 50 WG, a gift from Syngenta Agro S.A. Spain) was prepared in water for each treatment and applied with a household sprayer. When indicated, a mock inoculation of distilled water was performed. The treatments were conducted on the 8th, 11th, 15th, and 18th day (day 0 is when plants are transferred to growing conditions), and the weight of the plants recorded on the 21st day. For each genotype and treatment, 15 plants were weighed in 3 groups of 5. The mock treatment was considered to have a value of 100, and the average and standard deviation of the percentage of the fresh weight resulting from the BTH treatment are represented.

Western blot

Immunodetection of PR1 protein was carried out as described (Wang et al. 2005), using an Amersham ECL Plus Western Blotting Detection Reagents (GE HealthCare, Little Chalfont, UK). The second antibody was a 1:25000 dilution of Anti-Rabbit IgG HRP Conjugate (Promega, Madison, USA). Chemiluminescent signals were detected using a LA-3000 Luminescent Image Analyzer (Fujifilm Life Science, Stamford, CT, USA).

SA in plates and *in planta*

Arabidopsis seeds were surface-sterilized for 10 min in 70% ethanol and for 10 min in commercial bleach. Then, five washes were done with distilled water and the seeds were distributed on agar plates. The medium contains 0.5x Murashige and Skoog salts (Duchefa BV, Haarlem, the Netherlands), 0.6% (w/v) Phyto Agar (Duchefa), 2% (w/v) sucrose, with or without SA 500 μM (final concentration). The result was evaluated 10 days after transferring to growing conditions. For the measurement of SA *in planta*, three samples of 250 mg were frozen in liquid nitrogen. SA extraction was performed as described by (Mayda et al. 2000).

QTL mapping

Plants of Arabidopsis were treated with either mock or benzothiadiazole (BTH) 350 μM as described above. The weight of five plants per line and the genotype of each line (provided by the authors of the corresponding references, see below) were used as input for the program WinQTLCart (Wang et al. 2007b), that calculates the probability that a QTL is link to a particular region of the genome. The populations analysed were: Col-0 x Nd-1, 98 lines (Deslandes et al. 1998); Col-gl1 x Kas-1, 115 lines (Wilson et al. 2001); Cvi-1 x L*aer*-2, 50 lines (Alonso-Blanco et al. 1998a); L*aer*-0 x Sha-0, 114 lines (Clerkx et al. 2004); Bay-0 x Sha-0, 162 lines (data not shown, (Loudet et al. 2002); Col-4 x L*aer*-0, 85 lines (Lister and Dean 1993); and L*aer*-0 x No-0, 135 lines (Magliano et al. 2005).

Acknowledgements

This work was supported by a BIO2006-02168 grant of Ministerio de Ciencia e Innovación (MICINN) to PT, a JAE-CSIC Fellowship to JVC and a FPI-MICINN to AD. We thank the English translation service of the Universidad Politécnica de Valencia and the Statistics Service (CTI-CSIC). We appreciate the seeds provided by a great number of colleagues and the BTH provided by Syngenta. Thanks also to Carlos A. Blanco for advice in QTL mapping, and to José León and Pablo Vera for useful advice.

Supporting information

Table S1. List of ecotypes, populations and mutants tested.

S1A. List of ecotypes tested with BTH in Figure 4.

McKhann collection of 48 ecotypes		
Number Figure 4	**Ecotype**	**INRA Code**
EF-1	Pyl-1	8AV1B1
EF-2	Ran	21AV1B1
EF-3	Jea	25AV1B1
EF-4	Pi-0	40AV1B1
EF-5	Bl-1	42AV1B1
EF-6	Pa-1	50AV1B1
EF-7	Sp-0	53AV1B1
EF-8	Ta-0	56AV1B1
EF-9	St-0	62AV1B1
EF-10	Lip-0	63AV1B1
EF-11	Te-0	68AV1B1
EF-12	Kn-0	70AV1B1
EF-13	Edi-0	83AV1B1
EF-14	Tsu-0	91AV1B1
EF-15	Stw-0	92AV1B1
EF-16	Mt-0	94AV1B1
EF-17	Nok-1	95AV1B1
EF-18	Ge-0	101AV1B1
EF-19	Ita-0	157AV1B1
EF-20	Ri-0	160AV1B1
EF-21	Ct-1	162AV1B1
EF-22	Can-0	163AV1B1
EF-23	Cvi-0	166AV1B1
EF-24	Bur-0	172AV1B1
EF-25	Alc-0	178AV1B1
EF-26	Blh-1	180AV1B2
EF-27	Kondara	190AV1B1
EF-28	Enkheim-T	197AV1B1
EF-29	Gre-0	200AV1B1
EF-30	Jm-0	206AV1B1
EF-31	Mh-1	215AV1B1
EF-32	Oy-0	224AV1B1
EF-33	Rld-2	229AV1B1
EF-34	Rubezhnoe-1	231AV1B1

EF-35	Sap-0	234AV1B1
EF-36	Sav-0	235AV1B1
EF-37	Shahdara	236AV1B1
EF-38	Yo-0	250AV1B1
EF-39	Akita	252AV1B1
EF-40	Ishikawa	253AV1B1
EF-41	262 AV	262AV1B1
EF-42	263 AV	263AV1B1
EF-43	266 AV	266AV1B1
EF-44	Sah-0	233AV1B1
EF-45	Sakata	257AV1B2
EF-46	267 AV	267AV1B1
EF-48	Bla-1	76AV
EF-49	Ms-0	93AV
Nordborg collection of 96 ecotypes		
Number Figure 4	**Ecotype**	**NASC Code**
AB-1	RRS-7	N22564
AB-2	RRS-10	N22565
AB-3	Know-10	N22566
AB-4	Know-18	N22567
AB-5	Rmx-A02	N22568
AB-6	Rmx-A180	N22569
AB-7	Pna-17	N22570
AB-8	Pna-10	N22571
AB-9	Eden-1	N22572
AB-10	Eden-2	N22573
AB-11	Lov-1	N22574
AB-12	Lov-5	N22575
AB-13	Fab-2	N22576
AB-14	Fab-4	N22577
AB-15	Bil-5	N22578
AB-16	Bil-7	N22579
AB-17	Var2-1	N22580
AB-18	Var2-6	N22581
AB-19	Spr1-2	N22582
AB-20	Spr1-6	N22583
AB-21	Omo2-1	N22584
AB-22	Omo2-3	N22585
AB-23	Ull2-5	N22586
AB-24	Ull2-3	N22587
AB-25	Zdr-1	N22588
AB-26	Zdr-6	N22589
AB-27	Bor-1	N22590
AB-28	Bor-4	N22591

AB-29	Pu2-7	N22592
AB-30	Pu2-23	N22593
AB-31	Lp2-2	N22594
AB-32	Lp2-6	N22595
AB-33	HR-5	N22596
AB-34	HR-10	N22597
AB-35	NFA-8	N22598
AB-36	NFA-10	N22599
AB-37	Sq-1	N22600
AB-38	Sq-8	N22601
AB-39	CIBC-5	N22602
AB-40	CIBC-17	N22603
AB-41	Tamm-2	N22604
AB-42	Tamm-27	N22605
AB-43	Kz-1	N22606
AB-44	Kz-9	N22607
AB-45	Got-7	N22608
AB-46	Got-22	N22609
AB-47	Ren-1	N22610
AB-48	Ren-11	N22611
AB-49	Uod-1	N22612
AB-50	Uod-7	N22613
AB-51	Cvi-0	N22614
AB-52	Lz-0	N22615
AB-53	Ei-2	N22616
AB-54	Gu-0	N22617
AB-55	Ler-1	N22618
AB-56	Nd-1	N22619
AB-57	C24	N22620
AB-58	CS22491	N22621
AB-59	Wei-0	N22622
AB-60	Ws-0	N22623
AB-61	Yo-0	N22624
AB-62	Col-0	N22625
AB-63	An-1	N22626
AB-64	Van-0	N22627
AB-65	Br-0	N22628
AB-66	Est-1	N22629
AB-67	Ag-0	N22630
AB-68	Gy-0	N22631
AB-69	Ra-0	N22632
AB-70	Bay-0	N22633
AB-71	Ga-0	N22634
AB-72	Mrk-0	N22635
AB-73	Mz-0	N22636

AB-74	Wt-5	N22637
AB-75	Kas-1	N22638
AB-76	Ct-1	N22639
AB-77	Mr-0	N22640
AB-78	Tsu-0	N22641
AB-79	Mt-0	N22642
AB-80	Nok-3	N22643
AB-81	Wa-1	N22644
AB-82	Fei-0	N22645
AB-83	Se-0	N22646
AB-84	Ts-1	N22647
AB-85	Ts-5	N22648
AB-86	Pro-0	N22649
AB-87	LL-0	N22650
AB-88	Kondora	N22651
AB-89	Sakhadara	N22652
AB-90	Sorbo	N22653
AB-91	Kin-0	N22654
AB-92	Ms-0	N22655
AB-93	Bur-0	N22656
AB-94	Edi-0	N22657
AB-95	Oy-0	N22658
AB-96	Ws-2	N22659
Other ecotypes analyzed		
La-0	RLD-0	Aa-0
Bah-0	Bch-0	Bla-2
Chi-0	Fe-1	Ke-0
Ksk-1	Nd-0	Oy-1
Pla-0	No-0	

S1B. List of mutants tested with BTH.

Mutant (*)	F6	Keyword	Reference
35S:HAB1	d	ABA	Saez et al. 2004
aba2-1 (L)	d	ABA	Leon-Kloosterziel et al. 1996
aba3-2 (L)	d	ABA	Leon-Kloosterziel et al. 1996
abi1-2/*hab1*-2	d	ABA	Saez et al. 2006
abi5-3 (L)	d	ABA	Finkelstein and Lynch 2000
arf11-1	c	Auxins	Okushima et al. 2005
arf1-2	c	Auxins	Okushima et al. 2005
arf12-1	c	Auxins	Okushima et al. 2005
arf13-1	c	Auxins	Okushima et al. 2005
arf15-1	c	Auxins	Okushima et al. 2005
arf16-1	c	Auxins	Okushima et al. 2005
arf19-1	c	Auxins	Okushima et al. 2005
arf20-1	c	Auxins	Okushima et al. 2005
arf21-1	c	Auxins	Okushima et al. 2005
arf3-1	c	Auxins	Okushima et al. 2005
arf6-1	c	Auxins	Okushima et al. 2005
arf8-2	c	Auxins	Okushima et al. 2005
arf9-1	c	Auxins	Okushima et al. 2005
axr1-3	c	Auxins	Leyser et al. 1993
axr2-1	c	Auxins	Timpte et al. 1995
axr3-1	c	Auxins	Ouellet et al. 2001
ixr1-1	c	Auxins	Scheible et al. 2001
tir1-1	c	Auxins	Dharmasiri et al. 2005
yucca	c	Auxins	Zhao et al. 2001
35S-FMO1	a	Defence: SAR	Bartsch et al. 2006
fmo1		Defence: SAR	Bartsch et al. 2006
ald1		Defence: indep. NPR1	Song et al. 2004
dth9		Defence: basal; indep. NPR1; SAR	Mayda et al. 2000
eds1-1 (W)		Defence: basal, R genes	Parker et al. 1996
eds12	b	Defence: basal	Glazebrook et al. 1996
eds1-2 (L)		Defence: basal; R genes	Parker et al. 1996
eds1-2	a	Defence: basal; R genes	Parker et al. 1996
eds4	b	Defence: basal	Glazebrook et al. 1996
eds5-1	a	Defence: basal, SA	Nawrath et al. 2002
eds5-3		Defence: basal, SA	Nawrath et al. 2002
eds8-1	b	Defence: basal	Glazebrook et al. 1996

eds9-1	b	Defence: basal	Glazebrook et al. 1996
ocp11		Defence: basal	Agorio and Vera 2007
pad1-1	b	Defence	Glazebrook and Ausubel 1994
pad2-1	b	Defence	Glazebrook and Ausubel 1994
pad3-1	b	Defence	Glazebrook and Ausubel 1994
pad4-1	a	Defence: basal; R genes	Glazebrook and Ausubel 1994
pad5-1	b	Defence	Glazebrook and Ausubel 1994
phs3		Defence basal. Auxins	Warren et al. 1999
sgt1a (W)		Defence	Azevedo et al. 2002
why		Defence: basal; indep. NPR1	Desveaux et al. 2004
cim3	a	Defence	Maleck et al. 2002
cpr1	a	Defence: constitutive	Bowling et al. 1994
cpr5		Defence: constitutive	Bowling et al. 1997
dnd1		Defence: constitutive	Yu et al. 1998
edr1	a	Defence: constitutive	Frye and Innes 1998
nho1		Defence: non-host	Lu et al. 2001
pen1-1		Defence: non-host	Collins et al. 2003
pen2-1		Defence: non-host	Lipka et al. 2005
pmr1-1	b	Defence: non-host	Vogel, J. and Somerville 2000
pmr2-1	b	Defence: non-host	Vogel, J. and Somerville 2000
pmr3-1	b	Defence: non-host	Vogel, J. and Somerville 2000
pmr4-1	b	Defence: non-host	Vogel, J. and Somerville 2000
hsp90-2		Defence: R genes	Hubert et al. 2003
rar1/ndr1		Defence: R genes	Tornero et al. 2002b
rar1-21		Defence: R genes	Tornero et al. 2002b
rpm1-1		Defence: R genes	Grant et al. 1995
rps5-2		Defence: R genes	Warren et al. 1998
sgt1b	b	Defence: R genes; JA-necrotrophs	Tör et al. 2002
c-SAS-10	a	Defence: SA	Mauch et al. 2001
NahG (L)		Defence: basal; SA; SAR	Lawton et al. 1995
NahG	a	Defence: basal; SA; SAR	Lawton et al. 1995
NahG (W)		Defence: basal; SA; SAR	Lawton et al. 1995
sid2	a	Defence: basal; SA; SAR	Wildermuth et al. 2001
35S-DIR1	a	Defence: SAR	Maldonado et al. 2002
dir1	b	Defence: SAR	Maldonado et al. 2002
ndr1-1	b	Defence: R genes; SAR	Century et al. 1995
sfd1-1	b	Defence: SAR	Nandi et al. 2004
sfd1-2		Defence: SAR	Nandi et al. 2004
35S-ERF1	d	Ethylene	Berrocal-Lobo et al. 2002
ein2-1		Ethylene	Alonso et al. 1999

ein2-5		Ethylene	Alonso et al. 1999
eto1-1	d	Ethylene	Guzmán and Ecker 1990
eto2		Ethylene	Vogel, J.P. et al. 1998
eto3		Ethylene	Woeste et al. 1999
etr1-3	d	Ethylene	Guzmán and Ecker 1990
hls1-1	d	Ethylene	Lehman et al. 1996
bik1	e	JA- necrotrophs	Veronese et al. 2006
bos1	e	JA- necrotrophs	Mengiste et al. 2003
coi1	e	JA- necrotrophs	Xie et al. 1998
jin1	e	JA- necrotrophs	Berger et al. 1996
jin4	e	JA- necrotrophs	Berger et al. 1996
ocp3	e	JA- necrotrophs	Coego et al. 2005
cop1-4	c	Light	McNellis et al. 1994
det1-1	c	Light	Chory et al. 1989
hy5-215	c	Light	Oyama et al. 1997
phyA/*phyB* (L)	c	Light	Reed et al. 1993
phyA-201 (L)	c	Light	Nagatani et al. 1993
phyB-5 (L)	c	Light	Reed et al. 1993
uvr2-1	c	Light	Jiang et al. 1997
atrboh-D	d	ROS	Torres et al. 2002
atrboh-F	d	ROS	Torres et al. 2002
rcd1		ROS	Ahlfors et al. 2004

(*) Ecotype is only indicated when different than Col-0. L stands for L*a*e*r*-0 and W is Ws-0.

F6 Plot indicated for mutants whose corresponding result is shown in Figure 6. Otherwise, left blank.

ABA is abscisic acid, SA is salicylic acid, JA is jasmonic acid, ROS is reactive oxygen species, and SAR is systemic acquired resistance.

S1C. List of mutants in *NPR1*-related genotypes tested with BTH.

Mutant (*)	Keyword	Reference
npr1-1	*npr1* allele	Cao et al. 1994
npr1-2	*npr1* allele	Glazebrook et al. 1996
npr1-3	*npr1* allele	Glazebrook et al. 1996
npr1-5 (N)	*npr1* allele	Shah et al. 1997
35S:NPR1	*NPR1* overexpressor	Cao et al. 1998
35S:NPR1:HBD	*NPR1* overexpressor	Wang et al. 2005
npr2-1	*NPR1* paralog	SALK_006564
npr3 npr4	*NPR1* paralogs	Zhang et al. 2006
bop1 bop2	*NPR1* paralog	Ha et al. 2007; McKim et al. 2008
tga1	*NPR1* interactor	SALK_028212
tga7	*NPR1* interactor	GK-434F04
tga6-1 *tga2*-1 *tga5*-1	*NPR1* interactors	Zhang et al. 2003
sni1 npr1	*npr1* suppressor	Li et al. 1999

(*) Ecotype is only indicated when different than Col-0. N stands for No-0. All mutants in wildtype background, with the only exception of *35S:NPR1:HBD* (*npr1*-3 background).

S1D. List of T3 seeds from insertions in *NPR1*-related genotypes tested with BTH.

Genes	Reference
NIMIN1	SALK_086460
NIMIN2	SALK_098399
NIMIN3	SAIL_619_E06
TGA3	SALK_081158
TGA4	SALK_127923
SSI2	SALK_036854
SON1	SM_3_554 y SALK_009280
SNI1	SAIL_298_H07
BOP1	GK-386G09
BOP2	SALK_088943

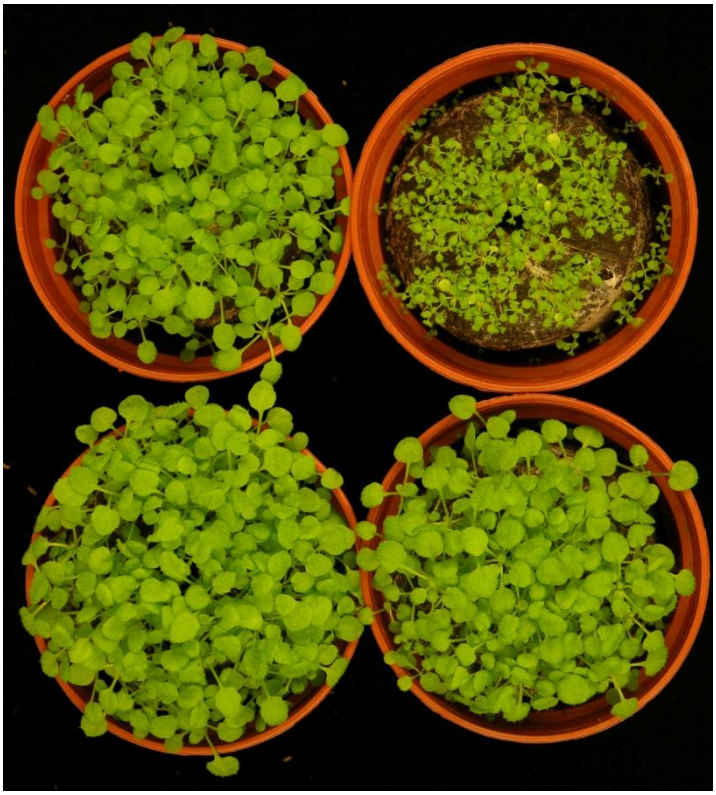

Figure S1. Picture of Col-0 and *npr1*-1 after mock and BTH 350 µM treatments. Plants were mock treated (left) or BTH 350 µM treated (right) as described in Experimental procedures. The genotypes are Col-0 (up) and *npr1*-1 (down). Part of this picture is shown in Figure 2A.

Artículo 2

Structure-function analysis of *npr1* alleles in Arabidopsis reveals a role for its paralogs in the perception of salicylic acid

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Este artículo ha sido publicado en la revista:
Plant, Cell and Environment (2010) 33, 1911-1922

Abstract

Salicylic Acid (SA) is necessary for plant defence against some pathogens, and NPR1 is necessary for SA perception. Plant defence can be induced to an extreme by several applications of benzothiadiazole (BTH), an analogue of SA. Then, plants that do not perceive BTH grow unaffected, while wild type plants are smaller. This feature allows us to screen for mutants in *Arabidopsis thaliana* that show insensitivity to BTH in a high-throughput fashion. Most of the mutants are *npr1* alleles with similar phenotype in plant fresh weight reduction and pathogen growth upon SA or BTH application. No obvious null alleles were recovered in our screening, but most of the mutations are clustered in the carboxyl-terminal part of the protein. These facts have prompted a search for knockouts in the *NPR1* gene. Two of these KO alleles identified are null and have an intermediate phenotype. All the evidence lead us to propose a redundancy in BTH/SA perception, with the paralogs of *NPR1* taking part in this signalling. We show that the mutations recovered in the screening genetically interact with the paralogs preventing their function in BTH/SA signalling.

Keywords: BTH, defence, NPR1, *Pseudomonas*.

Introduction

Salicylic acid (SA) is a plant hormone required for full resistance against biotrophic pathogens. It is important in two essential defence process; basal resistance and systemic acquired resistance (SAR; Vlot et al. 2008a). Basal resistance is triggered by compatible pathogens that do not trigger gene for gene resistance and produce disease (Jones and Dangl 2006). Thus, plants with less SA are more susceptible to compatible pathogens, and plants with more SA (either by genetic engineering or exogenous applications) are more resistant (Reviewed by Nawrath et al. 2005). SAR is the resistance that is established in non-inoculated leaves of a plant that has previously been inoculated with a pathogen, generally requiring cell death (Reviewed by Vlot et al. 2008a). SA is necessary but not sufficient for SAR.

SA research is hampered by its chemical properties, since the exogenous applications that produce a strong effect in 0.2-1 mM in *Arabidopsis thaliana* (Arabidopsis) are close to the phytotoxic ones (van Leeuwen et al. 2007). To avoid the phytotoxicity, and with a clear biotechnological focus, several analogues have been proposed, like 2,6-Dichloroisonicotinic acid (INA; Ward et al. 1991) and benzothiadiazole (BTH; Lawton et al. 1996). In the case of BTH, applications as low as 0.12 mM have an effect, but applications as high as 1.2 mM are not phytotoxic (Lawton et al. 1996 and data not shown).

SA is the only plant hormone where a receptor has not been described yet (Nawrath et al. 2005). It is plausible that its perception is through indirect means, like redox balances (see below). *NPR1* (*Non expresser of Pathogenesis Related proteins* 1) is the only gene that has been found to have a profound impact on SA signalling when mutated (Cao et al. 1994); Delaney et al. 1995; Glazebrook et al. 1996; Shah et al. 1997). There are two families of NPR1 interactors: *TGAs* (Zhang et al. 1999) and *NIMINs* (Weigel et al. 2001). Only when knocking out several genes (*TGAs,* Zhang et al. 2003), or expressing dominant members (*NIMINs,* Weigel et al. 2005), is a measurable phenotype observed in SA signalling.

When the plant is not under biotic stress, NPR1 is mainly present in the cytosol as oligomers (Mou et al. 2003). Upon SA perception, monomers are released from the oligomers, and move into the nucleus. NPR1 is degraded by the proteosome in the nucleus, as a requirement for defence activation (Spoel et al. 2009).

SA is mechanistically involved in the release of NPR1 monomers by triggering the expression of a thioredoxin (Tada et al. 2008). It is suggested that the fine regulation of these events depends on the redox balance of the cell, a balance that is altered when molecules such SA are more abundant (Mou et al. 2003).

There are five paralogs of *NPR1* in Arabidopsis (www.arabidopsis.org). *Blade On Petiole1* (*BOP1*) and *BOP2* have an important role in development (Ha et al. 2007; McKim et al. 2008). *NPR3* and *NPR4* are reported to have a role in defence (Liu et al. 2005; Zhang et al. 2006), but no function for *NPR2* has been described yet. In this context, we proposed to screen for genotypes that do not recognize SA; applications of BTH on separate dates produce a small plant in

the case of wild type Arabidopsis, while *npr1* plants look unaffected (Canet et al. 2010a). This prompted us to do a structure-function analysis of a full range of *npr1* alleles. The implications of their phenotype lead us to test also the behaviour of the *NPR1* paralogs in SA perception.

Materials and methods

Plant growth and inoculation

Arabidopsis thaliana was sown and grown as described (Canet et al. 2010a), in phytochambers with days 8 h at 21°C, 150 μmol m^{-2} s^{-1} and nights of 16 h. at 19°C. The treatments, inoculations, and sampling started 30 minutes after the initiation of the artificial day to ensure reproducibility. *Pseudomonas syringae pv. tomato* DC3000 (*Pto*) containing pVSP61 (empty vector) were maintained as described (Ritter and Dangl 1996). The bacteria were grown, inoculated and measured as described (Tornero and Dangl 2001). For all the experiments, at least three independent treatments were performed (three independent sets of plants sown and treated on different dates).

BTH and fresh weight

Benzothiadiazole (BTH, CGA 245704), in the form of a commercial product (Bion® 50 WG, a gift from Syngenta Agro S.A. Spain) was prepared in water for each treatment and applied with a household sprayer. The BTH treatments were done as described in (Canet et al. 2010a). For the screening of mutants, plants that seemed unaffected by BTH were rescued (First selection) and their progeny phenotyped both in mock and in BTH treatments (Second selection). A third selection consisted of a treatment of an F1 of each mutant with *npr1*-1 along with its parents.

Western blot

Immunodetection of PR1 protein was carried out as described (Wang et al. 2005), using an Amersham ECL Plus Western Blotting Detection Reagent (GE HealthCare, Little Chalfont, UK). The second antibody was a 1:25000 dilution

of Anti-Rabbit IgG HRP Conjugate (Promega, Madison, USA). Chemiluminescent signals were detected using a LA-3000 Luminescent Image Analyzer (Fujifilm Life Science, Stamford, USA).

Chemical treatments

For measuring the effect in *Pto* growth, water, 500 μM SA (in the form of sodium salicylate, S3007 SIGMA, St Louis, USA), and 350 μM BTH were applied by spray one day previous to pathogen inoculation. For in vitro culture, MS plates were prepared and sown as described (Canet et al. 2010a) with or without 500 μM SA. The results were evaluated between 10 and 14 days of growth.

Mutagenesis

Seeds were mutagenized with 0.15% ethyl methanesulfonate (M0880, SIGMA) for 8 hr, and M2 seed collected from ~ 100 M1 plants. For EMS, different backgrounds were used (*PR1:LUC*, Maleck et al. 2002; *rar1*-21 and *rar1*-21 *ndr1*-1, Tornero et al. 2002b; *rpm1*-1, Grant et al. 1995; *NahG*, Lawton et al. 1995, *RPM1-MYC*, Boyes et al. 1998 and others). Seeds of Col-0 treated with fast neutrons or with gamma ray were purchased (M1F-02-04 and M1G-02-02 respectively, Lehle seeds, Round Rock, TX, USA), and their progeny screened as with EMS. In the case of the activation tagging seeds, the references N31400, N31402, N31404, N21995, N21998, N21991, N23153, N84450, and N31100 were obtained from NASC (www.arabidopsis.info) and directly screened. The insertions *npr1*-63 (N831344, Alonso et al. 2003), *npr1*-71 (N163245, Sundaresan et al. 1995), and *npr2*-2 (N622643), were obtained from NASC, and *npr1*-70 (ET5232) from CSHL (www.cshl.edu). The insertions were followed to homozygosis by PCR, and their exact position checked by sequence.

Sequence and statistical analysis of the mutations

PCRs of the NPR1 gene were done with the oligonucleotides TP412 (5' TGCTTCTTCATATCTCACCACCACTCTCG 3') and TP413 (5'CACCTTACACGCCCACTCAGTGTTCTCT 3') (SIGMA). Four independent reactions were pooled and sequenced with internal

oligonucleotides. The analysis of the mutations was done with Excel software (Microsoft, Redmond, USA) and custom Perl scripts. The data for the conservation of each AA in the NPR1 paralogs was obtained from CLUSTAW (Chenna et al. 2003). The data for the different AA characteristics or mutations was downloaded from www.genome.jp/aaindex, and the background was considered to be all the possible mutations or only the EMS-induced mutations. A T-test was performed between the background and the mutations experimentally obtained, followed by a Benjamini-Hochberg False Discovery Rate correction (Benjamini and Hochberg 1995) with $p<0.05$.

Results

Searching for mutations in SA signalling

An important question in the field of plant-pathogen interactions is if *NPR1* is the only gene necessary for SA recognition. We used the model of BTH previously described (Canet et al. 2010a) to answer this question and to further characterize the role of NPR1 by mutagenesis. Figure 1a shows the proof of concept of the model in a screening setting. 3 seeds of *npr1*-1 were mixed with c. 3600 seeds of Col-0. After BTH treatments, the *npr1*-1 plants are easily identified in a wild type (wt) background (only a quarter of the experiment is shown in Figure 1a, with a single *npr1*-1). Our previous work suggests that mainly *npr1* alleles would appear in this kind of screening, so we set up the most difficult scenario. This scenario would be to find lack of complementation in an F2 between *npr1*-1 (recessive) and a dominant mutation, or a proportion of 3 wt plants and 13 *npr1*-like. Such a proportion is detectable (data not shown). To be sure that no mutation is misclassified, MS plates with 500 μM SA were used (Figure 1b). Although not all the wt plants are recovered, they are easily identified as green plants over a white background of *npr1*-1 plants.

A high-throughput screening was performed (Figure 1c) with several mutagens to obtain different types of mutations. Thus, besides using Ethyl Methane Sulfonate (EMS; Sega 1984), we also used Activation tagging, Gamma Rays, and Fast Neutrons (Table 1). In the case of EMS, a mutagenesis was performed in the *PR1:LUC* background (Maleck et al. 2002). Since different mutant backgrounds could provide unusual results, several mutants or transgenic lines

were used as parental lines for EMS mutagenesis. There is no evidence that these mutants provided a different number or kind of mutations (see below). More than 3,600 plants per family were analyzed to ensure the recovery of all mutations with a 95% probability (Malmberg 1993).

Three hundred seventy-seven plants that seemed unaffected by BTH were picked in the first selection (M2s), some of them from the same family of M1s. The progeny of these plants was retested with a mock treated pot of plants simultaneously with a BTH treated pot. 157 M3s were unaffected by BTH when compared with their mock treatment and with the Col-0 control. Figure S1 shows a picture of one candidate.

The 157 candidates were crossed with *npr1*-1, and their F1s tested with BTH as described in Figure 1. Throughout this third selection of candidates, it was decided to discard those with a weaker phenotype. With such a restrictive criterion, 93 candidates remained. Their F2s were tested with BTH and SA as described in Figure 1 (with the exception of *NahG* background, where growth on SA plates is not informative). Eventually, counting only one allele per independent family when several were found, 43 alleles of *npr1* were identified. These 43 alleles are hereafter named as a group as "new" alleles. The remaining 13 mutants that complement *npr1*-1 will be described elsewhere.

Mutagens	Families	M2s (x10E6)	First selection	Second selection	Third selection	*npr1* alleles
EMS	600	2.16	166	104	69	37
ADN-Ts	543	1.96	46	21	7	2
Gamma	140	0.50	95	13	7	2
FN	180	0.65	70	19	10	2
Total	1463	5.27	377	157	93	43

Table 1. Mutants obtained in this screening. Seeds from plants treated with different mutagens (first column) were selected by BTH and then by size as described in Figure 1. The second column provides the number of independent families, and the third column is the number of seeds screened. The fourth column gives the number of mutants recovered as M2s and the fifth the number of mutants retested in M3 and selected to cross with *npr1*-1. The sixth column shows the mutants that still have a strong phenotype and the seventh the number of *npr1* alleles found from independent families. EMS, ethyl methane sulfonate; FN, fast neutrons; Gamma, gamma rays; T-DNAs, activation tagging.

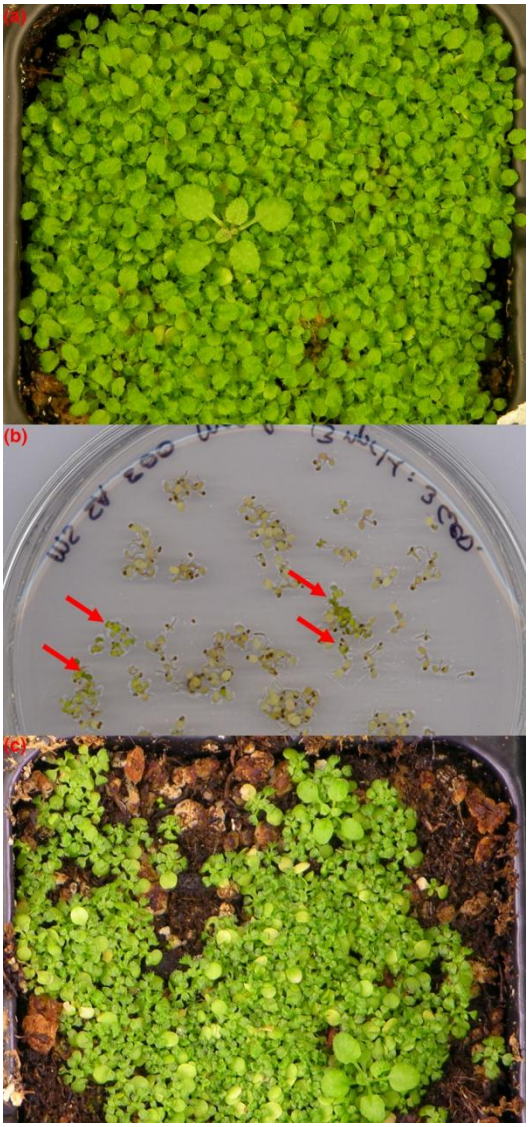

Figure 1. Screening for non-recognition of benzothiadiazole (BTH). (a) Proof of concept in soil. three seeds of *npr1*-1 were mixed with c. 3600 seeds of Col-0 and sown. Plants were treated with 350 µM BTH four times, and the picture taken at 3 weeks of growth. Only a quarter of the experiment is shown, with a single *npr1*-1 plant. (b) Proof of concept *in vitro*. Seeds of Col-0 and *npr1*-1 were mixed in a proportion of 3 to 13, respectively, and sown in MS plates containing 500 µM salicylic acid (SA). The picture was taken at 12 days of growth. The arrows point to wt plants. (c) Screening with mutagenized plants. An M2 family with a putative mutant is showed, at three weeks of growth.

The new *npr1* alleles share some phenotypes

Once the *npr1* alleles were identified visually, a more detailed quantification was made. We used the percentage of fresh weight (PFW), that is, fresh weight of plants treated with 350 μM BTH divided by fresh weight of mock treated plants, expressed in percentage. Figure 2a shows that Col-0 has a low PFW, while *npr1*-1 is unaffected. Since all the new alleles behaved similarly, only the first nine are presented. There is some variation in the PFW, but there are no reproducible differences with *npr1*-1.

It is plausible that a selection by BTH based on size could produce mutations that still recognize BTH in terms of *Pseudomonas syringae pv tomato* DC3000 (*Pto*) growth. Similarly, a lack of response to BTH does not necessarily imply a lack of response to SA and vice versa (e.g. *NahG*; Lawton et al. 1995). At least for the new *npr1* alleles, four phenotypes (response to BTH in PFW, response to BTH in *Pto* growth, response to SA in *Pto* growth, and tolerance to SA in vitro) were absent in the same mutants (Figure 2b and data not shown). Since all the new alleles behaved similarly, only the first nine are presented.

SA perception is sometimes checked by the expression of Pathogen Related proteins (PRs; van Loon et al. 1994). In fact, some *npr1* alleles were identified by the lack of *PR2* (Cao et al. 1994) or *PR1* (Shah et al. 1997) expression in inductive conditions. This last protein is a reliable marker of plant distress, so a western blot was performed with the new *npr1* alleles (Figure 2c) detecting PR1. Mock treated plants show no PR1 expression (data not shown). In contrast, upon *Pto* infection, Col-0 showed a strong signal three days after inoculation, while *npr1*-1 showed no signal at all. From the new *npr1* alleles, only *npr1*-20 and *npr1*-41 consistently produced some PR1 protein, always in lower levels than Col-0 (Figure 2c shows the first nine alleles and *npr1*-41). The levels of PR1 in these two alleles are not correlated with a significant defence response (Figure 2b and data not shown), likely because the amount of protein is low enough that a threshold is not reached, and/or because other defence molecules are required.

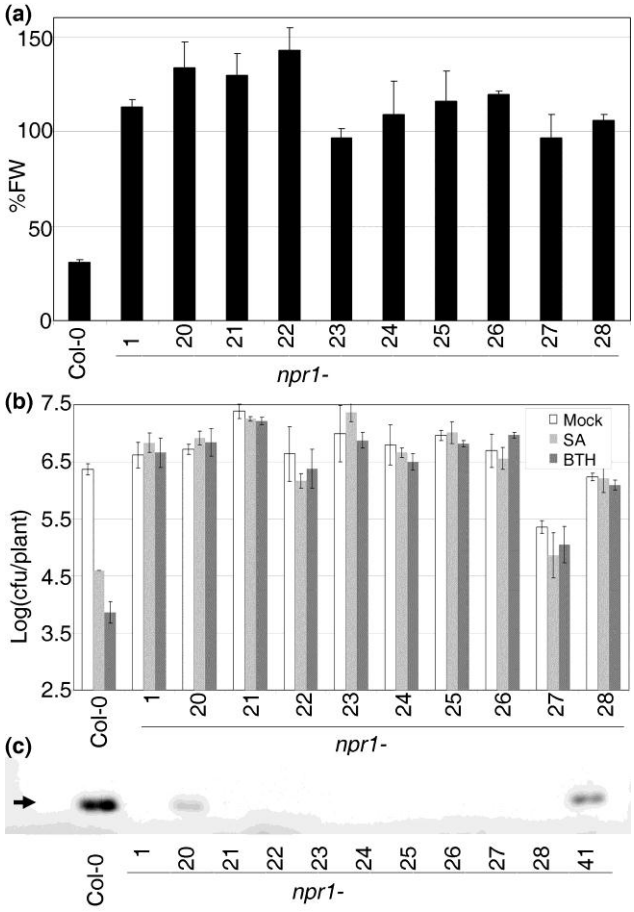

Figure 2. All the *npr1* alleles show a similar phenotype in response to SA or BTH. (a) Plants were treated with either mock or 350 µM BTH as described in Figure 1a, their weight recorded, and the ratio between the BTH and mock treated plants represented (average and SD of 15 plants in three groups of five). The ratio is expressed as percentage of fresh weight (FW). (b) Plants were pretreated with mock, 500 µM SA, or 350 µM BTH, inoculated with *Pseudomonas syringae* pv *tomato* DC3000 (*Pto*), and the growth of *Pto* measured three days later. The bacteria are measured in Logarithm of colony forming units per plant (Logs(cfu/plant)). (c) Plants were inoculated with *Pto*, and tissue was sampled three days later. The defence marker PR1 was detected by western blot. Only two alleles, *npr1*-20 and *npr1*-41, show consistent expression of PR1. The arrow points to the expected size of PR1 (14 kDa). For simplicity, the first nine alleles are shown in (a) and (b) plus *npr1*-41 in (c). Col-0 is a positive control and *npr1*-1 a negative one for response to SA and BTH.

Sequence and analysis of the alleles

The next step was to sequence the *NPR1* gene in these alleles to identify the mutations. Table 2 shows the result of the sequence, presenting the nucleotide change, AA change, mutagen, and genetic background. There was a mutation in the structural gene in all the alleles, except in the case of *npr1*-62. All the attempts to amplify a part of this allele by PCR failed regardless of the pair of primers, or the origin of the DNA (data not shown), likely due to a large deletion or rearrangement. Some mutations are repeated two or three times. Although we cannot rule out that these repetitions are caused by contamination from one family to another, we consider them independent events based on our screening procedure described above. Note that some of these were isolated from different backgrounds, and we did not recover the *npr1*-1 control (Table 2), even though it was present in all the screening batches.

Allele	Nt mutation	AA mutation	Mutagen	Background
npr1-20	G → A	V501M and *splicing* 4th exon	EMS	*rar1*-21
npr1-21	C → T	Q384*	EMS	*rar1*-21
npr1-22	G → A	R544K	EMS	*PR1:LUC*
npr1-23	G → A	*Splicing* 2nd exon	EMS	*PR1:LUC*
npr1-24	C → T	L497F	EMS	*PR1:LUC*
npr1-25	G → A	E443K	EMS	*PR1:LUC*
npr1-26	G → A	*Splicing* 4th exon	EMS	*PR1:LUC*
npr1-27	G → A	D428N	EMS	*PR1:LUC*
npr1-28	C → T	R538*	EMS	*PR1:LUC*
npr1-29	G → A	*Splicing* 4th exon	EMS	*PR1:LUC*
npr1-30	C → T	A451V	EMS	*PR1:LUC*
npr1-31	G → A	E449K	EMS	*PR1:LUC*
npr1-32	G → A	R432K	EMS	*PR1:LUC*
npr1-33	G → A	R432K	EMS	*PR1:LUC*
npr1-34	C → T	Q526*	EMS	*PR1:LUC*
npr1-35	G → A	C155Y	EMS	*PR1:LUC*
npr1-36	G → A	E449K	EMS	*PR1:LUC*
npr1-37	C → T	Q491*	EMS	*PR1:LUC*

npr1-38	G → A	R493K	EMS	*RPM1-MYC*
npr1-39	C → T	S512L	EMS	*rpm1*-1
npr1-40	G → A	C306Y	EMS	*rpm1*-1
npr1-41	G → A	E288K	EMS	*rpm1*-1
npr1-42	G → A	G504E	EMS	*rpm1*-1
npr1-43	G → A	*Splicing* 4th exon	EMS	*NahG*
npr1-44	C → T	H80Y	EMS	*NahG*
npr1-45	C → T	Q 371*	EMS	*NahG*
npr1-46	C → T	L497F	EMS	*NahG*
npr1-47	C → T	L497F	EMS	*NahG*
npr1-48	C → T	L515F	EMS	*NahG*
npr1-49	C → T	Q491*	EMS	*NahG*
npr1-50	C → T	L274F	EMS	*NahG*
npr1-51	G → A	R432K	EMS	*rar1*-21 *ndr1*-1
npr1-52	Deletion	N210FS	EMS	*rar1*-21 *ndr1*-1
npr1-53	C → T	Q343*	EMS	*rar1*-21 *ndr1*-1
npr1-54	G → A	*Splicing* 2nd exon	EMS	*rar1*-21 *ndr1*-1
npr1-55	C → T	Q491*	EMS	*rar1*-21 *ndr1*-1
npr1-56	G → C	A496P	EMS	*rar1*-21 *ndr1*-1
npr1-57	Deletion	Δ VDFML 164-168	T-DNA	Col-0
npr1-58	T → A	Y64N	T-DNA	Col-0
npr1-59	Deleción	V194*	Gamma	Col-0
npr1-60	C → T	P342S	Gamma	Col-0
npr1-61	Deletion	V194*	FN	Col-0
npr1-62	Unknown	Unknown	FN	Col-0

Table 2. *npr1* alleles obtained in this screening. The allele number (first column) is followed by the nucleotide mutation (second), the amino acid mutation (third), the mutagen used (fourth) and the background of each allele (fifth). Where a change in nucleotide produces a change in splicing, it is noted as "splicing" and the exon affected in the amino acid column. * Stands for stop codon. FS, frame shift. We could not get a PCR product for the *npr1*-62 allele. *rar1*-21 and *rar1*-21 *ndr1*-1 are described in Tornero et al. 2002b, *PR1:LUC* in Maleck et al. 2002, *rpm1*-1 in Grant et al. 1995, *RPM1-MYC* in Boyes et al. 1998 and *NahG* in Lawton et al. 1995.

The position and kind of mutation allow for a deep analysis, searching for correlations between the molecular data and the phenotype. Firstly, the simplest correlation would be a bias in the nature of the wt AA that has been mutated (e.g. more mutated polar AA than the average). There are 544 AA indices (www.genome.jp/aaindex/, V9.1), but no bias was found for any index when the values of the wt AA subject to mutation were compared with the average value all the AAs that make up NPR1 (see Methods for details). Since EMS is able to mutate AAs to a different extent (Sega 1984), and a significant amount of the mutations recovered are produced by EMS, an additional calculation was made. This time only the canonical point mutations caused by EMS (22) were compared with all the AAs that could be mutated by EMS in NPR1 (413). Again, no bias was detected (data not shown).

Secondly, the nature of the change was considered. Thus, the mutations could be biased in the properties of the new AAs (e.g. a trend towards more polar AAs). There are 94 AA substitution matrices (www.genome.jp/aaindex/, V9.1), but no bias was found. Note that we can only consider the EMS mutations that produce a point mutation in order to have a background (all the possible new AAs that EMS can produce in NPR1, 516) that can be contrasted with the observable mutations.

Thirdly, the conservation of the AA could also mark the mutations. There are five paralogs of *NPR1* in Arabidopsis, *NPR2, NPR3, NPR4, BOP1* and *BOP2*. The predicted sequences of the proteins were aligned with CLUSTALW (Chenna et al. 2003), which provides a numerical value for each AA based on its conservation. The AAs mutated in the alleles recovered in this work (EMS point mutations) are more likely to be conserved among the paralogs, in a modest but significant fashion (Student´s T test, $p \approx 0.03$).

Fourthly, we also analyzed whether the mutations were clustered. Figure 3a shows the localization of the mutations in the backbone of the protein. It is obvious that the mutations are not clustered in any domain, but were more frequent in a small region, between the ankyrin repeats and the nuclear localization signals (see below). Some of the mutations are in introns (Figure 3b), but since they are at the beginning or the end of an intron, they affect the splicing and the coding sequence. Figure 3c shows RT-PCR with primers designed to amplify exon junctions. *npr1*-54 (also *npr1*-23, data not shown) does

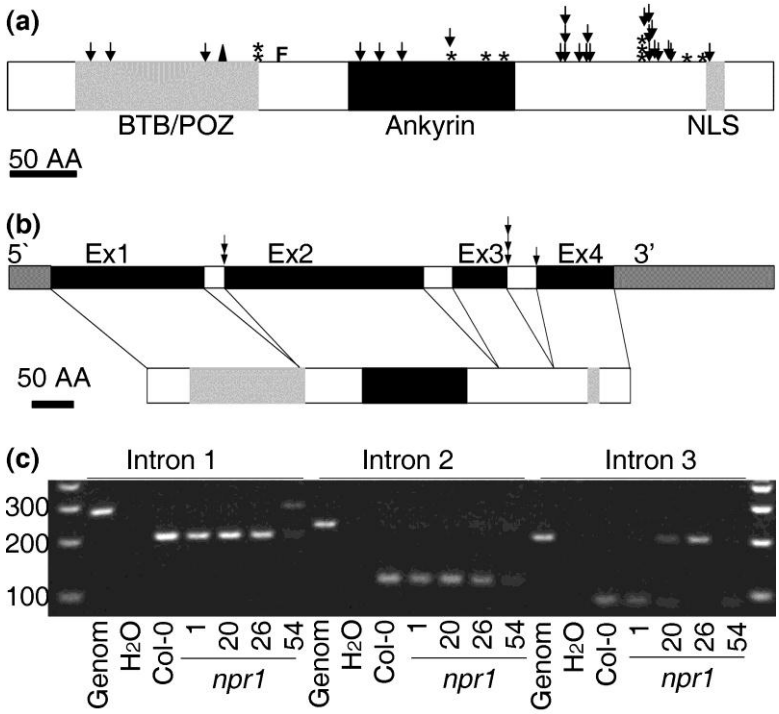

Figure 3. The distribution of mutations in *npr1* is biased. The localization of the mutations found is represented along the structure of NPR1. (a) Mutations in exons of *NPR1* found in this screening. The structure of NPR1 with its domains is shown, along with a 50 AA scale. The arrows indicate point mutations, the asterisks stop codons, the letter "F" frameshift, and a triangle an internal deletion. (b) Mutations in introns of *NPR1* found in this screening. The structure of introns and exons of *NPR1* is shown, with the position of the mutations that alter the splicing. "Ex" stands for Exon. Note that *npr1*-20 is in both (a) and (b), since it belongs to both categories. (c) RT-PCR of the mutations in introns. The picture shows an ethidium bromide agarose gel with its molecular weights in nucleotides on the left side. Specific PCR probes for each exon-exon junction were used (labelled Intron 1 to 3) in Genomic DNA, as a positive control; H_2O, as a negative control, and RTs from the labelled genotypes. *npr1*-29*,* *npr1*-43*,* and *npr1*-26 have the same mutation, and only the last one is showed. Also, *npr1*-23 and *npr1*-54 share the same change and the second is showed. Ankyrin, Ankyrin Repeat Motifs (four of them); BTB/POZ, Broad-Complex, Tramtrack and Bric-a-brac proteins, Pox virus and Zinc finger proteins; NLS, nuclear localization signal.

not splice the first intron properly, introducing a frameshift in the coding sequence. In *npr1*-26 (also *npr1*-29 and *npr1*-43, data not shown) the third intron is not spliced, again creating a frameshift. A peculiar case occurs with *npr1*-20. In this allele, the mutation is a change from Valine to Methionine in the position 501 (Table 2). Since this is a conservative change, the wt AA had to be a very important one or have an additional effect. Indeed, the mutated nucleotide is the first one of the fourth exon, and it does alter the splicing of the fourth exon (Figure 3c).

The stop codons obtained are not randomly distributed, but clustered in a small region. The coding sequence of NPR1 was divided into windows of 50 AAs (Tornero et al. 2002a), and the number of stop codons found in each window plotted (Figure 4a). The tenth window is enriched in stop codons, and the statistical significance of this enrichment can be calculated with a Poisson distribution (for 3 alleles/50 AA, $p \approx 0.042$). The frameshifts are not point mutations, nor stop codons, since there are additional AAs introduced before a stop codon is found. However, if the frameshifts were to be considered equal to the stop codons, the same window (plus an additional one between 150 to 200 AA) is significantly enriched (Figure S2a). Extending the argument, if we were to consider not only the *npr1* alleles described here, but also the ones obtained before (5 alleles between stops and frameshifts, Cao et al. 1994; Delaney et al. 1995; Glazebrook et al. 1996; Shah et al. 1997), the result still holds (Figure S2b).

There are 25 point mutations in this screening. Surprisingly, there is no enrichment in the described domains of NPR1, but in the region between the ankyrin repeats and the NLS (Figure 4b, windows from AA 400 to 550). In this case, the probability of this clustering being a random event is lower than before (Poisson distribution for 7 alleles/50 AA, $p \approx 0.004$). As happened with the stop codons, the biased distribution still holds with the inclusions of point mutations from previous screenings (Figure S2c). The only difference is that two windows show mutations clustered instead of three, but with a higher significance ($p \approx 0.001$).

The last two points (conservation and localization of the mutations) are related. Thus, 68% of the point mutations are found in 15% of NPR1 (17 out of 25 mutations in the region between AAs 428 and 515). This region is highly conserved in NPR2, NPR3 and NPR4, but not with BOP1 and BOP2 (Figure

4c). Although there is no homology with a described domain, this region has been described as a repression region of NPR1 (Rochon et al. 2006).

Fifthly, there could be more quantitative information in the position. Since there is some variation in the strength of alleles in terms of PFW (Figure 2a), we searched for a correlation with the position. There is no significant correlation between stops and point mutations when ordered by phenotype (data not shown) and their localization in the protein.

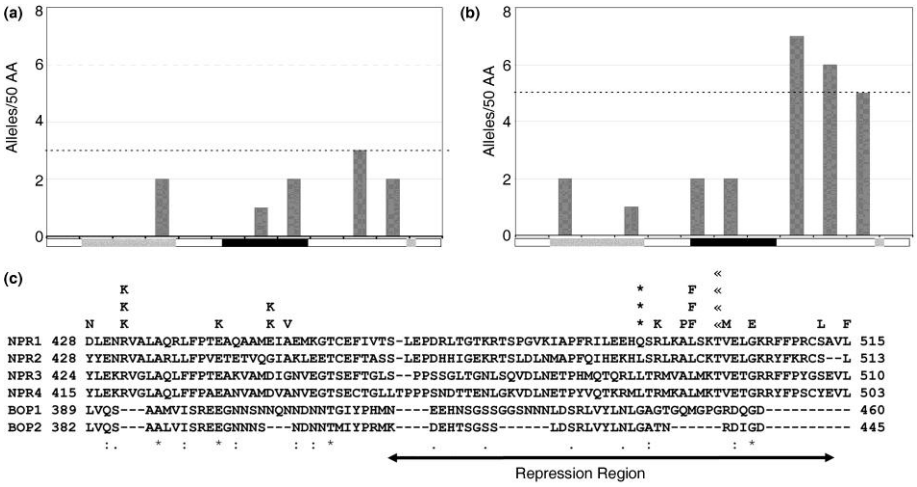

Figure 4. Statistics of the distribution of mutations. (a) Distribution of stop codons. In each 50 AA window (X- axis), the number of stop codons is represented as a bar (Y-axis). The schematic structure of NPR1 is represented in the X-axis for visualization of the domains. The dotted line shows where the distribution is not random (Poisson distribution, 3 alleles/50 AA $p<0.05$). (b) Distribution of point mutations, as described in (a). In this case, the dotted line is at 5 alleles/50 AA (Poisson distribution, $p<0.05$). (c) Distribution of mutations in a selected region (AA428 to 515) of NPR1. The mutations are showed over the wt AA, with the alignment of the NPR1 clade. The stop codons are marked with an asterisk, and the frameshifts (due to altered intron splicing) with a "«". The repression region described by Rochon et al. 2006 is marked with an arrow.

npr1 null alleles

From the information presented we conclude two important points. The screening did not produce a clear *npr1* null allele, and the point mutations obtained are clustered. The logical step was to check if there was a question of lethality (i.e. NPR1 has an essential role and only mutations that do not alter that role are recovered) or redundancy (SA perception is accomplished by other proteins, and only mutations that affect NPR1 and other proteins are recovered). It follows that it would be a partial redundancy since the screening provided a majority of *npr1* alleles (43 out of 56 or 77%, considering only one mutant per M2 family). We reasoned that the existence of *npr1* null alleles would discern between these two possibilities, so a search in the databases for knockouts in genes was initiated. Three insertions that could be taken to homozygosis were identified at the time of the search (Figure 5a). *npr1*-63 is in Col-0 background, while *npr1*-70 and *npr1*-71 are in La*er*-0 (see Methods for details). While *npr1*-63 could produce a protein with the two known domains, *npr1*-70 and *npr1*-71 contain transposon insertions at AA 55 and 40, respectively. Using the same set of primers of Figure 3c, no mRNA was detected in *npr1*-70 and *npr1*-71 (data not shown), so both were considered as null alleles.

The phenotype of response to BTH in terms of fresh weight is strikingly intermediate in *npr1*-70 and *npr1*-71 (Figure 5b), low enough to avoid selection in our screening. This intermediate response could be attributed to a difference in ecotypes, since both null alleles are in La*er*-0 background. An F2 between *npr1*-1 and La*er*-0 analyzed with BTH as in Figure 1a did not deviate from the expected results (Figure S3a), so if there is any difference between the alleles, it is not detectable to the naked eye. In order to have a proper control, *npr1*-1 was crossed with La*er*-0 three successive times with marker selection. Four independent lines were selected in the F2 of the third cross for *npr1*-1 homozygosis, and all four behave similarly to *npr1*-1 in response to BTH (Figure S3b). Therefore, only one line was included in the experiments, named as *npr1*-1_L (Figure 5b). The effect of BTH in the biomass of the null *npr1* alleles set them far apart from the other alleles (Figure 5b), but when SA or BTH treatment is followed by *Pto* growth, the differences are smaller, but still significant (Student´s T test, Figure 5c). Qualitatively, the alleles are also intermediates in their behaviour on MS-SA plates (Figure 5d; there are no differences between Col-0 and La*er*-0 in these plates, data not shown). As in

npr1-1 and in most of the alleles presented here, the expression of PR1 in inductive conditions is not detectable in *npr1*-70 and *npr1*-71 (data not shown).

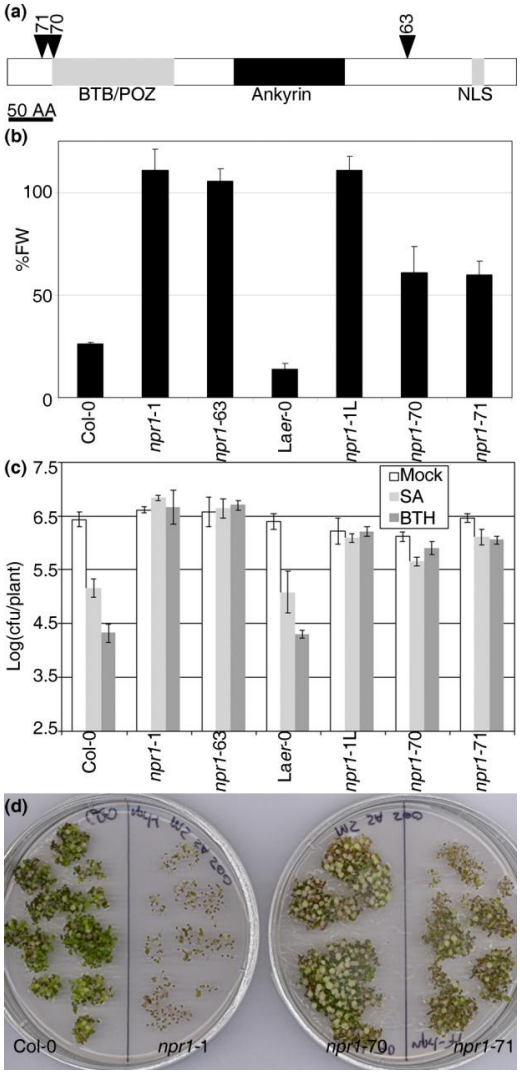

Figure 5. Phenotype of null npr1s. (a) Three insertions (T-DNA and transposons) were characterized, and their localization is shown. Note that *npr1*-70 and *npr1*-71 are in background La*er*-0. (b) Response to BTH in terms of fresh weight, as in Figure 2a. *npr1*-1_L is an introgression of *npr1*-1 in the background La*er*-0. (c) Response to SA and BTH in terms of *Pto* growth, as in Figure 2b. The response of *npr1*-70 and *npr1*-71 to SA and BTH, although small, is statistically significant (T-test, $p<0.05$). (d) Response to SA in MS-SA plates. Col-0, *npr1*-1, *npr1*-70, and *npr1*-71 as in Figure 1b.

NPR1 paralogs

The conclusions from the previous results are that the null alleles exist and express an intermediate phenotype. Hence the lethality hypothesis to explain the lack of null alleles in our screen is not valid, and the results are consistent with a partial redundancy in SA signalling. So the question that arises is the identification of these protein(s). The simplest explanation is that proteins with a similar sequence perform similar functions. In other words, the paralogs of *NPR1* could have a role in SA signalling. There are five paralogs of *NPR1* in Arabidopsis, and the knockouts of these genes in a *NPR1* wt background do not have a strong impact on SA signalling (Canet et al. 2010a). Regarding *NPR2*, our initial characterization was carried out with *npr2*-1, which has a T-DNA in an intron. Since this could be a functional allele, a second allele, *npr2-2,* with a T-DNA inserted in the first exon was used (Figure S4). Crosses of *npr1*-70 and *npr1*-71 with *npr2*-2, *npr3 npr4*, and *bop1 bop2* were carried out. The resulting F2s are a mixture of ecotypes, so we analyzed by weight across F2 pools to take into account the ecotype variation. The BTH treatment guarantees that the null alleles are selected, and by picking up the biggest plants in each family any additional effect of the paralogs will be detected (if such effect exists). Figure 6a shows these F2s, including the F2 with Col-0 as a baseline. All the F2s of *npr1* nulls with paralogs have more biomass than the F2s with Col-0. To further confirm this result, F2 plants were selected by BTH and PCR to found the double mutants between *npr1*-70 and the paralogs. 8 independent lines of *npr1*-70 *npr2*-2 were characterized, along with 8 independent introgressions of *npr1*-70 in Col-0 as a control. Figure 6b shows the maximal and minimal values of each set of lines. A T-test with the pooled values of all the lines indicates that *NPR2* has an impact in SA perception ($p \approx$ 3E-5). This impact is also found in the other paralogs, from which 4 (*npr1*-70 *npr3, npr1*-70 *npr4 npr1*-70 *bop2*) and 2 (*npr1*-70 *bop1*) independent lines were selected. The degree of certainty is not as strong as in *npr2*, but it is still significant (*npr3*, $p \approx$4E-3; *npr4*, $p \approx$5E-3; *bop2*, $p \approx$5E-3; *bop1* $p \approx 0.04$). Therefore, all the paralogs have a measurable function in SA perception in an *npr1* null background. The increase in biomass is small, and yet the five paralogs explain the difference between *npr1* nulls and alleles like *npr1*-1.

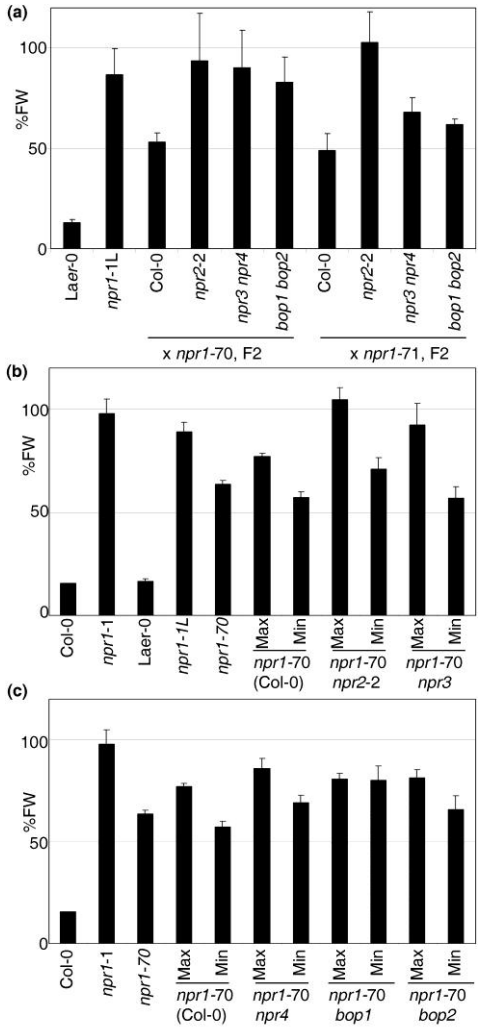

Figure 6. Role of the paralogs in response to BTH. (a) F2s of the indicated genotypes were analyzed as in Figure 2a, but selecting the biggest plants in each family. The baseline is the F2 of the null *npr1* alleles with Col-0. (b) Double mutants between *npr1*-70 and the paralogs were selected by phenotype and PCR in F2, and their progeny tested as in Figure 2a. Only the maximal and minimal values are showed from 8 (Col-0 and *npr2*-2) and 4 (*npr3*) independent lines. (c) Similar to b, but showing the response of the rest of paralogs. 4 (*npr4* and *bop2*) and 2 independent lines (*bop1*) were analyzed. For each double mutant, the pooled values of all the tested lines were found to be significantly higher than the pooled values of all the *npr1*-70 (Col-0) lines (T-test, $p<0.05$).

Discussion

Genes required for SA signalling

The screening described in Figure 1 and Table 1 has provided us with a wealth of information about the genes required for SA signalling. It is difficult to compare different mutagenesis, even when the same mutagen at the same dose was used. Nevertheless, and considering only EMS, there are 69 mutants in 600 M2 families. In a similar selection, 110 mutants in the recognition of *avrRpm1* were detected in 172 M2 families (Tornero et al. 2002a), more than five times the amount presented here. The main reason for the abundance of mutations was the 95 alleles of *RPM1*, a gene that is deleted in some ecotypes (Grant et al. 1998), so is not essential for the plant. Assuming that we recover all the mutants of *npr1* (as in the proof of concept of Figure 1a), the low numbers of mutants found indicate that lethality and/or redundancy limits their number.

We cannot discard the idea that the model used, loss of biomass upon BTH treatment, is limiting the scope of mutants obtained. Our previous work done with the model suggests that, upon BTH treatment, one branch stops the growth of the plant ("scorched earth defence"), while another produces active defences against the pathogen (Canet et al. 2010a). Therefore, we could lose mutants that are impaired in pathogen response, but not in biomass response. In any case, the mutants in SA recognition itself would be enriched, even if we do not gain knowledge about the branch that stops the pathogen. Regarding the possible bias in the mutants, previous screenings using pathogens (Glazebrook et al. 1996) or gene expression (Cao et al. 1994; Shah et al. 1997) gave only *npr1* alleles, so it seems that we have not introduced any bias, but expanded the identification of *npr1* alleles and other genes. It is also important to notice that all the new *npr1* alleles obtained with BTH are not responsive to SA in terms of pathogen growth and in growth in MS-SA plates. Therefore, and for the data here presented, a screen for lack of response to BTH leads to mutants that do not perceive SA.

The mutations in *npr1* are clustered

All the alleles recovered show a similar phenotype in weight and in *Pto* growth upon defence induction (Figure 2a and b). Also, there is no discrimination of

the chemicals SA and BTH by the new *npr1* alleles. The main difference is the phytotoxicity of SA (Figure 1b and data not shown), and the stronger resistance triggered by BTH with respect to SA (Figure 2a).

The results obtained with the marker PR1 were unexpected (Figure 2c), since there are two alleles that produce a low but detectable amount of this defence marker in all the experiments. The case of *npr1*-20 is unique, since the nucleotide mutation leads to a prediction of a conserved change (Valine to Methionine), but experimentally there is evidence for a different splicing (Figure 3c). The mutation could produce a fraction of *NPR1* mRNA with the right splicing and a conserved change which would explain PR1 production. Regarding *npr1*-41, the mutation is a change from Glutamic acid to Lysine, a change from acidic to basic AA that is recovered in other mutated AAs (Table 2). The only particular feature of this allele is that it is located in the ankyrin repeats, where few mutations are localized. A mutation like this in one of the ankyrin repeats may compromise the overall function of the protein, but not abolish the induction of PR1.

The new *npr1* alleles are not biased in a considerable number of features (www.genome.jp/aaindex). There is no preference for any property of AA to be mutated from or to, including the cysteines that have been reported to be critical for the oligomerization (two cysteines out of the nine tested by (Mou et al. 2003). More importantly, the known domains of NPR1 do not have more mutations than the rest of the protein. Note that we assume that all the nucleotides have an equal probability of being affected by the mutagen, and what changes is the selection based on phenotype (lethal, wt, or mutant). In the case of EMS (86% of the alleles) this is experimentally true, since all the regions of the genome have a similar probability of being mutated. There is a local compositional bias, but it does not produce any significant difference between triplets (Greene et al. 2003). Since the mutagens do not have a preference for any region, it follows that there are a number of alleles that are not recovered. They could be detrimental to the plant, or could produce a phenotype similar to the wt allele. We searched in the databases for alleles of *NPR1* (thus without selection) that could differentiate between the two explanations; lethality vs. redundancy. Two of these alleles have an insertion very early in the gene and they can be taken to homozygosis (Figure 5a). Furthermore, their phenotype in terms of weight is intermediate (Figure 5b).

Although the intermediate phenotype is quantitative strong, it is close to the threshold of detection in a screening (Figure 1c), explaining why no null alleles were recovered in this screening. We speculate that only mutations that affect *NPR1* and have a supplementary negative effect are recovered. This additional effect would be to inhibit other proteins that could work in the SA signalling pathway. In any case, lethality can be ruled out, and the results fit with a partial redundancy in SA perception. It is partial because the null alleles of *NPR1* produce a quantitative strong effect, and *npr1* alleles were 77 % of the mutants recovered.

The *npr1* null alleles are in L*a*e*r*-0 background. We discarded the possibility that the intermediate phenotype is due to the difference between ecotypes based on several facts. There are no significant QTLs between Col-0 and L*a*e*r*-0 in terms of PFW (Canet et al. 2010a). Besides, there is no observable variation of *npr1*-1 in L*a*e*r*-0, with segregations that fit the expected value (Figure S3a), and the introgression lines of *npr1*-1 in L*a*e*r*-0 behave similarly (Figure S3b). Lastly, the F2 or F3s of the nulls with Col-0 show that, while there is variation due to different lines isolated, there is no difference between ecotypes when the null alleles are present (Figure 6a and 6b). Therefore, there is no intrinsic difference between the ecotypes and there is no specific interaction of the alleles with one ecotype.

NPR1 paralogs have a role in BTH perception

The existence of *npr1* nulls with an intermediate phenotype reveals that other proteins besides NPR1 are acting in BTH perception at the same point. The mutations are enriched in AA conserved among the NPR1 clade, and are clustered in a conserved region (Figure 4c). This region coincides almost perfectly to a repression region described in NPR1 (AAs 463 to 513, Rochon et al. 2006). Although the repression region was not functional in SA stimulated cells, the bias observed in the mutations could indicate a stronger repression, perhaps extending its function in SA stimulated cells.

Thus, a simple model that can explain the bias in the mutations is that they could enhance the mentioned repression region, locking the protein in a repression state when it is close to the target DNA. The problem with this hypothesis is that -while explaining the observed bias- it does not explain how

some mutations have a strong phenotype, without having a repression region (i.e. stops and frameshifts). A second alternative is that a mutated npr1 protein could interact negatively with interactors of NPR1 reducing the availability of these proteins. There are two families of NPR1 interactors described so far. *NIMINs* are repressors of *NPR1* (Weigel et al. 2005), and there is no known interaction with the *NPR1* paralogs, so there is no simple model that takes into account all the requirements. The *TGAs*, on the other hand, are members of a multigenic family (Jakoby et al. 2002), that also interact with NPR3, NPR4 (Zhang et al. 2006), BOP1, and BOP2 (Hepworth et al. 2005), so they seem a clear candidate to be titred out by the npr1 mutated proteins. A third option can be postulated without requiring interactors. If the paralogs have the same mechanisms of oligomerization as NPR1 does (Tada et al. 2008), an npr1 defective protein could interfere with the monomerization of the paralogs, reducing their activity.

The behaviour of the new *npr1* alleles does not fit into conventional recessive or dominant negative alleles, but into a mixture of properties. When considering only *NPR1*, they are recessive, but when two copies of the allele are mutated, they interact with the paralogs as dominant negatives. A similar mechanism has been described before for closely related paralogs. Five recessive alleles of *hsp90.2* show a phenotype of loss of recognition of *avrRpm1*, while the knockout does not have any distinctive phenotype (Hubert et al. 2003; Hubert et al. 2009). The model proposed was that the HSP90s interact with RPM1, and a knockout is functionally replaced by the remaining paralogs. In this model, only mutations that abolish the function of HSP90.2 and interact negatively with other paralogs (HSP90s form heterodimers, Richter et al. 2001) are recovered. The fact that all the alleles recovered are in the same gene leads the authors to propose a certain specificity or preference. The analogies with NPR1 are not complete, but notable. The differences are that NPR1 nulls have a measurable phenotype and the number of NPR1 alleles recovered is one order of magnitude higher.

Following a similar model, we propose that all the members of the *NPR1* clade participate in the perception of BTH, with a strong preference for *NPR1*. This model explains the results of the screening: the low number of *NPR1* alleles, the lack of null alleles, and the clustering of the mutations (see below). There is a precedent in this family of an allele with an analogous behaviour to the new

npr1 alleles; *bop1*-1 has a stronger phenotype than several *bop1* nulls (Ha et al. 2004).

It is tempting to speculate with a correlation between the homology with NPR1 and the importance of the paralogs in BTH/SA perception. The order from more homology to less is *NPR2*, *NPR4*, *NPR3, BOP1* and *BOP2* (Liu et al. 2005). The F2 and F3 data of Figure 6 predict a role for all the paralogs with different degrees, from a strong one for *NPR2*, to a weaker, yet significant, for *BOP1*. There are several arguments in favour of a possible degree of functionality. Thus, *NPR2, NPR3* and *NPR4* are slightly induced upon biotic stress (like *NPR1*), while *BOP1* and *BOP2* are repressed (www.arabidopsis.org). Also, the clustering of the mutations in *NPR1* is stronger in a region that it is highly conserved with *NPR2, NPR3* and *NPR4*, but not with *BOP1* or *BOP2* (Figure 4c), so there is ample evidence for a predominant role of the three closer paralogs. Of course the model proposed does not exclude that other genes are required in BTH/SA perception, and they could be the mutations that complement *npr1*.

Acknowledgments

This work was supported by a BIO2006-02168 grant from Ministerio de Ciencia e Innovación (MICINN) of Spain to PT, a JAE-CSIC Fellowship to JVC and a FPI-MICINN to AD. We thank the English translation service of the Universidad Politécnica de Valencia. We appreciate the BTH provided by Syngenta. Thanks also to Jeff Dangl, Pablo Vera and José León for helpful comments on the manuscript.

Supporting information

Figure S1. Screening for non-recognition of BTH. Picture of a candidate of the screening after the second selection. The original name of the candidate is AT26, and it was later confirmed to be an *npr1* allele (*npr1*-57). Also shown Col-0 as a positive control and *npr1*-1 as a negative control. Note that the plants have no particular phenotype upon mock treatment.

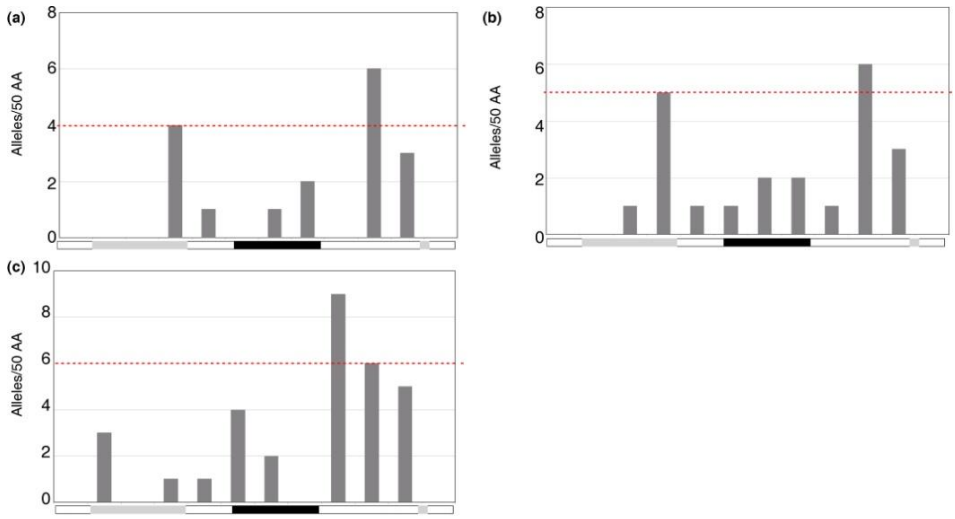

Figure S2. Extending the analysis of the distribution of mutations.(a) Distribution of stop codons. In each 50 AA window (X- axis), the number of stop codons is represented as a bar (Y-axis). The schematic structure of NPR1 is represented in the X-axis for visualization of the domains. The difference with Figure 4a is that here we include also frameshifts and mutations in introns that alter the splicing. The dotted line shows where the distribution is not random. (b) Similar to (a), including stops, frameshifts and mutations that alter the splicing found in other screenings (Cao et al. 1994; Delaney et al. 1995; Glazebrook et al. 1996; Shah et al. 1997). (c) Distribution of point mutations. The difference with Figure 4b is that here we include point mutations found in other screenings.

(a)

F2 npr1-1 x Laer-0

First experiment

	wt	mut	TOTAL		
Observed	163	58	221		
	wt	mut	Chi square		Maximum Chi square
Expected	165.75	55.25	0.1825038	<	3.8415
	(x3/4)	(x1/4)			

Second experiment

	wt	mut	TOTAL		
Observed	150	52	202		
	wt	mut	Chi square		Maximum Chi square
Expected	151.5	50.5	0.0594059	<	3.8415

(b)

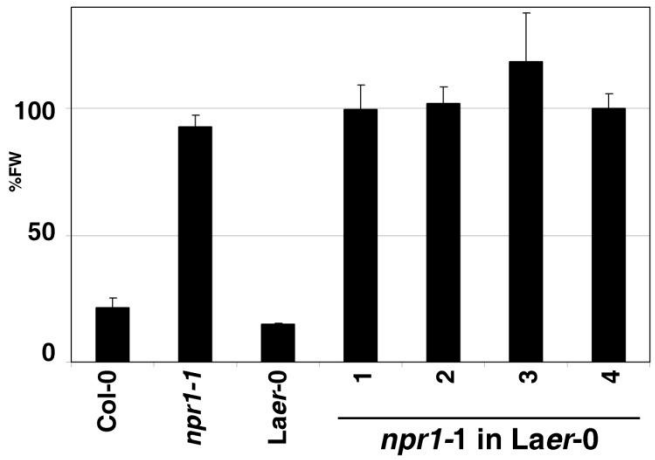

Figure S3. Introgression of *npr1*-1 in La*er*-0. **(a)** Chi square distribution of an F2 from *npr1*-1 x La*er*-0. Two independent experiments are shown, and in both cases, the distribution fits in a single recessive gene. **(b)** Characterization of four introgression lines as described in Figure 2a. *npr1*-1 was crossed to La*er*-0, the F1s selected by PCR marker for heterozygosis at the *npr1*-1 locus, and then crossed again with La*er*-0, repeating this process three times. Then, an F2 was selected by PCR marker for *npr1*-1 homozygous plants. The four lines behaved similarly. These experiments were repeated two times with similar results.

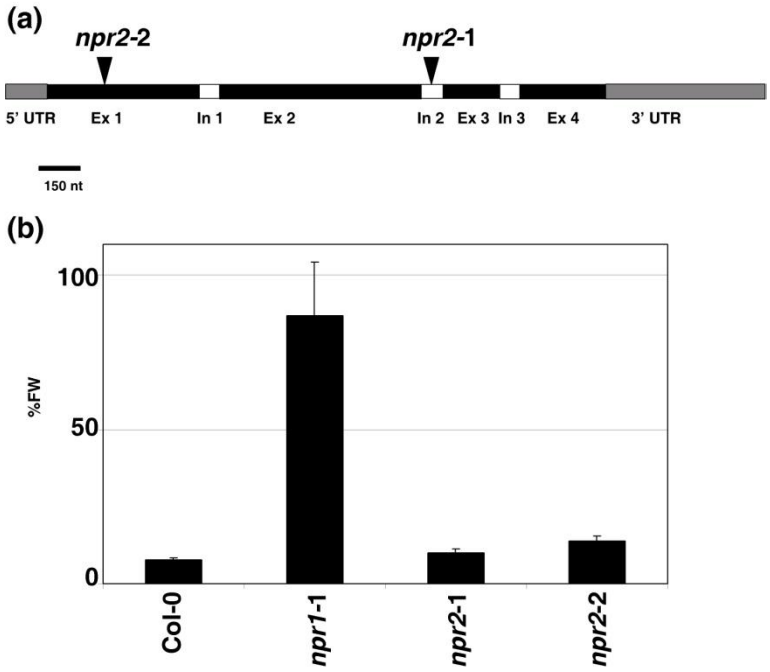

Figure S4. Figure S4.-T-DNA insertions in *NPR2*. (a) Two T-DNA insertions are shown, along the structure of NPR2. *npr2*-1 has been previously described (Canet et al. 2010a). **(b)** Phenotype of PFW upon BTH treatments of *npr2*-1 and *npr2*-2, as described in Figure 2a.

Artículo 3

The *Blade-On-Petiole* genes of Arabidopsis are essential for resistance induced by methyl jasmonate

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Este artículo ha sido enviado para
su publicación (mayo 2012)

Abstract

Background: *NPR1* is a gene of *Arabidopsis thaliana* required for the perception of salicylic acid. This perception triggers a defense response and negatively regulates the perception of jasmonates. Surprisingly, the application of methyl jasmonate also induces resistance, and *NPR1* is also suspected to be relevant. Since an allelic series of *npr1* was recently described, the behavior of these alleles was tested in response to methyl jasmonate.

Results: The response to methyl jasmonate of different *npr1s* and *NPR1* paralogs was measured by the growth of a pathogen. We have also tested the subcelular localization of some npr1s, along with the protein-protein interactions that can be measured in yeast. The localization of the protein in *npr1* alleles does not affect the response to methyl jasmonate. In fact, *NPR1* is not required. The genes that are required in a redundant fashion are the *BOPs*. The *BOPs* are paralogs of *NPR1*, and they physically interact with the TGA family of transcription factors.

Conclusions: Some *npr1* alleles have a phenotype in this response likely because they are affecting the interaction between BOPs and TGAs, and these two family of proteins are responsible for the resistance induced by methyl jasmonate in wild type plants.

Keywords: methyl jasmonate, salicylic acid, Arabidopsis, NPR1, BOPs, defense.

Background

Plants are constantly defending themselves against pathogens by means of a wide array of mechanisms. Some of them are pre-existing (or non inducible) and others are induced in response to the pathogen attack. Salicylic acid (SA, reviewed by Vlot et al. 2009) is a plant hormone which is crucial for the inducible response of *Arabidopsis thaliana* (Arabidopsis) to biotrophic pathogens like *Pseudomonas spp* (Katagiri et al. 2002). When a pathogen is perceived, SA is produced and accumulated, producing a proper defense. This SA signaling

occurs not only where the attack takes place, since defense is also enhanced in leaves different from the one inoculated. This is called Systemic Acquired Resistance (SAR, Ross 1961). SA has an intricate crosstalk with other hormones, showing an overall negative crosstalk with auxins, ethylene (ET), and methyl jasmonate (MeJA, crosstalk of hormones reviewed by Lopez et al. 2008). In the case of JA, it has been shown that the active form *in planta* is JA-Ile (reviewed by Browse 2009), while in the laboratory is used exogenously as Methyl Jasmonate (MeJA).

NON-EXPRESSER OF PATHOGENESIS-RELATED GENES1 (*NPR1*) is the main gene required for SA perception (Dong 2004). There are five paralogs of *NPR1* in Arabidopsis (Zhang et al. 2006), *BLADE-ON-PETIOLE1* (*BOP1*) and *BOP2* have an important role in development (Ha et al. 2003), *NPR3* and *NPR4* have a role in defense (Liu et al. 2005), probably through SA perception (Fu et al. 2012), and no specific function for *NPR2* has been described, besides a secondary role in SA perception (Canet et al. 2010b). There are other genes that are relevant for signal transduction, like the family of *TGA* transcription factors whose products interact with NPR1 (Zhang et al. 1999), but they are required in a redundant fashion.

NPR1 has been described as having more than one role in defense, since it is also important in the Induced Systemic Resistance (ISR, Pieterse et al. 1998). ISR is defined as the resistance triggered at the leaves by a non pathogenic organism inoculated in the roots, and while SAR requires SA signaling, ISR requires MeJA and ET signaling. As with SA, exogenous applications of MeJA and ET trigger resistance in Arabidopsis towards some biotrophs, like *Pseudomonas* (Ton et al. 2002). It has been proposed that *NPR1* is relevant for the MeJA induced resistance (Pieterse et al. 1998, hereafter abbreviated as MIR), although MIR it is not necessarily equivalent to ISR. While the role of *NPR1* in SA perception takes place in the nucleus (Kinkema et al. 2000), its function in MIR is not so clearly understood. It has been described a cytosolic function of NPR1 crucial in cross-talk between SA and JA signaling (Spoel et al. 2003). Furthermore, Arabidopsis transcriptome analysis upon pathogen infection has suggested that such cytosolic function is also involved in the modulation of JA-dependent defenses (Glazebrook et al. 2003). The *npr1*-3 mutant, which produces a truncated cytoplasmatically localized npr1 protein with no nuclear localization signal, has been reported to be affected only in SA-

dependent gene expression, not in JA and ET dependent genes. In contrast, the *npr1*-1 mutant, which has a mutation in a key domain, is affected in the expression of SA, JA and ET-dependent genes (Glazebrook et al. 2003). More recent studies support such cytosolic NPR1 function as regulator of JA-dependent defense responses (Johansson et al. 2006; Leon-Reyes et al. 2009; Ramirez et al. 2010).

Since a series of *npr1* alleles has recently been available (Canet et al. 2010b), we tested the hypothesis that the role of *NPR1* in MIR is cytosolic. In this work, we show that *NPR1* has no role in MIR in wild type conditions, since the genes responsible for MIR are *BOP1* and *BOP2*, with an important part being played by the *TGAs*. Therefore, two genes required for the normal development of the leaf, are also required for plant defense.

Results

Role of NPR1 in MIR

NPR1 has been characterized as a result of observing the response to SA of the great number of alleles described for it (Cao et al. 1994). *NPR1* has also been described as essential for MeJA induced resistance (MIR), but there are differences between alleles, since *npr1*-1 and *npr1*-3 have different MIR (Ramirez et al. 2010; Dobón et al. 2011). *npr1*-1 and *npr1*-3 have other differences in phenotypes related to MeJA. Thus, the SA-JA antagonism is not present in *npr1*-1, but it is active in *npr1*-3 (Spoel et al. 2003). Other difference is the gene expression, whereas *npr1*-1 was affected in SA, JA, and ET dependent genes upon *Pto* inoculation, *npr1*-3 was only affected in SA dependent genes (Glazebrook et al. 2003). These different phenotypes have been attributed to the lack of nuclear localization in *npr1*-3, since the truncated cytosolic protein would be functional to modulated JA-dependent defense response (Leon-Reyes et al. 2009).

In order to determine the precise role of *NPR1* in MIR, and to asses the role of the cytosolic vs. nuclear localization, we tested an allelic series of 43 *npr1* alleles (Canet et al. 2010b). The *npr1* alleles produced a mixture of phenotypes in MIR (Fig. 1), and out of the 43 alleles tested, 11 had a significant MIR (MIR+, Fig.

1a), while the others showed no response (MIR-, 11 out of 32 are shown in Fig. 1b). These two categories of alleles did not share any obvious feature and had the same proportion of stops and point mutations in each category. In fact, in the MIR+ alleles tested, six point mutations were widely scattered along the protein (Fig. 1c, *npr1*-3 is included as reference).

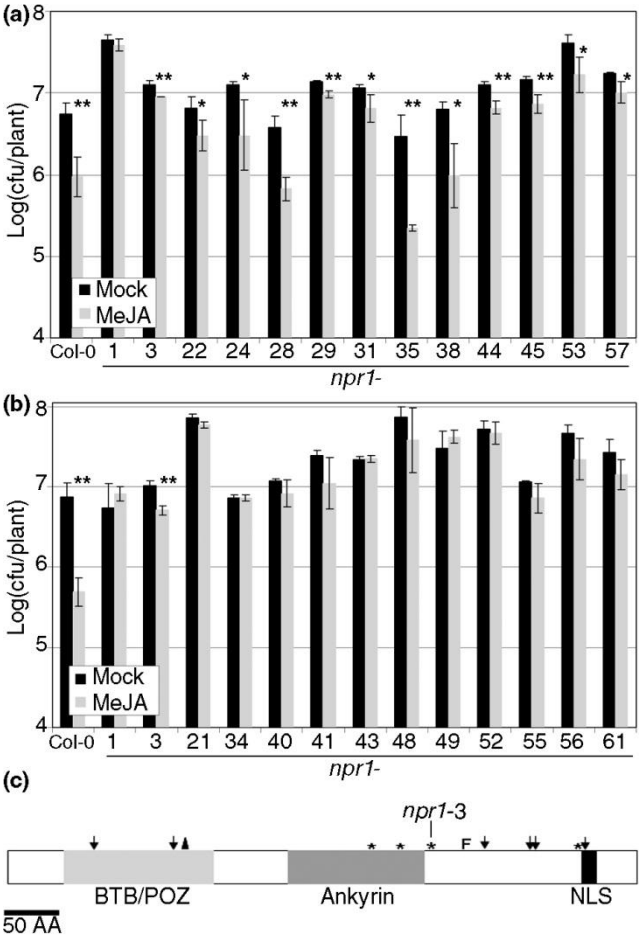
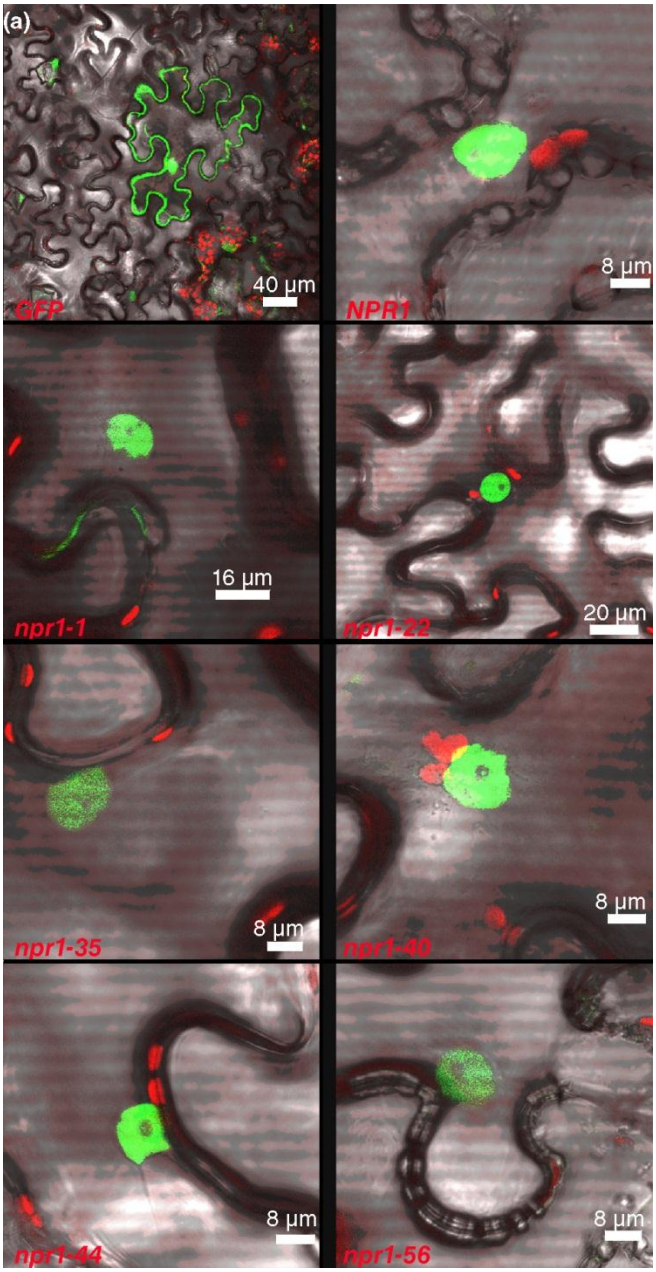


Fig. 1 *npr1* alleles differ in their response to MeJA. Forty-three *npr1* alleles were treated with 100 µM methyl jasmonate (MeJA), with 0.1% DMSO and 0.02% Silwet L-77 or a mock treatment. One day later, *Pseudomonas syringae pv. tomato DC3000* (*Pto*) was inoculated and its growth measured three days later in a logarithmic scale. For each genotype and treatment three samples with 5 plants per sample were taken. The bars show the average ± SD and *npr1*-1 and *npr1*-3 are included as

controls for negative and positive response to MeJA respectively. (a) *npr1* alleles that show resistance induced by MeJA. (b) *npr1* alleles that do not show resistance induced by MeJA. (c) Schematic representation of the *npr1* alleles that showed resistance induced by MeJA along the structure of NPR1. BTB/POZ stands for Broad-Complex, Tramtrack and Bric-a-brac proteins, Pox virus and Zinc finger proteins. Ankyrin for Ankyrin Repeat Motifs (4 of them) and NLS for Nuclear Localization Signal. The arrows indicate point mutations, the asterisks stop codons, the letter "F" frameshift, and a triangle an internal deletion. In all figures, the experiments were repeated at least three times with similar results. One asterisk means a significant difference with $P<0.05$, and two asterisks means $P<0.01$ (Student's test of one tail).

These alleles could somehow affect the localization of the protein inside the cell, even if the mutation was not in the NLS. To check this possibility, cDNAs of three MIR+ alleles (*npr1*-22, -35 and -44, Fig. S1), and three MIR- alleles (*npr1*-1, -40, and -56, Fig. S1), chosen among the point mutations, were cloned along with the wild type *NPR1*. Then, these seven cDNAs were transiently expressed in *N. benthamiana* under the control of the 35S promoter and with the GFP marker. The free GFP was detectable in the nucleus and in the cytoplasm. But when the wild type and the six alleles of *npr1* were expressed in the same conditions, GFP was detected mainly in the nucleus, with no difference existing between the two classes of alleles (Fig. 2a).

As a complementary approach, we took advantage of the transgenic line that overexpresses NPR1 fused to the steroid hormone binding domain of the rat glucocorticoid receptor (HBD, and the transgenic plants are known as NPR1-HBD, Kinkema et al. 2000). NPR1-HBD remains exclusively in the cytosol in mock conditions and should be functional in MIR. The original line is in an *npr1*-3 background (MIR+), and therefore the transgene was transferred to an *npr1*-1 background (MIR-) to check for complementation. Treatments with BTH with and without glucocorticoid dexamethasone (DEX) showed that NPR1-HBD was functional (Fig. S2). NPR1-HBD, even under the control of the 35S promoter, did not complement the lack of MIR in *npr1*-1 (Fig. 2b). When DEX was applied, NPR1-HBD moved to the nucleus and *npr1*-1 was complemented in the MIR phenotype. Note that the presence of cytosolic NPR1-HBD in an *npr1*-3 background did not enhance MIR in comparison to *npr1*-3 alone.


(a)
40 µm
GFP
8 µm
NPR1
16 µm
npr1-1
20 µm
npr1-22
8 µm
npr1-35
8 µm
npr1-40
8 µm
npr1-44
8 µm
npr1-56

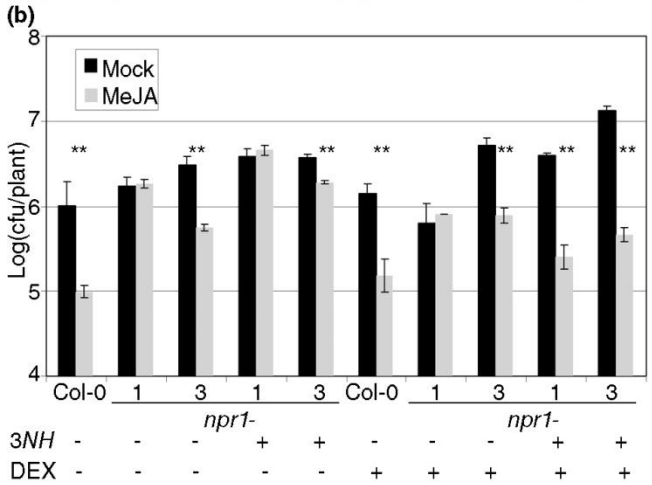


Fig. 2 The nuclear localization of *npr1* alleles is not relevant for resistance induced by MeJA. (a) Transient expression of *GFP*, *NPR1:GFP,* and six *npr1* alleles (three from Fig. 1a and three from Fig. 1b). *Agrobacterium tumefaciens* containing the mentioned genes under the promoter 35S were infiltrated in leaves of *Nicotiana benthamiana*, and the expression was detected with a confocal microscopy four days later. (b) The cytoplasmic anchoring of NPR1 does not complement *npr1*-1 in its response to MeJA. Plants with the transgene *35S:NPR1:HBD* (abbreviated as 3*NH*) in *npr1*-1 or *npr1*-3 background and its controls were treated with either dexamethasone (DEX) or mock solution, and then treated with either MeJA or mock solution. One day later, *Pto* was inoculated and measured as described in Fig. 1.

The *npr1* alleles MIR+ or MIR- did not share any obvious feature, so it would be difficult to assign a precise role to the wild type gene. A critical genetic resource to discern the role of a gene is the null allele. Therefore, the response of two null alleles of *npr1* to MIR was measured (Fig. 3a). Both *npr1*-70 and *npr1*-71 are in Laer-0 background, so an introgression of *npr1*-1 in Laer-0 was used as control (Canet et al. 2010b). These two null alleles responded to MIR in all experiments like the wild type. *npr1*-70 introgressed in Col-0 responded again like the wild type, which ruled out any ecotype effect (Fig. 3b). Since the direct role of *NPR1* in MIR was in question as a consequence of the aforementioned results, we included an independent MIR- control, *coi1*-40 (see Methods).

The role of NPR1 in this response might be indirect. Thus, one scenario would be a reinforcement of the negative crosstalking between SA and MeJA. *npr1* alleles produced more SA when infected with *Pto* (Zhang et al. 2010b) and seemed unable to metabolize it (Cao et al. 1997). MIR- alleles -defective in terms of SA perception- might have left intact the negative crosstalk between SA and MeJA, and an excess of SA repressed the response to MeJA beyond the wild type levels. Therefore, the MIR+ alleles would be defective in terms of both SA perception and SA-MeJA crosstalking, an explanation that would also be in agreement with the behavior of the null alleles. .

To test this hypothesis, the double mutants between *npr1*-1 and *NahG* (a transgenic plant that degrades SA, Lawton et al. 1995), *eds5* (a mutant in SA transport, Nawrath et al. 2002), and *sid2* (a mutant in SA biosynthesis, Wildermuth et al. 2001), were constructed and tested for MIR. *npr1*-1 did not respond to MeJA even if the levels of SA were low (Fig. 3c), so the hypothesis of a reinforcement of the negative crosstalk was not supported.

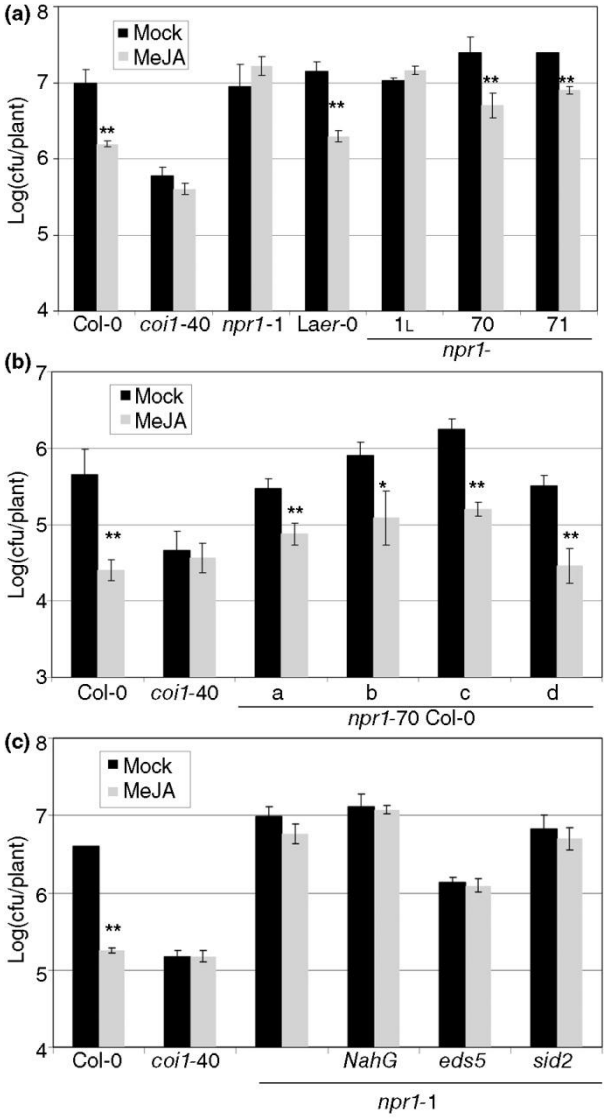
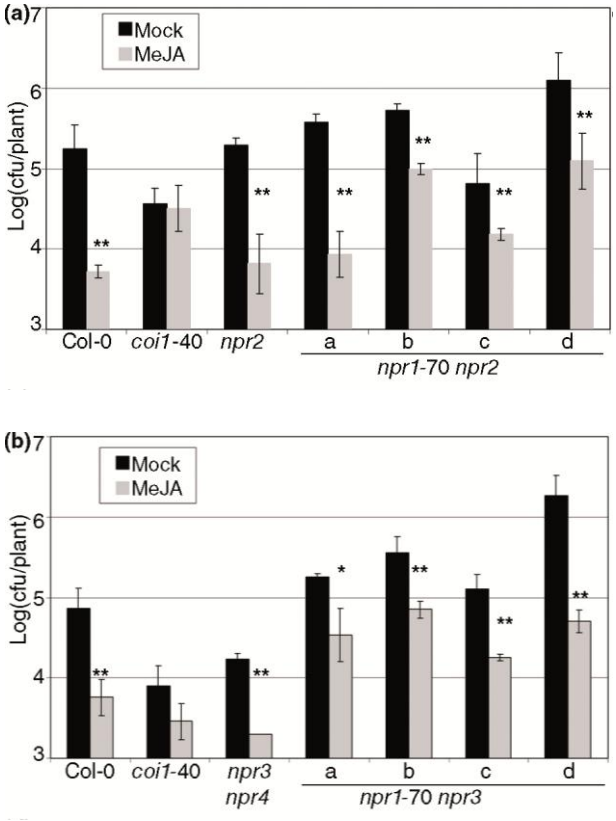


Fig. 3 *NPR1* is not necessary for resistance triggered by MeJA. (a) Two null *npr1* alleles (*npr1*-70 and *npr1*-71, both in La*er*-0 background), plus their controls were tested for resistance induced by MeJA as described in Fig. 1. *coi1*-40 is introduced as negative control for resistance triggered by MeJA. (b) Introgressed lines of *npr1*-70 in Col-0 show the same phenotype than the original *npr1*-70. (c) The effect of some *npr1* alleles on resistance triggered by MeJA is not due to an excess of salicylic acid. Double mutants of *npr1*-1 with *NahG*, *eds5*, and *sid2* were tested for their response to MeJA in resistance.

BOP1 and *BOP2* and their role in MIR

The experiments with the null alleles showed that *NPR1* was not necessary for MIR. Perhaps *NPR1* and other gene(s) would be redundantly responsible of MIR, and while null *npr1* alleles would have a MIR+ phenotype, some *npr1* alleles could be MIR- by interacting with other protein(s) negatively. The most likely candidates for these interactions were the *NPR1* paralogs, since their proteins shared the same overall structure. There are five additional paralogs of *NPR1* in the Arabidopsis genome (Zhang et al. 2006), and we analyzed double mutants of *npr1*-70 with different paralogs (Fig. 4). There was no proof of *NPR1* having a redundant role in MIR. *NPR2* did not play a significant role in MIR (Fig. 4a) and the same was true for the double *npr3 npr4* (Fig. 4b, c, and Zhang et al. 2006). Strikingly, the double *bop1 bop2* (Hepworth et al. 2005) was MIR- (Fig.4d).



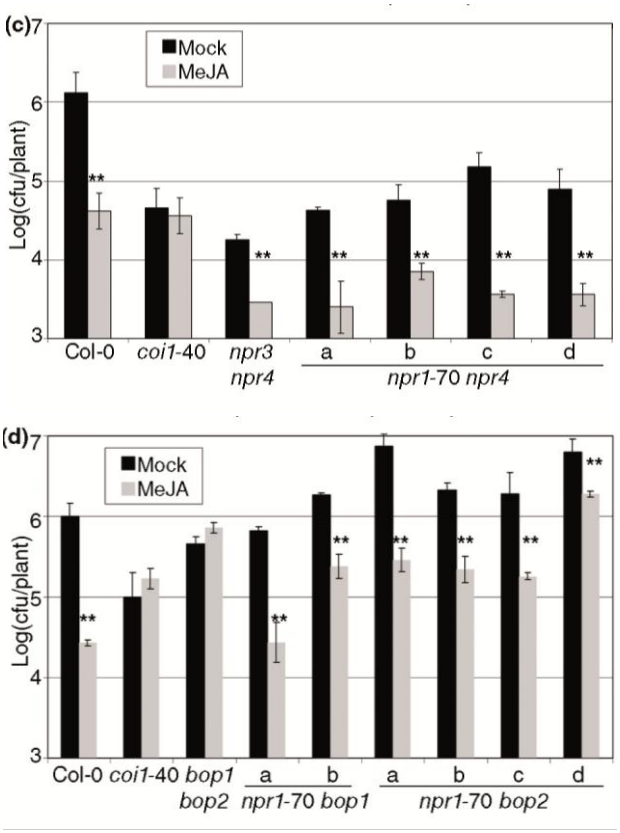
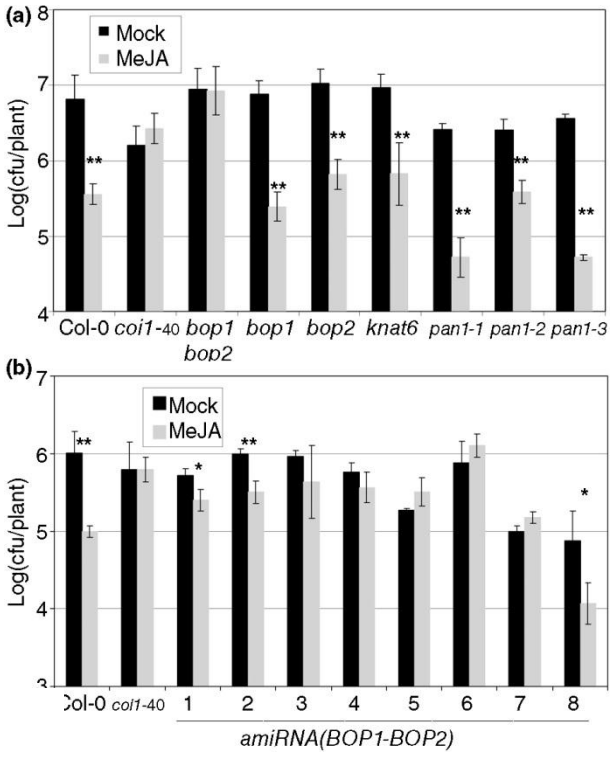


Fig. 4 A role for the *NPR1* paralogs in resistance triggered by MeJA. Double mutants of *npr1*-70 with *NPR1* paralogs and their corresponding controls were tested for resistance triggered by MeJA as described in Fig. 1. (a) Double mutants with *npr2*. (b) *npr3*. (c) *npr4*. (d) *bop1* and *bop2*. Four independent lines were tested in each case, except with *bop1*, where only two were obtained.

Single *bop1* and *bop2* were also tested for MIR and showed to be wild type (Fig. 5a). BOP1 and BOP2 exert their function in part through transcriptional regulation of KNAT6 and physical interaction with PAN (TGA8, Khan et al. 2012), but T-DNA insertions predicted to disrupt KNAT6 or PAN activity did not have an effect on MIR (Fig. 5a). To rule out the possibility that other mutations besides *bop1* and *bop2* were producing this MIR- phenotype, we constructed an artificial microRNA (amiRNA, Schwab et al. 2006) to deplete the levels of *BOP1* and *BOP2* at the same time. Eight independent homozygous transgenic lines for *amiRNA* (*BOP1 - BOP2*) were analyzed for MIR (Fig. 5b). Five out of eight lines were MIR-, and the remaining three responded less than the wild type control. The levels of both genes were partially depleted in the eight lines (Fig. 5c); two of the MIR- lines had both genes significantly reduced, and all had *BOP2* significantly reduced. None of these lines had the characteristic blade-on-petiole macroscopic phenotype, not even as the subtle phenotype of *bop1* alone.



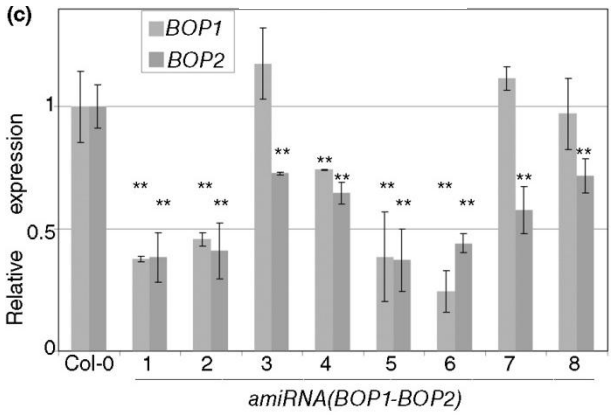


Fig. 5 Only the reduction of both *bop1* and *bop2* expression affects resistance triggered by MeJA. (a) The double *bop1 bop2*, the single mutants, T-DNAs insertions in *KNAT6* and *PAN1*, and their controls were tested for resistance induced by MeJA as described in Fig. 1. *KNAT6* and *PAN1* are genes that interact with *BOP1* and *BOP2*. (b) Reduction of *BOP1* and *BOP2* expression partially phenocopies the double *bop1 bop2*. Eight independent transgenic lines of an artificial micro RNA designed to reduce the levels of *BOP1* and *BOP2* (*amiRNA(BOP1-BOP2))* were tested as described in Fig. 1. The lines did not show any macroscopic Blade-on-petiole phenotype. (c) RNA was extracted from 3-week-old plants of the lines described in (b), and transcript levels for *BOP1* and *BOP2* were measured by means of RT-qPCR. Levels of expression are normalized to three reference genes and to the level of Col-0 in mock. Asterisks mark the significance of the difference between the levels of expression of each line with Col-0.

The previous experiments had shown that *BOP1* and *BOP2* were acting redundantly in MIR. Therefore, increasing the amount of any of them should have an effect on MIR, especially since normal levels of *BOP1* and *BOP2* are quite low (Fig. S3, Hepworth et al. 2005). The overexpression lines of *BOP1* and *BOP2* described (Norberg et al. 2005) were analyzed for MIR. *35S:BOP1* had a stronger MIR than Col-0, and *35S:BOP2* had a strong variation in the MeJA treated plants (Fig. 6a). At the time of the experiments, each population looked homogeneous, but when these plants were grown to set seeds, two phenotypes could be observed in each transgenic line. Approximately half of the plants showed a wild type phenotype, and the other half reproduced the

dwarf plants described (Norberg et al. 2005). Seeds from both lines and from both phenotypes reproduced the two phenotypes. It seems that it was an issue of silencing, since RNA taken from plants classified by their mentioned phenotype diverged widely in the transgene expression (Fig. 6b).

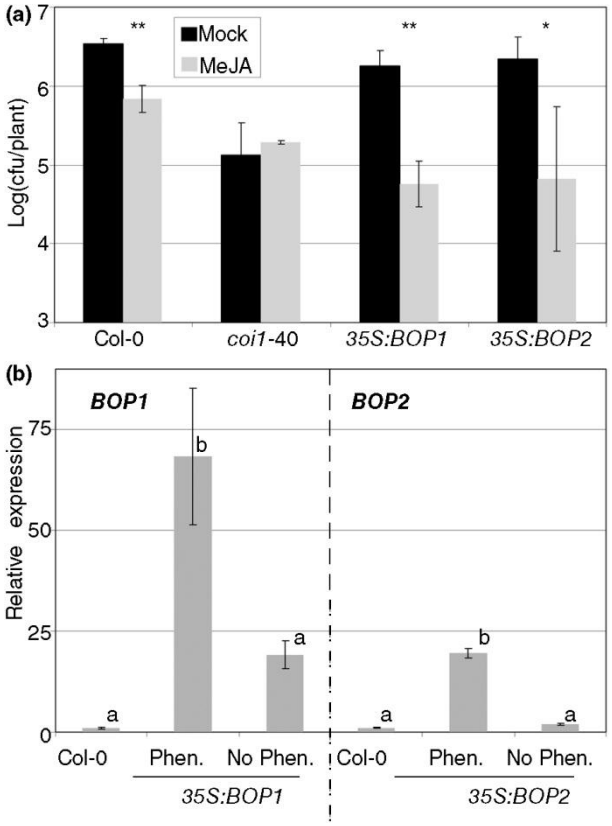
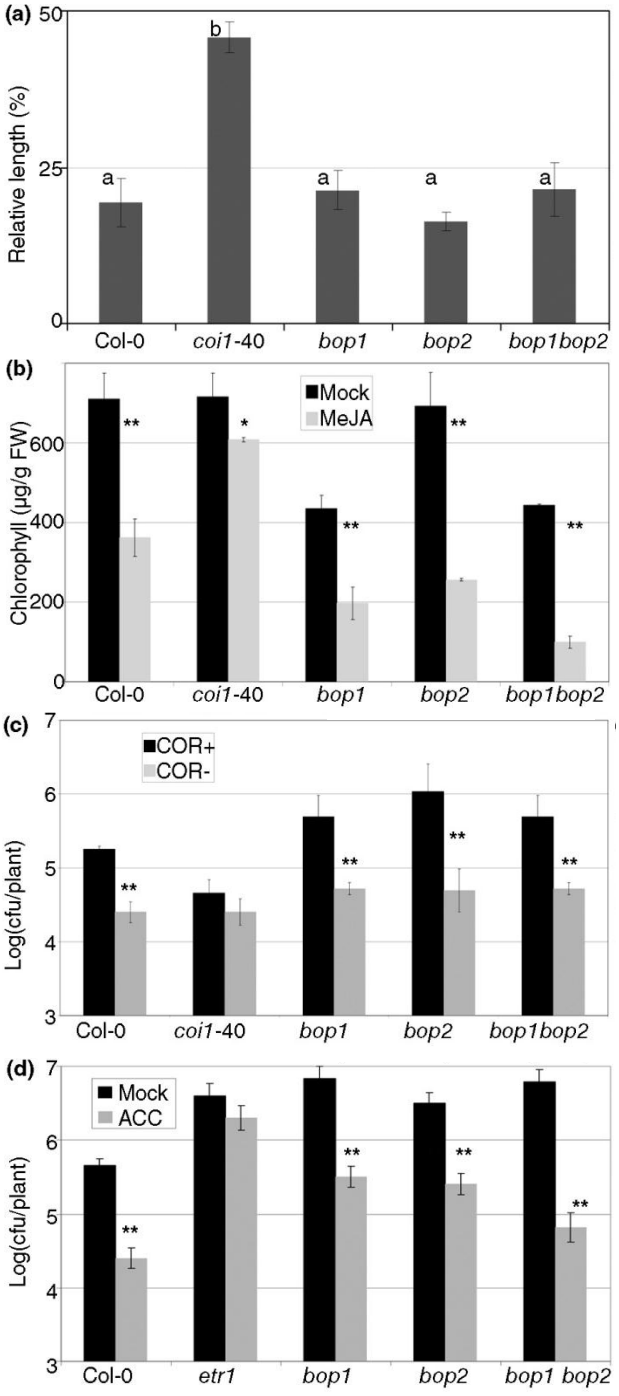


Fig. 6 The overexpression of *BOP1* or *BOP2* produces more response to MeJA. (a) *35S:BOP1* and *35S:BOP2* lines (Norberg et al. 2005) and their controls were tested as described in Fig. 1. (b) Silencing of the overexpression lines. *35S:BOP1* and *35S:BOP2* lines were phenotyped at the time of bolting as having a characteristic phenotype (Phen.) (Norberg et al. 2005) or being wild type (No Phen.). Then, RNA was extracted from several plants and the levels of the transgenes quantified as described in Fig. 5c. On the left side of the plot, relative expression of *BOP1* and, on the right, relative expression of *BOP2*. Means with the same letter are not significantly different (Fisher's LSD test, $P<0.05$). The test was performed separately for each gene.

bop1 bop2 specificity in MIR

The *bop1 bop2* double mutant lacked a MIR response, but this failure in MeJA signaling might occur at different points of the signal transduction. For example, the defect could target a general signaling component affecting all MeJA responses (e.g. *coi1,* Xie et al. 1998), or a specialized part of the pathway, affecting a subset of MeJA responses (e.g. *jin1,* Dombrecht et al. 2007). When *bop1 bop2* plants were grown in plates containing MeJA, the growth of the roots was similar to the wild type controls (Fig. 7a). Other phenotypes of *bop1 bop2* plants growing in MeJA plates were similar to the wild type controls (carotenoids production, size of aerial part, number of trichomes, etc; data not shown). Another effect of MeJA is the increase of senescence in detached leaves, measured as chlorophyll production (He et al. 2002). *bop1 bop2* responded as the wild type in this particular system (Fig. 7b). Coronatine is a virulence factor of several *Pseudomonas* isolates with structural and functional similarities to JA-Ile (the functional form of Jasmonate in planta, (Browse 2009)), therefore a mutant *Pto* that lacks coronatine (Mittal and Davis 1995) grows less in Col-0 than the wild type *Pto. bop1 bop2* was also wild type in response to *Pto* with and without coronatine (Fig. 7c). Inoculations with *Plectospharaella cucumerina*, a fungus that causes more disease in MeJA mutants than in wild type plants (Ton and Mauch-Mani 2004), did not cause any more disease in *bop1 bop2* than in Col-0 (data not shown). If *bop1 bop2* was not a MeJA signaling mutant, but specifically in MIR, it could be defective in the other signaling required for ISR; ET. It was not; when 1 mM of 1-aminocyclopropane-1-carboxylic acid (ACC, an ET precursor) was sprayed to *bop1 bop2*, the resistance triggered was similar to that triggered in the wild type controls (Fig. 7d). *etr1* (*ETHYLENE RESPONSE 1,* Chang et al. 1993) was included as a negative control of resistance induced by ethylene.


(a)
Relative length (%)
50
25
0
a
b
a
a
a
Col-0
coi1-40
bop1
bop2
bop1bop2
(b)
Chlorophyll (µg/g FW)
600
400
200
0
Mock
MeJA
**
*
**
**
**
Col-0
coi1-40
bop1
bop2
bop1bop2
(c)
Log(cfu/plant)
7
6
5
4
3
COR+
COR-
**
**
**
**
Col-0
coi1-40
bop1
bop2
bop1bop2
(d)
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7
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ACC
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Col-0
etr1
bop1
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Fig. 7 The double *bop1 bop2* is specifically affected in the resistance induced by MeJA. (a) Length of primary root. *bop1 bop2* and their controls were grown in plates with Johnson´s Media (Johnson et al. 1957) with or without 50 µM MeJA. At the age of 10 days, the lengths of the roots were measured in both conditions and their ratio (MeJA treated divided by mock treated) expressed as percentage. (b) Senescence induced by MeJA. The indicated genotypes were grown in soil and mature leaves from 6-week-old plants were cut and floated on water with or without 100 µM MeJA. The amount of chlorophyll (in µg/g fresh weight) was measured after four days of darkness, with three groups of leaves of *c.* 1 g each. (c) Coronatine as a virulence factor. Bacteria with coronatine (*Pto*, COR+) or without coronatine (*Pto(cfa⁻),* COR-) were inoculated and their growth measured as in Fig. 1. (d) Resistance induced by ethylene. The plants were treated with 1 mM 1-aminocyclopropane-1-carboxylic acid (ACC) or a mock treatment, and then *Pto* was inoculated and measured as in Fig. 1. *etr1* is a negative control of resistance induced by ethylene.

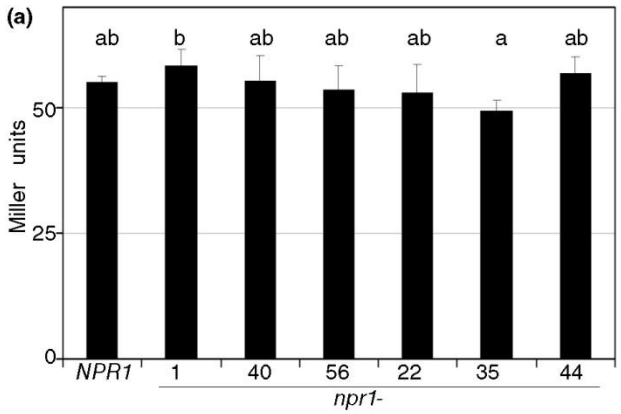
NPR1 and BOPs interactions

Once it was clear that both BOP genes are required for MIR, we tested the model that *npr1* MIR- alleles could have a dominant negative effect on BOP activity, either directly or indirectly. To first test whether NPR1 had an effect in the interaction between BOP proteins, we used a yeast two-hybrid assay. As reported, BOP1 and BOP2 interacted with each other (Jun et al. 2010). Next, we introduced in a third plasmid containing wt *NPR1* or various mutant *npr1* alleles presented in Fig. 2a. If the effect of *npr1* on MIR were a direct interaction between NPR1 and the two BOPs, the alleles that diverge in their MIR phenotype would diverge in their ability to interfere in the interaction of BOP1 and BOP2. The two classes of *npr1* alleles did not have a distinct behavior (Fig. 8a, the first three *npr1* alleles are MIR-, and the last three are MIR+), therefore the dominant negative effect did not seem to be direct.

All the NPR1 paralogs tested interact with members of the TGA family in a different degree (Hepworth et al. 2005; Zhang et al. 2006). Therefore, the TGAs would be a reasonable candidate for being the third component, and their interaction with MIR- alleles would indirectly affect the function of BOP1 and BOP2. As a control, single mutants in *TGA1* and *TGA7* produced a

significant MIR (Fig. 8b), but when three specific *tgas* are knocked out at the same time (a triple which phenocopies an *npr1* mutant in SA response, Zhang et al. 2003), there is no MIR (Fig. 8b).

We reasoned that one or several of these three TGAs (TGA2, 5, and 6) might have a functional interaction with the BOPs, which might be affected by the MIR- alleles. To test this hypothesis *BOP1* and each of the mentioned *TGAs* were introduced in the yeast two-hybrid system with the *npr1* alleles mentioned above in a third plasmid. TGA2 and TGA6 interact differentially with BOP1 depending on the npr1 protein present (Fig. 8c). There was an enhancement of the interaction in two out of the three MIR- alleles, and no interference in two out of the three MIR+ alleles. The interaction TGA5-BOP1 was not affected by the presence of npr1 proteins (data not shown). The experiments were repeated with BOP2 with similar results (data not shown). In sum, the data indicated that BOPs and TGA2, TGA5 and TGA6 are required for MIR, that BOPs interact with these (and other) TGAs, and that NPR1 may modulate the affinity or stability of the interactions.



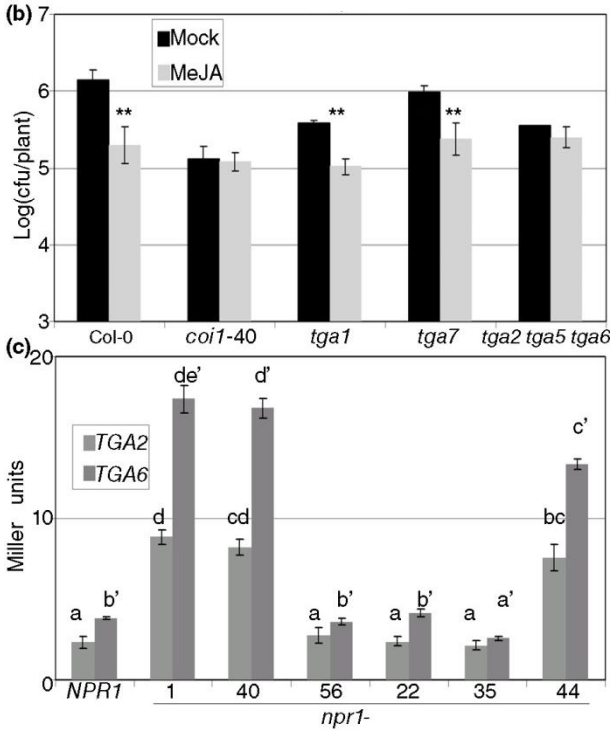


Fig. 8 The TGA family plays an important role in resistance induced by MeJA. (a) Interaction of BOP1 and BOP2 in the yeast two hybrid system in presence of several *npr1* alleles cloned in a third plasmid. NPR1 is included as control, the next three alleles do not respond to MeJA in defense, and the remaining three respond as wild type. The interaction is measured in Miller Units (Miller 1972). (b) Null alleles of several *TGAs* alleles were analyzed as in Fig. 1. *tga2,5,6* stands for the triple *tga2 tga5 tga6*. (c) Interaction between BOP1 and TGA2, and TGA6 in the yeast two hybrid system in the presence of the same *npr1* alleles of (a).

Discussion

NPR1 is not required for MIR

NPR1 is an essential gene for SAR and SA perception (Dong 2004). *npr1*-1, the most widely used allele, is also impaired in MIR (Pieterse et al. 1998). We speculated that since *npr1*-3 is wild type for MIR (Ramirez et al. 2010; Dobón et al. 2011), and it has been reported that the difference of some phenotypes between *npr1*-1 and *npr1*-3 was due to the lack of NLS in *npr1*-3 (Glazebrook et al. 2003; Leon-Reyes et al. 2009), the same could be true for MIR. However, we show here that the nuclear localization of the alleles makes no difference. This conclusion is supported by multiple lines of evidence. First, the *npr1* alleles with MIR+ are not structurally similar to *npr1*-3; not all of them are affected in the NLS (Fig. 1c). Even an allele with a point mutation in the NLS (*npr1*-22, Fig. S1) should be partially localized in the nucleus (Kinkema et al. 2000). Second, three MIR- and three MIR+ alleles do not differ in their nuclear localization or stability when transitorily expressed in *N. benthamiana* (Fig. 2a, S1). While these proteins are no longer functional, they respond to the signals of a wild type background by localizing in the nucleus. Third, when a functional NPR1 is anchored in the cytoplasm there is no complementation of the MIR- phenotype in an *npr1*-1 background (Fig. 2b), nor there is an increase in MIR phenotype in an *npr1*-3 background. In fact, the application of DEX triggered an increase the MIR+ in both backgrounds (discussed below). But, most importantly, NPR1 is not required for MIR, since the null *npr1* alleles are MIR+ regardless of the background (Fig. 3a, b). We also discarded that *NPR1* could be a part of MIR in a redundant fashion with its paralogs (Fig. 4).

An interesting alternative for the role of *NPR1* in MIR would be an effect on the crosstalking between SA and MeJA. *NPR1* has been described as a key point in the negative regulation between SA and MeJA. Thus, the MIR+ alleles could be defective in both SA perception and in SA-MeJA crosstalk, while the MIR- alleles would be defective only in SA perception but not in SA-MeJA crosstalk. The inoculation with *Pto* triggers an increase in the levels of SA, and in the case of the *npr1* alleles, there is more SA than in the wild type (Delaney et al. 1995). Although this hypothesis would explain the phenotype of the null alleles, it was rejected after the experiment of Fig. 3c, where a severe reduction of SA levels in a MIR- allele did not have any effect on the phenotype.

BOP1 and *BOP2* are redundant in MIR

The redundant functions of *BOP1* and *BOP2* are essential for normal development. Previous work has shown that the double mutant has numerous defects in plant architecture including altered leaf morphology (Ha et al. 2007), changes in floral patterning (Hepworth et al. 2005), defects in the conversion of shoots to flowers (Xu et al. 2010) and loss of floral-organ abscission (McKim et al. 2008). The double mutant was tested for basal defense (Hepworth et al. 2005) and SA perception (Fig. S4) but no difference from wild type was found. We show here that both genes are also redundantly required in defense against pathogens triggered by MeJA. Interestingly, whereas significant loss of BOP activity is required to exert changes in development (Hepworth et al. 2005), MIR is abolished in plants that are only partially silenced for the BOP genes (Fig. 5b, c). Thus, the levels of gene expression required for MIR are higher than those required for normal development. Compatible with this, *BOPs* expression in plants is highly localized, restricted to young organ primordia, leaf petioles, and lateral organ boundaries, which may make systemic responses to MeJA sensitive relatively minor changes in BOP transcript abundance. Both NPR1 and the BOPs localize to the cytoplasm as well as nucleus and interact with members of the TGA family of bZIP transcription factors, albeit with different affinities (e.g., Zhang et al. 1999). In development, BOP1 and BOP2 form a nuclear complex with TGA8/PERIANTHIA (PAN) to regulate number of sepals and petals in flowers and potentially to promote floral meristem fate (Hepworth et al. 2005). Given that *pan* loss-of-function did not reproduce the MIR- phenotype (Fig. 5a) other genes, perhaps *TGAs*, are involved in this phenotype, as shown for SA perception (Canet et al. 2010a). Given that BOPs play both positive and negative roles in transcriptional regulation of the KNOX (Knotted1-like homeobox) gene *KNAT6* (Ha et al. 2010), we also tested if MIR was affected by *knat6* loss-of-function, but again, no difference was observed (Fig. 5a). This may reflect redundancy with other KNOX genes, or more likely, that BOP regulation of MIR is independent of KNAT6.

Whether *bop1 bop2* recapitulates or not all the phenotypes of the MIR- *npr1* alleles (e.g. ISR, (Pieterse et al. 1998); *Verticilium* resistance, (Johansson et al. 2006); resistance induced by *Piriformospora indica,* (Stein et al. 2008); etc.) remains to be assessed. We did check that there were similar phenotypes in the

specificity of response to MeJA as well as the fact that *bop1 bop2* and *npr1*-3 were wild type for the rest of MeJA phenotypes (Fig. 7). But there were strong differences, since *npr1*-3 is affected in basal defense and SA perception while *bop1 bop2* is wild type for both phenotypes (Hepworth et al. 2005). Regarding ET, the other hormone relevant for ISR, it has been proposed that applications of this hormone could render the crosstalk between SA and MeJA independent of NPR1 (Leon-Reyes et al. 2009). It seems plausible that the ET induced resistance works as the MeJA induced resistance and other proteins -perhaps NPR1 paralogs, but not the BOPs (Fig. 7d) - might also be affected by some alleles of *npr1*.

Some *npr1* alleles interfere in the BOPs-TGAs interaction

The MIR- *npr1* alleles were the majority of the alleles found (32 MIR- vs. 11 MIR+). How is this compatible with the fact that the null *npr1* alleles are MIR+? A possible explanation was the selection used in the screening. Since the selection was made for complete loss of SA perception, perhaps most of the MIR+ alleles had a phenotype of partial SA perception, as the null alleles. Then, the prediction would be that a good number of random alleles of *npr1* would be MIR+ and partially receptive to SA. We previously showed that for SA perception, there are genetic interactions between the *npr1* alleles and the *NPR1* paralogs (Canet et al. 2010b). The work reported herein points to a genetic interaction too, this being between *npr1* alleles on one side and the *BOPs* on the other. Thus, the MIR- alleles were a phenocopy of the *bop1 bop2* mutant in defense but not in development. This discrimination was a consequence of the different thresholds for the phenotype in development and defense (Fig. 5b, c).

Mechanistically, the levels of expression of the *BOPs* were low in comparison to *NPR1* (Fig. S3), so a direct or indirect negative interference of NPR1 with the BOPs would be favored stoichiometrically. Once the pathogen was inoculated, the levels of SA would rise and in a wild type plant NPR1 is degraded as part of the signaling process (Spoel et al. 2009). In an *npr1* background, this signaling would not be transmitted and perhaps the npr1 proteins would be able to interfere longer in MIR. This would explain the behavior of *NPR1-HBD* in *npr1*-1 (Fig. 2b); NPR1-HBD in the cytoplasm did not complement *npr1*-1 in the MIR phenotype, but when DEX was applied there was complementation of

the phenotype. Likely, when no DEX was present npr1-1 would somehow interfere with the function of the BOPs. When DEX was present, the presence of NPR1-HBD in the nucleus would trigger the degradation of both NPR1-HBD and npr1-1. If npr1-1 was degraded, the BOPs would function normally.

There was no evidence for a direct interaction in yeast, since the presence of NPR1 or mutated versions of this protein did not interfere in the interaction between BOP1 and BOP2 in a consistent manner with the phenotype (Fig. 8a). A first alternative was that the interference of the MIR- alleles would occur with the BOPs without affecting the interaction between the BOPs. A second alternative would be that the MIR- alleles would interfere with other proteins that normally interact with the BOPs. In both cases there is a family of proteins that interacts with both NPR1 and the BOPs, the transcription factors TGAs (Jakoby et al. 2002), with -again- functional redundancy (Fig. 8b). Two out of three MIR- alleles enhanced or stabilized the BOPs-TGAs interaction, while two out of three MIR+ alleles did not (Fig. 8c and data not shown). It was clear that the npr1 mutated proteins had an unpredicted effect on the BOPs-TGAs interaction, but the yeast experiments did not produce definitive answers about the role of npr1 proteins in MIR. Perhaps the combination of these interactions with the fact that there are ten *TGAs* (Jakoby et al. 2002), and the fact that *NPR1* is expressed between 3 and 18 times *in planta* more than *BOP1* + *BOP2* (Fig. S3) will definitively explain the role of npr1 in MIR.

Conclusions

In sum, we have shown that, in wild type conditions, the *BOPs* and the *TGAs* (but not *NPR1*) are required for the resistance triggered by methyl jasmonate against *Pto*. We propose that the phenotype of the *npr1* MIR- alleles is caused by their interference between BOPs and TGAs.

Methods

Plant growth and inoculation

Arabidopsis thaliana (L.) Heynh. was sown and grown as described (Canet et al. 2010a) in controlled environment rooms with days of 8 h at 21°C, 150 µmol m^{-2} s^{-1}, and nights of 16 h at 19°C. Treatments, inoculations, and sampling started 30 minutes after the initiation of the artificial day to ensure reproducibility. The following genotypes were used: *npr1*-1 and *npr1*-3 (Cao et al. 1997); *npr2*, *npr1*-20 to *npr1*-71, and combinations of *npr1*-70 with other genotypes (Canet et al. 2010b); *35S:NPR1HBD* (Kinkema et al. 2000); *sid2* (Wildermuth et al. 2001); *eds5* (Nawrath et al. 2002); *NahG* (Lawton et al. 1995); *npr3* and *npr4* (Zhang et al. 2006); *bop1*-3 and *bop2*-1 (Hepworth et al. 2005); *coi1*-40 (Dobón, Wulff, Canet and Tornero, to be published elsewhere); *kant6, pan1*-1 *to pan1*-3, *tga1*, and *tga7* (Alonso et al. 2003); *35S:BOP1* and *35S:BOP2* (Norberg et al. 2005); *etr1*-3 (Chang et al. 1993); *tga2 tga5 tga6* (Zhang et al. 2003). *Pseudomonas syringae pv. tomato* DC3000 (*Pto*) was grown, inoculated and measured as described (Tornero and Dangl 2001). Briefly, plants of 14 days were inoculated by spray with *Pto* at OD_{600}=0.1 with 0.02% Silwet L-77 (Crompton Europe Ltd, Evesham, UK). Three days later, the amount of colony forming units (cfu) per plant was quantified and represented in a logarithmic scale. When indicated, a strain of *Pto* lacking coronatine was used (*Pto(cfa⁻),* Mittal and Davis 1995). For all the experiments, at least three independent treatments were performed (three independent sets of plants sown and treated on different dates).

Expression *in planta* and in yeast

NPR1 and six alleles of this gene were cloned in pDONR222 or pDONR221 (Invitrogen, Barcelona, Spain) and then transferred to pMDC43 (Curtis and Grossniklaus 2003) for expression *in planta* with GFP and to pARC352 (Ciannamea et al. 2006) for expression in yeast. Similarly*, BOP1, BOP2, TGA2, TGA5,* and *TGA6*, were cloned and then transferred to pDEST22 and pDEST32 (Invitrogen) for expression in yeast. Yeast n-hybrid analyses were done as described (Vidal and Legrain 1999), and the interactions were quantified as described (Miller 1972). *N. benthamiana* leaf tissue was mounted in water under a coverslip 4 days after infiltration with *Agrobacterium tumefaciens* containing the constructs. All imaging was conducted with a Leica TCS SL

confocal laser scanning microscope (Leica, Barcelona, Spain) using an HCX PL APO CS 40X/1.25 oil objective to study the subcellular localization of the fluorescence-tagged proteins. Green fluorescent protein was visualized by 488-nm excitation with an Ar laser, and its emissions were examined with a band-pass filter for 500 to 530 nm. The primers used are included as Supplemental Table S1. Primers and chemical products were purchased from SIGMA (St. Louis, MO, USA) unless otherwise is stated. For the construction of *amiRNA(BOP1-BOP2)*, the plasmid pRS300 was modified (Schwab et al. 2006), cloned in pGW14 (Nakagawa et al. 2007), and plants were transformed as described (Clough and Bent 1998).

Chemical treatments

To measure the effect in *Pto* growth 100 μM methyl jasmonate (MeJA) in 0.1% DMSO and 0.02% Silwet L-77 (Crompton Europe Ltd) was applied by spray one day previous to pathogen inoculation (Dobón et al. 2011). Dexamethasone was applied at 2 μM diluted in water from a stock of 20 mM in EtOH. 1-Aminocyclo- propane-1-carboxylic acid (ACC) was sprayed at 1 mM in water with 0.02% Silwet L-77.

In vitro growth

For *in vitro* culture, plants were grown in Johnson's media (Johnson et al. 1957) with 1 mM KH_2PO_4. When indicated, the plates were supplemented with 50 μM MeJA. The length of the roots was measured with ImageJ software (Abramoff et al. 2004). Senescence induced by MeJA was measured as described (He et al. 2002).

RT-qPCR

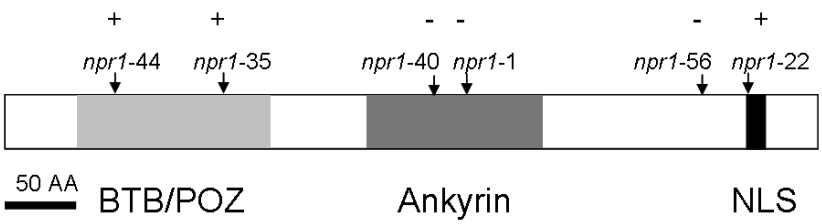
Total RNA from 3-week-old (Fig. 5c) or 6-week-old plants (Fig. 6b) was extracted with Trizol (Invitrogen), following the manufacturer's instructions. cDNA was synthesized with RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Madrid, Spain), and the quantitative PCR performed with LuminoCt Sybr Green qPCR Ready Mix (SIGMA) in a 7000 RT-PCR Systems machine (Applied Biosystems, Madrid, Spain), following the manufacturer's instructions. For each measurement three biological replicates were done. The

obtained values were referred to the geometric average of three reference genes (At3G18780, At1G49240, and At5G60390), as described (Vandesompele et al. 2002), and normalized, being the value of Col-0 in mock equal to one. The list of primers used is provided in Supplemental Table S1.

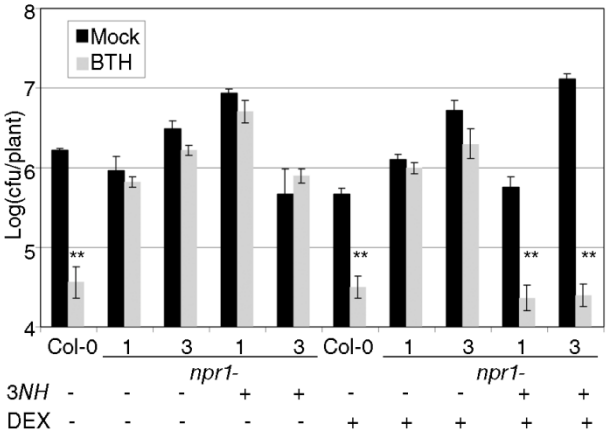
Acknowledgments

This work was supported by the Ministerio de Ciencia e Innovación (MICINN) of Spain (grant BIO201018896 to PT, a JAE-CSIC Fellowship to JVC and a FPI-MICINN to AD). Thanks to Dr. Xinnian Dong for *NPR1* overexpression lines and to Dr. Ove Nilsson for *BOPs* overexpression lines. We appreciate the opinions and generous help of Drs. Vicente Ramirez, Pablo Vera, and Shelley Hepworth about the manuscript. The authors declare that they have no conflict of interest.

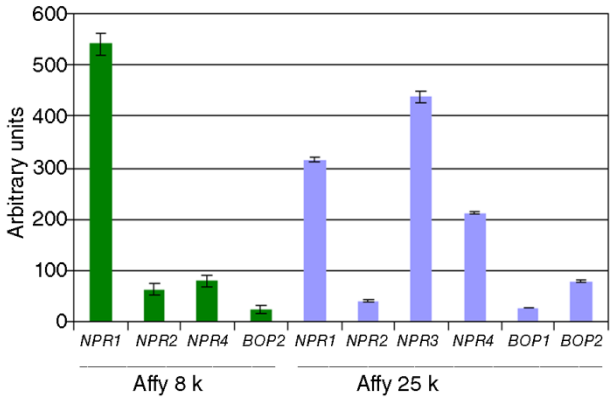
Supporting information



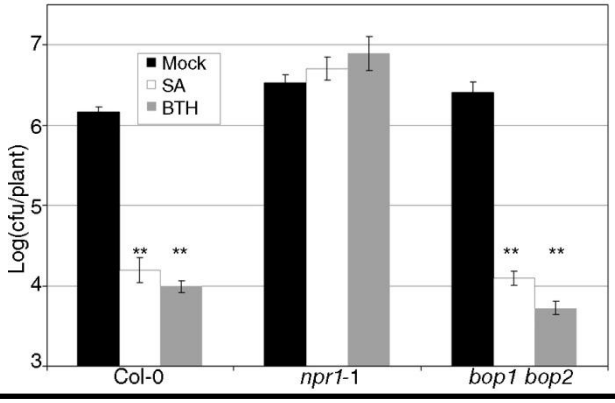
Supplemental Fig. 1 Localization of cloned *npr1* alleles mentioned in the text. These alleles are used in Figures 2 and 8, see Canet et al. 2010b, for a detailed list of the point mutations. As in Figure 1c, BTB/POZ stands for Broad-Complex, Tramtrack and Bric-a-brac proteins, Pox virus and Zinc finger proteins. Ankyrin for Ankyrin Repeat Motifs (4 of them) and NLS for Nuclear Localization Signal. The arrows indicate point mutations.



Supplemental Fig. 2 NPR1HBD treated with DEX is more resistant to BTH. As a control, the same lines described in Figure 2B were treated with and without 350 µM BTH, and with and without DEX. BTH (Benzothiadiazole, in the form Bion® 50 WG, www.syngenta.com) is an analogue of SA that triggers a strong resistance in plants with a functional NPR1 protein. DEX stands for dexamethasone and *3NH* for *35S:NPR1-HBD*.



Supplemental Fig. 3 Expression levels of NPR1 paralogs. The levels of expression of NPR1 and its paralogs were obtained from TAIR (www.arabidopsi.org) for the Affymetrix AG 8 k microarray (green) and for the Affymetrix ATH1 25 k microarray (blue).



Supplemental Fig. 4 *bop1 bop2* is able to perceive SA and BTH. The response of the double mutant *bop1 bop2* to SA and BTH was tested, along Col-0 and *npr1*-1. SA was applied as 500 µM, and BTH as 350 µM, one day prior to *Pto* inoculation.

Table S1. List of primers used.

Name	Sequence	Description
NPR1GWF2	GGGGACAAGTTTGTACAAAAA AGCAGGCTTCATGGACACCACC ATTGATGG	Clonning NPR1 in pDONR222
NPR1GWR2	GGGGACCACTTTGTACAAGAAA GCTGGGTTTTACCGACGACGAT GAGAGAGT	Clonning NPR1 in pDONR222
TGA2GWF2	GGGGACAAGTTTGTACAAAAA AGCAGGCTTCATGGCTGATACC AGTCCGAG	Clonning TGA2 in pDONR221
TGA2GWR2	GGGGACCACTTTGTACAAGAAA GCTGGGTTTTACTCTCTGGGTC GAGCAAGCC	Clonning TGA2 in pDONR221
TGA5GWF	GGGGACAAGTTTGTACAAAAA AGCAGGCTTCATGGGAGATAC TAGTCCAAGAAC	Clonning TGA5in pDONR222
TGA5GWR	GGGGACCACTTTGTACAAGAAA GCTGGGTTTCACTCTCTTGGTC TGGCAAGCCATAG	Clonning TGA5 in pDONR222

TGA6GWF	GGGGACAAGTTTGTACAAAAA AGCAGGCTTCATGGCTGATACC AGTTCAAGGAC	Clonning TGA6 in pDONR222
TGA6GWR	GGGGACCACTTTGTACAAGAAA GCTGGGTTTCACTCTCTTGGCC GGGCAAGCCACAAG	Clonning TGA6 in pDONR222
BOP1GWF	GGGGACAAGTTTGTACAAAAA AGCAGGCTTCATGAGCAATACT TTCGAAGA	Clonning BOP1 in pDONR222
BOP1GWR	GGGGACCACTTTGTACAAGAAA GCTGGGTTCTAGAAATGGTGG TGGTGGTGA	Clonning BOP1 in pDONR222
BOP2GWF	GGGGACAAGTTTGTACAAAAA AGCAGGCTTCATGAGCAATCTT GAAGAATC	Clonning BOP2 in pDONR222
BOP2GWR	GGGGACCACTTTGTACAAGAAA GCTGGGTTCTAGAAGTGATGTT GATGATGG	Clonning BOP2 in pDONR222
3rd_set_I	GATTTCAATGTGAGTCTATCCT GTCTCTCTTTTGTATTCC	amiRNA(BOP1+BOP2)
3rd_set_II	GACAGGATAGACTCACATTGAA ATCAAAGAGAATCAATGA	amiRNA(BOP1+BOP2)
3rd_set_III	GACAAGATAGACTCAGATTGAA TTCACAGGTCGTGATATG	amiRNA(BOP1+BOP2)
3rd_set_IV	GAATTCAATCTGAGTCTATCTT GTCTACATATATATTCCT	amiRNA(BOP1+BOP2)
BOP1qF	GCTCGCTACTTTGGCGTCG	RT-qPCR BOP1
BOP1qR	CTTCAATGGAGGCTTTCTCCAC	RT-qPCR BOP1
BOP2qF	GTTACTTCGGCGTCGAGCAG	RT-qPCR BOP2
BOP2qR	CATCTTCGATAGAGGCTTTCTC CA	RT-qPCR BOP2

Artículo 4

Non-Recognition-of-BTH-4, an Arabidopsis *Mediator* gene homolog, is necessary for development and response to salicylic acid

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Este artículo ha sido enviado para
su publicación (mayo 2012)

Abstract

Salicylic acid is a hormone relevant for defense and for certain processes in plant development. The only gene demonstrated to be required for response to SA is *NPR1*. Mutations in *npr1* derived from several genetic screens of Arabidopsis are insensitive to SA, all to the same degree. By focusing on the effect of analogs of SA on normal plant development, we isolated additional complementation groups relevant for SA response. In this work, we describe another gene necessary for SA response, *NRB4*. Three *nrb4* alleles recovered from the screen are similar to wild type, except in SA-related phenotypes. While mostly insensitive to SA, the alleles differ in some particular phenotypes, like systemic acquired resistance or pathogen growth restriction. *NRB4* null alleles express profound insensitivity to SA in terms of defense, even more than *npr1*. *NRB4* null mutants are also sterile and their growth is severely affected. When weak alleles are used, *NPR1* and *NRB4* mutations are additive, but we did not find evidence of genetic interaction in F1, nor biochemical interaction in yeast or *in planta*. *NRB4* is predicted to be a subunit of *Mediator*, the ortholog of *MED15* in Arabidopsis. Mechanistically, NRB4 functions downstream of NPR1 to regulate SA response.

Keywords: salicylic acid, BTH, Arabidopsis, NPR1, *Mediator*, *Pseudomonas*, defense.

Introduction

Plants mount several types of resistance against different pathogens. Some types of defense consist of preexisting barriers, while others are inducible. Among the inducible defenses against biotrophic pathogens, the hormone salicylic acid (SA) is of capital importance (reviewed by Vlot et al. 2009). Upon pathogen perception, SA biosynthesis is increased, and its response leads to a proper defense. There are other hormones in plant defense, such as methyl jasmonate (MeJA) and ethylene (ET), and there are complex interactions between them (reviewed by Robert-Seilaniantz et al. 2011). Broadly speaking, MeJA and ET act synergistically, while SA and MeJA negatively regulate each other.

A key player in the recognition of SA is *NPR1* (*Non expresser of Pathogenesis Related 1*, reviewed by Dong 2004). Different genetic screens aimed to identify components of SA response have exclusively rendered *npr1* alleles (Cao et al. 1994; Delaney et al. 1995; Glazebrook et al. 1996; Shah et al. 1997), suggesting that it is the only gene responsible for SA response, or the only one accessible through mutagenesis. Additional work, searching for protein-protein interaction in yeast, was useful to identify components that interact with *NPR1*, as *NIMINs* (Weigel et al. 2001) and *TGAs* (Zhang et al. 1999; Després et al. 2000).

Benzothiadiazole (BTH) is an analog of SA used in the laboratory because it is not as phytotoxic as SA (Lawton et al. 1996). Repeated applications of BTH have the effect of decreasing the size and weight of the plant (Canet et al. 2010a), and this feature was used to perform an *en masse* screening for non recognition of BTH mutants (NRBs). The first complementation group derived from this screen, not surprisingly, was NPR1 (Canet et al. 2010b). The second complementation group, *NRB4*, is the focus of this report.

As mentioned, SA is at the center of inducible responses to the pathogens, and most of these responses involve gene expression. Part of this gene expression is regulated by DNA repair proteins (Song et al. 2011) and chromatin remodeling (Wang et al. 2010). This process has been revealed by the function of genes identified as suppressors of *npr1* or suppressors of the suppressors. There are also sets of transcription factors notably *TGAs* (Jakoby et al. 2002) and *WRKYs* (Eulgem et al. 2000) that act downstream of SA response to regulate gene expression leading to defense. Neither of these specific transcription factor sets have been demonstrated to interact directly with the RNA Pol II.

In yeast, the "*Mediator*" complex functions as a bridge between specific transcription factors and the core transcriptional machinery (Kelleher et al. 1990; Flanagan et al. 1991). *Mediator* is a complex of c. 22 proteins, divided in four modules: head, middle, tail, and a detachable kinase domain. The tail module interacts with the specific transcription factors, while the head module interacts with RNA Pol II (Cai et al. 2009). The presence of *Mediator* has been verified in all eukaryotes (Chadick and Asturias 2005), and has recently been described in Arabidopsis (Backstrom et al. 2007). Indeed, several reports of *Mediator* subunits of Arabidopsis affecting specific signaling process have appeared (reviewed by Kidd et al. 2011).

The *nrb4* missense mutations we found are only affected in SA response, while null alleles in *NRB4* express both severe defense and developmental phenotypes. NRB4 is predicted to be a subunit of the *Mediator* complex, and in this work we show that the missense mutations are clustered in the KIX domain. Importantly the orthologs in other species interacts with different receptors, some of them which bind salicylates. From the phenotypes presented, we infer an essential role for *NRB4* in plants. This essential function perhaps could reflect a role for response to SA in normal development, as previously suggested (Vanacker et al. 2001).

Results

NRB4 is required for SA response

We previously performed a genetic screen looking for genes involved in SA response, and the first locus identified was *NPR1*, with 43 alleles (Canet et al. 2010b). The second locus from this screen was named *NRB4* (*Non recognition of BTH 4*), defined by three alleles. These three alleles came from independent lots mutagenized with Ethyl methanesulfonate (EMS), and they were almost as insensitive to BTH as *npr1*-1, at least in terms of diminished fresh weight when grown in the presence of BTH (Figure 1A). The alleles were numbered chronologically, with *nrb4*-1 having a less severe phenotype than the other two (Figure 1A). The mutations were recessive (Figure 1A, χ^2 0.17, 0.60, and 0.81, <3.84 for n=335, 405, and 449 F2s analyzed, respectively) and did not interact genetically with *npr1* in F1 plants. The phenotypes of the F1s between the alleles were as insensitive to BTH as their parents (Figure 1A).

The screen and the quantification of the fresh weight were carried out with BTH. It was possible that these *nrb4* alleles were impaired in BTH response, but had no effect on SA response. To test this possibility, *nrb4* plants and their controls were grown in MS plates with 500 μM SA. *npr1* alleles are unable to grow on these plates, likely because they are unable to detoxify SA (Cao et al. 1997). *nrb4*-2 and *nrb4*-3 behaved as *npr1*-1, while *nrb4*-1 was intermediate between Col-0 and *npr1*-1 (Figure 1B). This observation was quantified by measuring the amount of chlorophyll per plant in three different situations (Figure 1C). The quantification corroborated the intermediate phenotype of

nrb4-1, while there was no difference in chlorophyll in mock conditions. In fact, there were no observable phenotypes in the three *nrb4* alleles in development, and they were indistinguishable from Col-0 in our growth conditions.

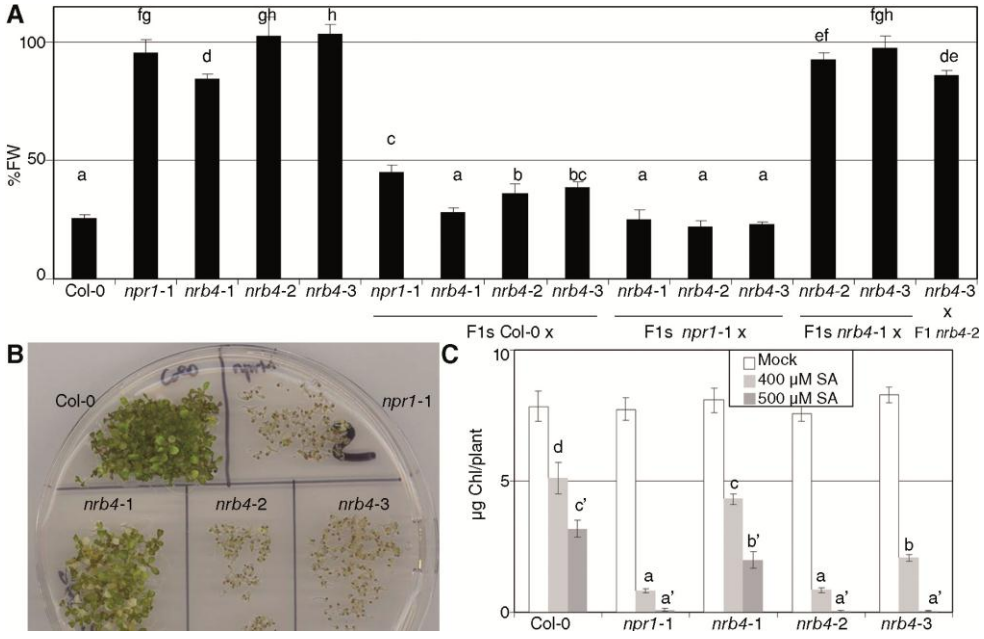


Figure 1. SA related phenotypes of *nrb4*. A Plants were treated with either mock or 350 µM BTH four times along three weeks, their weight recorded, and the ratio between the BTH and mock treated plants represented (15 plants in three groups of five). The ratio is expressed as percentage of fresh weight (%FW). **B** Plants were grown in MS plates supplied with 0, 400 and 500 µM SA, the picture shows the 500 µM SA plate at day 14. **C** The amount of chlorophyll of plants growing in the plates described in B was measured as an indication of the response to SA. Data represent the average and the standard deviation of three measurements of 10 plants. In all figures, the experiments were repeated three times with similar results. The letters above the bars indicate different homogeneous groups with statistically significant differences (Fisher's LSD Test, $P < 0.05$). In the C panel, the differences were evaluated among genotypes in the same treatment, 400 µM SA on one hand, 500 µM SA on the other (marked with the symbol prime).

The *nrb4* mutants, like *npr1*, were also affected in SA-dependent defense (Figure 2). For example, inoculations with *Pseudomonas syringae pv. tomato* DC3000 (*Pto*) after SA and BTH treatments induced a strong resistance in Col-0, but not in *npr1*-1 (Figure 2A). The *nrb4* alleles showed some residual resistance, but the difference with respect to Col-0 was always considerable. PR1 is a Pathogenesis Related protein used as a marker for stress in plants (Wang et al. 2005), so we produced PR1 Western blot of plants treated with *Pto* or BTH (Figure 2B, top and bottom, respectively). In both cases, strong expression of PR1 was observed only in Col-0. Therefore, even if SA and BTH induce a small amount of resistance, this resistance is not evidenced in the expression of PR1.

The similarities between *nrb4* and *npr1* extended beyond the initial characterization. When tested for enhanced disease susceptibility phenotypes (Glazebrook et al. 1996), *nrb4* alleles were as susceptible as *npr1* or more so (Figure 2C). Surprisingly, *nrb4*-2 is wild type for pathogen-induced SAR (while the other two alleles, like all *npr1* alleles, are SAR defective; Figure 2D) (e.g. Cao et al. 1994; Delaney et al. 1995). Since this was an important difference, it was repeated several times, always with the same result. All *nrb4* and *npr1* alleles expressed similar effector-triggered immune (ETI) responses (Figure 2E and F) and responses to non-host pathogens (Figure 2G and H). Only in the case of RPS2-dependent ETI triggered by *Pto(avrRpt2)* did we observe a decrease in resistance in some alleles; these paralleled the responses to *Pto* (Figure 2A, C, and D). Other pathogens tested included: *Pto(hrpC-)* (Deng et al. 1998) and *Plectosphaerella cucumerina* (Ton and Mauch-Mani 2004). In these cases, the *nrb4* alleles were not different from the wild type (Figure S1A and B, respectively).

NPR1 alleles differ in their response to MeJA induced resistance (Dobón et al. 2011). In the case of *NRB4*, it was wild type in their response to MeJA (Figure S1C). It was also wild type in MeJA plates (Figure S1D) and in growth of *Pto(cor-)*, (Mittal and Davis 1995, Figure S1E).

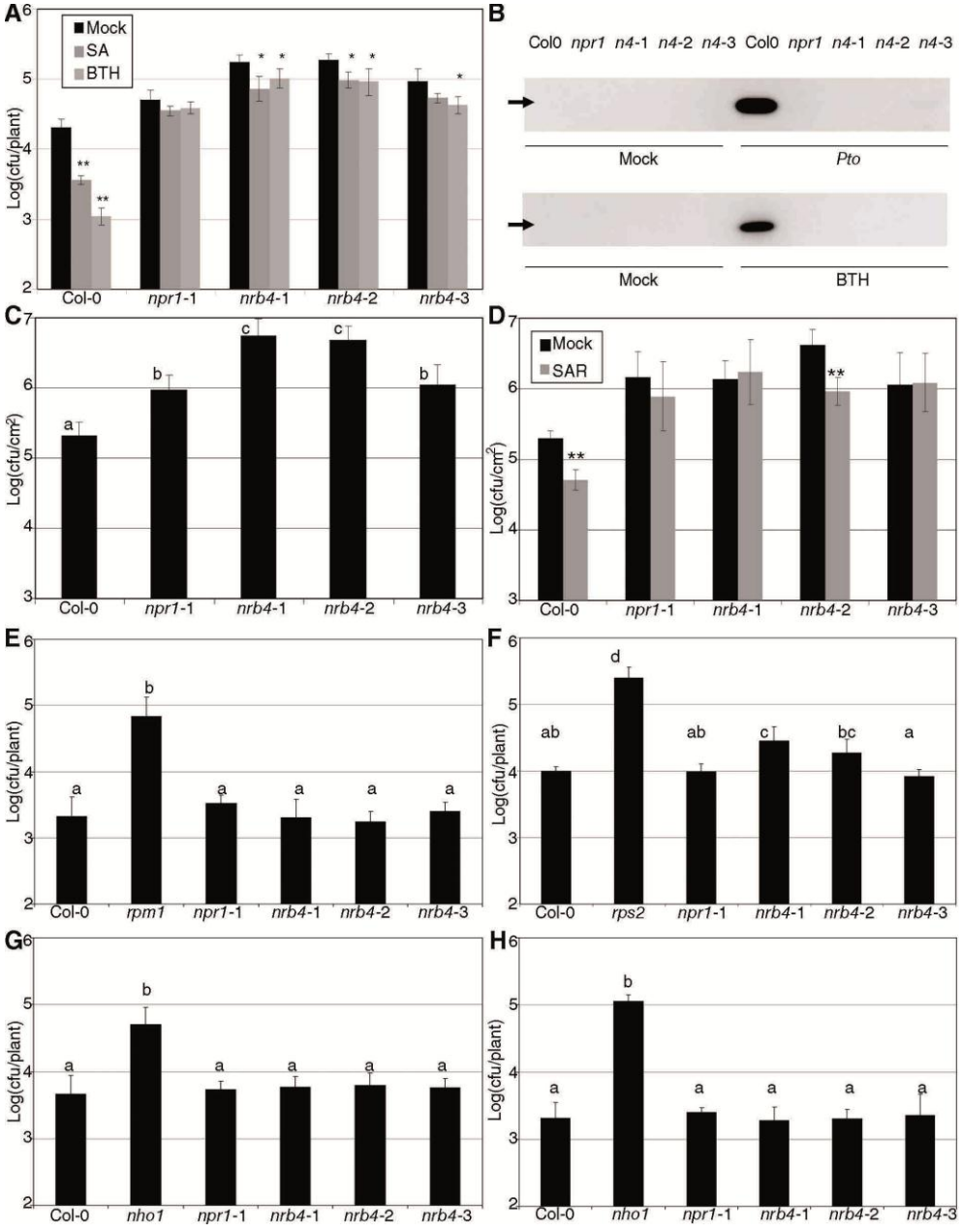

A
Mock
SA
BTH
Log(cfu/plant)
Col-0
npr1-1
nrb4-1
nrb4-2
nrb4-3
B
Col0 npr1 n4-1 n4-2 n4-3 Col0 npr1 n4-1 n4-2 n4-3
Mock
Pto
Mock
BTH
C
Log(cfu/cm2)
D
Mock
SAR
Log(cfu/cm2)
E
Log(cfu/plant)
rpm1
F
Log(cfu/plant)
rps2
G
Log(cfu/plant)
nho1
H
Log(cfu/plant)
nho1

Figure 2. Pathogenic phenotypes of *nrb4*. **A** Plants of 17 days were treated with either 500 µM SA, 350 µM BTH or a mock solution. One day later, the plants were inoculated with *Pseudomonas syringae* pv. *tomato* isolate DC3000 (*Pto*) at an OD_{600} of 0.1. Three days after inoculation, the growth of *Pto* was evaluated with three measurements, each one averaged from five plants, so the units are Logarithm of colony forming units (cfu) per plant. **B** PR1 Western blot of the indicated genotypes three days after mock or a *Pto* inoculation (top), and one day after mock or 350 µM BTH treatment (bottom). The genotypes -abbreviated- are the same as in A. The arrow indicates the position of PR1 (14 kDa). **C** Plants of 32 days were treated with *Pto* as in A. In these plants, only a sample of known surface is taken, so the units are Log(cfu/cm^2). **D** Three leaves of 30-day-old plants were hand infiltrated with either *Pto(avrRpm1)* or a mock solution. Two days later, *Pto* was inoculated and its growth in systemic leaves measured as in C. SAR stands for Systemic Acquired Resistance. **E** *Pto*(*avrRpm1*) was inoculated in *nrb4* as in A. *rpm1* is included as a control. **F** Idem with *Pto*(*avrRpt2*), with *rps2* added as a control. **G** *Pseudomonas syringae* pv. *phaseolicola* isolate NPS3121 was inoculated as in A, with *nho1* used as a control. **H** Idem with *Pseudomonas syringae* pv. *tabaci*. Asterisks indicate statistically significant differences from the mock treatment ($P < 0.05$ one asterisk, $P < 0.01$ two) using the Student's t test (one tail).

NRB4 and *NPR1* mutants shared most of the phenotypes attributed to SA-dependent defense and/or response to biotrophic pathogens. Thus, we addressed whether they act in the same pathway. To clarify this, double mutants between *nrb4* and *npr1*-1 were constructed. These lines showed no additional phenotype in response to BTH in fresh weight (Figure S2A). Therefore, the double was reconstructed with *npr1*-70, (a null allele with an intermediate response to BTH, Canet et al. 2010b), and *nrb4*-1 since it was the weakest allele (Figure 1A). *nrb4*-1 *npr1*-70 showed additive phenotypes, since the double mutant had a stronger phenotype than either weak allele alone (Figure 3A). Similar results were obtained with respect to growth of *Pto* in response to SA and BTH treatment (Figure S2B).

This additive relationship could be translated into several mechanistic models. NPR1 functions in the nucleus (Kinkema et al. 2000; Maier et al. 2011), and *nrb4* could affect its localization and, potentially, that of other proteins acting with NPR1. The trafficking of NPR1 can be manipulated with a transgenic line that over-expresses *NPR1* fused to the steroid hormone binding domain of the

rat glucocorticoid receptor (*NPR1-HBD*, Kinkema et al. 2000). Upon application of the glucocorticoid dexamethasone (DEX), NPR1-HBD is forced to the nucleus, while in mock conditions it is excluded from it. The double *nrb4*-2 *NPR1-HBD* did not respond to BTH when DEX was applied (Figure 3B). Thus, the presence of NPR1 in the nucleus, in the presence of BTH, was not enough to trigger the response. An alternative explanation of the previous experiment could be that NRB4 is a chaperone, required for NPR1 stability. This explanation was ruled out with the help of a line that expressed NPR1 fused to GFP (Kinkema et al. 2000). In control conditions, NPR1-GFP was weakly detected in the nucleus, likely because part of the protein was also in the cytosol (Figure 3C, compare with Figure 2A of Kinkema et al. 2000). The same localization was observed in this transgenic line in an *nrb4*-2 background (Figure 3D). Upon BTH application, NPR1-GFP was strongly detected in the nucleus, both in a *NRB4* wild type and in *nrb4*-2 (Figure 3E and 3F, respectively). Therefore NPR1 was not only stable in *nrb4*-2 but responded to the stimulus triggered by BTH and re-localized appropriately to the nucleus. In spite of this wild type NPR1 behavior, this line did not respond to BTH (Figure S2C). Thus, NRB4 functions downstream of NPR1 after NPR1 is re-localized to the nucleus.

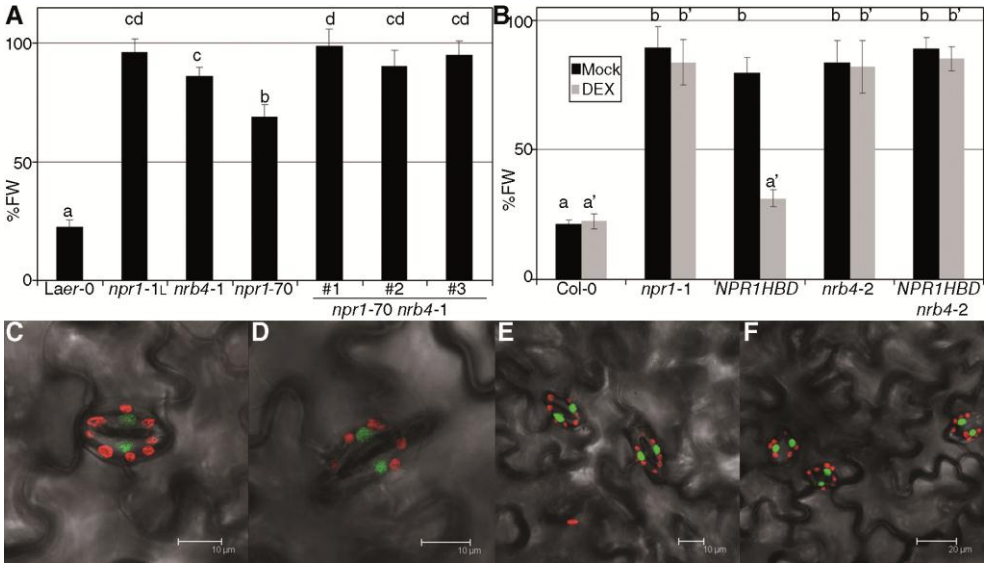


Figure 3. Epistasis of *NRB4* with *NPR1*. **A** Three double mutants *nrb4-1 npr1*-70 and their controls were tested as in Figure 1A. **B** *35S::NPR1-HBD* (*NPR1HBD*) in a *nrb4*-2 background was tested with and without dexamethasone (DEX) for its response to BTH. **C** Confocal image of Arabidopsis *35S::NPR1-GFP* in an *npr1*-1 background in mock conditions. D Same transgenic in an *nrb4*-2 *npr1*-1 background in mock conditions. E. The same line as in C, one day after 350 µM BTH treatment. F The same line as in D, one day after 350 µM BTH treatment.

Cloning of NRB4 and phenotypes of null alleles

Conventional mapping showed that *NRB4* is encoded by At1g15780, a gene labeled as "unknown" in TAIR. The predicted protein contains a KIX domain (Radhakrishnan et al. 1997) at the very beginning, and a glutamine rich region (Guo et al. 2007) in the middle (Figure 4A). The sequence of the three alleles revealed canonical EMS mutations. Each allele had a single point mutation in the KIX domain (Figure 4A, detail of the KIX domain in 4B). The mutations were not extreme in terms of physiochemical distances (Grantham 1974). In fact, the mutations introduced did not change the prediction of an alpha helix structure (Radhakrishnan et al. 1997). Therefore, mutations that introduced small changes in the protein produced a considerable change in phenotype.

Three independent T-DNAs insertions in *NRB4* were found in the databases; two of them are in introns that disrupt the KIX domain (*nrb4*-4 and *nrb4*-5, Figure 4B), and in the third one we did not find any insertion (see Methods for details). The progeny of plants heterozygous for *nrb4*-4 produced a quarter (χ^2 2.15 < 3.84, n=97) of plants of smaller size and more chlorotic than wild type, while heterozygous plants were wild type (Figure 5A). The smaller plants were confirmed to be homozygous *nrb4*-4 by PCR, and they grew very slowly in comparison with wild type plants (Figure 5A and S3A vs. S3B). *nrb4*-4 plants did not seem to be affected in the anatomy of the leaf, at least when observed with cryo-SEM (Figure S4A, B and C). We observed differences in the trichomes. While wild type plants had trichomes with papillae on their surface and prominent cells at their base (Figure 5B), *nrb4*-4 lacked these two elements. Additionally, the arms of the trichomes were different, irregular and chaotically arranged (Figure 5C and S4F). From several stainings, none revealed any difference in terms of cell death or callose deposition (Figure S5), with the

exception of DAPI (Kubista et al. 1987). *NPR1* and SA response are necessary for appropriate DNA content in the nucleus, with *npr1*-1 plants having more endoreplication than wild type (Vanacker et al. 2001). The point mutations in *nrb4* produced normal endoreplication using this assay, while *nrb4*-4 had the same or more DNA per cell than *npr1*-1 (Figure S6A). Therefore, NPR1 and NRB4 shared a role in controlling endoreplication of nuclear DNA.

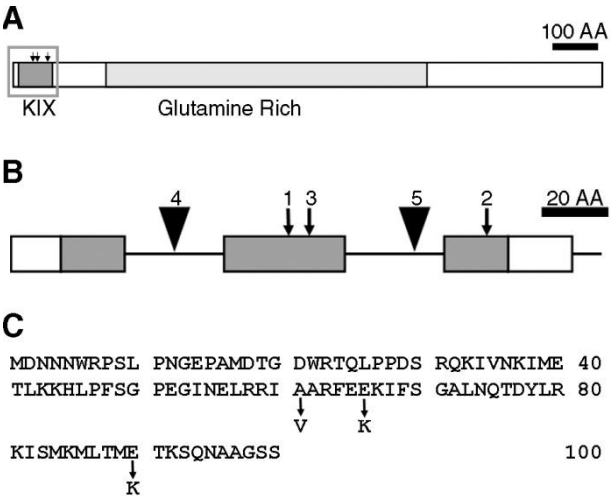


Figure 4.-Predicted structure of NRB4. A Drawing of the predicted NRB4 protein, showing the conserved KIX domain and the region rich in glutamine. **B** Magnification of the KIX domain, showing the introns (horizontal lines), the point mutations (arrows) and the T-DNAs insertions (triangles) found. The number above the mutation indicates the number of allele. The region shown corresponds to the grey rectangle in A. **C** Sequence of the first 100 AA of NRB4, indicating the point mutations.

When transferred to long day conditions to induce flowering, *nrb4*-4 bolted, but did not produce any seeds (Figure 5D and S3). In most plants, there was no production of flowers at all, while in few plants some flowers did appear (Figure S3D vs. S3E). These flowers did not have stamens, and the carpels did not enclose the ovules (Figure S3F). The growth habit of *nrb4*-4 was normal until several days after bolting. Then several additional stems appeared, and afterwards a next generation of stems appeared in the previous stems in a

pattern similar to a fractal (Figure 5D and S3C). Some plants kept growing up to 23 weeks, and when they died, they did not seem to be following the natural program of senescence.

nrb4-4 phenotypes were reproduced by *nrb4*-5 homozygous (Figure S6B). Similarly, F1s between each homozygous EMS allele and heterozygous *nrb4*-4 had a ratio of wild type *vs.* no response to BTH of 1:1 (χ^2 0.1<3.84, n=162), while the EMS alleles and the nulls were fully recessive (Figure 1A and segregation data mentioned). F1s between heterozygous *nrb4*-4 and heterozygous *nrb4*-5 had a ratio of wild type *vs.* no response to BTH of 3:1 (χ^2 0.32<3.84, n=51). Therefore, the phenotypes observed in *nrb4*-4 and *nrb4*-5 were caused only by the insertions of the T-DNAs in the *NRB4* gene, and one copy of the missense mutation was enough to complement the phenotypes of *nrb4*-4, besides the response to BTH.

The *nrb4*-4 homozygous plants were easily distinguished at two or three weeks, and some experiments could be carried out or adapted to this circumstance. *nrb4*-4 did not perceive BTH, either in terms of fresh weight (Figure 5E) or *Pto* growth (Figure 5F). The amount of symptoms in *nrb4*-4 after inoculation with *Pto* indicated that these plants were more susceptible than any other genotype, but perhaps the growth of *Pto* was already reaching a maximum. This extra susceptibility could be quantified with a weak pathogen, *Pseudomonas syringae* pv. *maculicola* CR299 (*Psm* CR299, Ritter and Dangl 1995). Thus, *Psm* CR299 was unable to grow in Col-0, *npr1*-1, or *nrb4*-2, but grew two log units (a hundred fold) in *nrb4*-4 (Figure 5G).

Since *nrb4*-4 had an extreme susceptibility to pathogens, we wondered if it had also an extreme phenotype with SA. The response to SA/BTH in terms of fresh weight and *Pto* growth were already maximized (Figures 5E and F, respectively), so we searched for another phenotype. SA content was considered a promising one, since it increases in plants under biotic stress -like *Pto* inoculation, but it is also increased in *npr1* with respect to wild type plants. *nrb4*-2 plants behaved like *npr1*-1, accumulating roughly the same SA amounts as the wild type in control conditions, and more than the wild type after *Pto* inoculation (Figure 5H). *nrb4*-4 behaved differently, accumulating SA in both free and total form (free plus conjugated) in control conditions. Upon *Pto* inoculation, levels of both forms of SA were strongly increased (Figure 5H). It

was possible to identify *nrb4*-4 homozygous plants in vitro (Figure S6C) and to test their growth in plates with SA. Growth of these plants were severely affected on SA plates, while heterozygous or wild type siblings were largely unaffected by SA (Figure S6D).

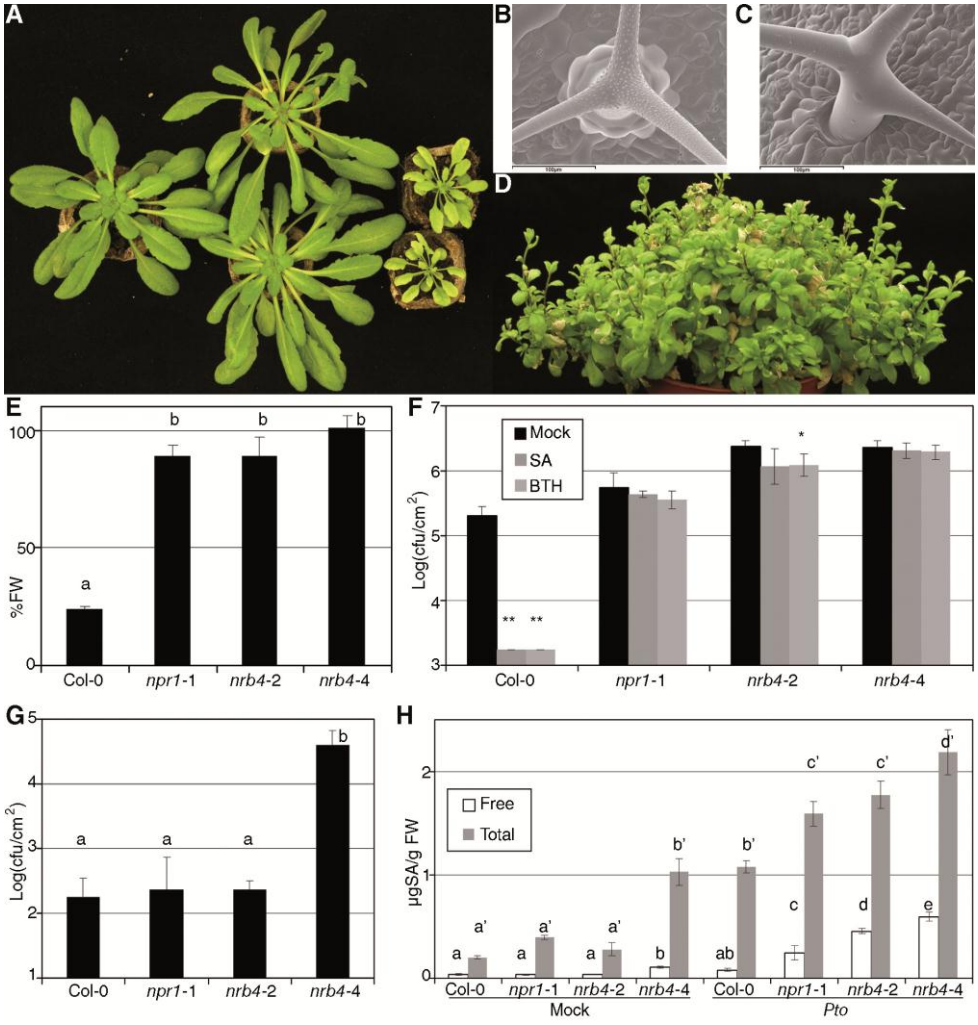


Figure 5.-*nrb4*-4 is a null allele. A From left to right, wild type plant, *NRB4/nrb4*-4 plants, and *nrb4*-4 homozygous plants. Picture taken after six weeks in short day conditions. **B** Cryo-SEM pictures of wild type trichomes. The leaves sampled were approximately 7 mm and plants were five weeks old. **C** Idem for *nrb4*-4 trichomes, although plants were seven weeks old in order to roughly sample leaves of the same size. The length of the bar (bottom of the picture) is 100 µm. **D** Picture of *nrb4*-4 taken after 18 weeks (seven in short day, eleven in long day). **E** Plants were tested as in Figure 1A, but with one more week of growing and two more treatments. **F** Response of *nrb4*-4 to SA and BTH in growth curves. The inoculations were done as in Figure 2C, with the particularity that *nrb4*-4 plants were seven weeks old. **G** *Pseudomonas syringae* pv. *maculicola* CR299 was inoculated and its growth measured as F. **H** The amount of SA (both free and total) was measured three days after a mock or a *Pto* inoculation, as in F. The differences were evaluated among genotypes in the same category, free SA on one hand, and total SA on the other (marked with the symbol prime).

NRB4 is an ortholog of *MED15*

NRB4 was co-immunoprecipitated in Arabidopsis with MED6 (Backstrom et al. 2007), a subunit of the *Mediator* Complex (reviewed by Taatjes 2010). Due to its homology to subunits of *Mediator* in other species, it was labeled MED15, and an *in silico* search claims that it is one of the three MED15 in Arabidopsis (Mathur et al. 2011). The role of MED15 in plants may be divided among these three genes, since the expression of *NRB4* in yeast lacking a functional GAL11/MED15 did not complement the mutant phenotypes (Figure S6E). The location of MED15 is in the *Mediator* complex tail module, and interacts with specific transcription factors (Taatjes 2010). Since *NRB4* is a subunit of the *Mediator* complex, and the *Mediator* complex is critical for global regulation of transcription (Boube et al. 2002), it seemed logical to test the behavior of other *Mediator* subunits in SA response. There were 51 additional potential *Mediator* complex-encoding genes when we cloned *NRB4* (TAIR V9, Swarbreck et al. 2008), and we tested the T-DNAs insertions available at that moment. Six genes had no insertion, 15 had one or more in heterozygosity, and 30 have one or more homozygous insertion. These populations were tested in the same conditions that allowed the identification of *nrb4*-4 homozygous plants, yet none of them produced any phenotype different from the wild type control (Table S1). Therefore, the role of NRB4 in SA response was unique among the *Mediator* subunits.

Molecular footprint of *nrb4*

The additional phenotypes of the null alleles *vs.* the EMS alleles of *NRB4* were striking, but they did not point to any obvious process (e.g. auxins or light) that could be altered besides SA response. A transcriptomic analysis was performed in *nrb4*-4 to identify possible physiological processes affected by the null mutation. Thus, RNAs from *nrb4*-2, and Col-0 three weeks old plants (without treatment or inoculation), plus *nrb4*-4 of the same size (five weeks old) were isolated and hybridized with a commercial oligonucleotide microarray (see Methods). Interestingly, the molecular footprints of the two *nrb4* alleles were quite different.

nrb4-2 had a very small impact on transcription, with eight genes statistically downregulated, and only one upregulated (Figure S7A). On the other hand, *nrb4*-4 had 243 genes statistically downregulated, and 106 upregulated (Figure S7A). Among the genes upregulated, there were genes related to SA biosynthesis (*EDS5* and *SID2*) and to defense (e.g. *PR1, PR2,* and *PR3*), although the levels of induction of the defense genes were quite low in comparison to pathogen inoculation of wild type plants (Figure S7A). This induction, though significant, was not strong enough to detect PR1 protein in a western blot (Figure S8A). The software package MapMan (Usadel et al. 2005) is able to identify groups of genes that are altered in one situation with respect to the control. In the case of *nrb4*-2, there were only two main groups (e.g. "signaling") strongly altered ($p<0.001$), while in *nrb4*-4 there were up to ten main groups strongly altered (Figure S7B), thus reflecting the severity of the pleiotropic phenotype of the mutant.

In spite of the main groups suggested by these and other analyses (see Methods), there was no evidence of specific processes being altered besides SA and defense. Using the global footprint of the transcriptome, one software package, AtCAST (Sasaki et al. 2011), indicated a weak correlation of the *nrb4*-4 transcriptome with the transcriptome of plants overexpressing *ARR22* and *ARR21* (Kiba et al. 2004 and Kiba et al. 2005, respectively). To put these correlations into perspective, several transcriptomic experiments were clustered along *nrb4*-2 and *nrb4*-4 (Figure 6A). *nrb4*-2 was closer to *eds1* (Falk et al. 1999) and *NahG* (Lawton et al. 1995), mutations that produced a decrease in defense, while *nrb4*-4 was closer to the treatments that induced defenses and to *ARR21*

and *ARR22* overexpression. The correlations with *ARR21* and *ARR22* overexpressor plants, although not very strong, might indicate altered cytokinin signaling in *nrb4*-4. When the alleles of *nrb4* were grown in presence of trans-zeatin, there were no phenotypes of cytokinin insensitivity (Figure 6B), so even if the overexpression of genes involved in cytokinin signaling produced the data closest to *nrb4*-4, there was no visible phenotype of the *nrb4* alleles in cytokinins.

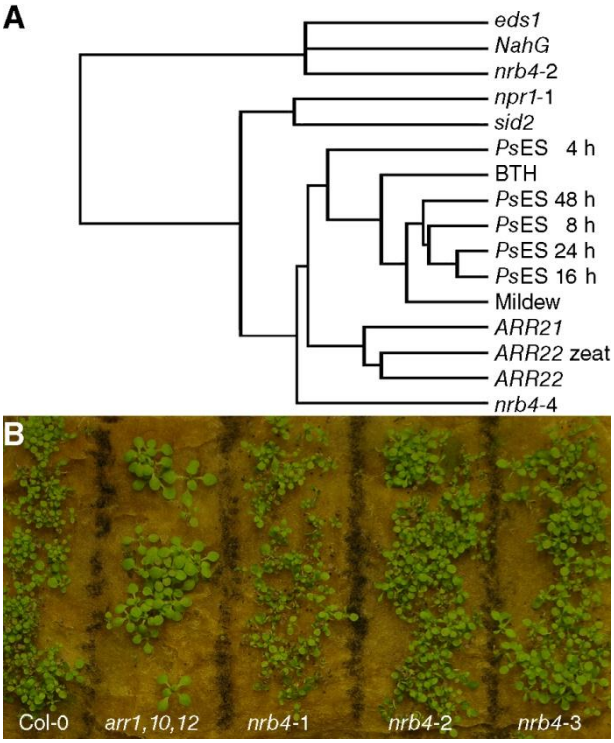


Figure 6.-Molecular footprint of *nrb4*. A The transcriptome of *nrb4*-2, *nrb4*-4, and Col-0 plants were determined, and then compared with different transcriptomic experiments by means of hierarchical clustering and visualization as a dendogram. The references of the experiments used are specified in Methods section. **B** Response to cytokinins in growth. Seeds of the indicated genotypes were grown in rock wool imbibed with 5 µM trans-zeatin. This picture was taken 21 days after growing. *arr1,10,12* stands for the triple *arr1 arr10 arr12*, used as a control for lack of response to cytokinins.

The application of high amounts of cytokinins has been reported to induce SA biosynthesis and therefore defenses (Choi et al. 2010). There is also a negative regulation of cytokinins by SA, which may help to fine tune the amplitude of the defense output (Argueso et al. 2012). We did not detect any difference between the EMS alleles of *nrb4* and the wild type in this regard (Figure S8B), nor did the *nrb4*-4 plants express cytokinin phenotypes (Figure S8C). Therefore, there was no evidence for a role of *NRB4* in cytokinin response, or in any other specific process besides SA response.

NRB4 expression and localization

NRB4 expression is apparently unaltered by stimuli covered in the available microarray data (Hruz et al. 2008). We confirmed this by measuring the levels of *NRB4* after several treatments by RT-qPCR, including *Pto* inoculation and chemical treatments (Figure 7A). There was a reproducible increase in the expression after several treatments, but even in the best conditions (SA) it was quite low (1.46 fold induction). We did not detect any interaction between NRB4 and NPR1 (or its interactors), in yeast two hybrid, regardless of the presence of SA in the media (Figure S9). The EMS alleles did not produce a measurable instability in the mutated mRNA, while the *nrb4*-4 rendered the mRNA below the threshold of detection (Figure 7B). The expression of *NRB4* was unaltered in *npr1*-1 plants (Figure 7B), and *NPR1* was detectable in *nrb4*-2 at normal levels (Figure S10A).

Although the *Mediator* complex is described to act in the nucleus, *NRB4* did not contain any obvious nuclear localization signal. To determine the localization of the protein, we transiently over-expressed the *NRB4* cDNA before and after the GFP protein in *N. benthamiana* (Figure 7C and D, respectively). In both cases, there was a strong localization in the nucleus, with the particularity of NRB4-GFP accumulating also outside the nucleus. The nuclear localization did not change by the application of 350 μM BTH (Figure S10B). The predicted size of NRB4 was 146 kDa, well above the free diffusion limit into the nucleus of 50 kDa (Talcott and Moore 1999).

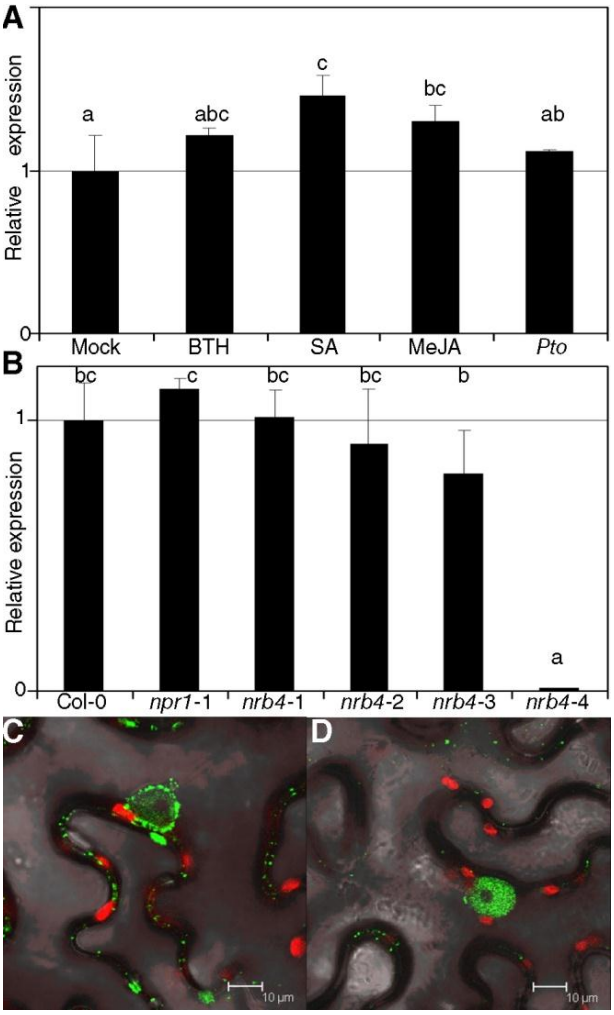


Figure 7.- Expression of NRB4 and cellular localization. A *NRB4* was measured one day after treatments of mock, 350 µM BTH, 500 µM SA, and 100 µM Methyl jasmonate (MeJA), or three days after a *Pto* inoculation. The levels of expression are normalized to three reference genes and to the level of Col-0. **B** RNA was extracted from three weeks old plants (five weeks for *nrb4*-4), and transcript levels for *NRB4* were measured by RT-qPCR. **C** *Agrobacterium tumefaciens* with the construct *35S:NRB4-GFP* was infiltrated in leaves of *Nicotiana benthamiana*, and the expression was detected with a confocal microscopy four days later. **D** Idem with the construct *35S:GFP-NRB4.*

A *35S:NRB4-GFP* construct was transformed in Arabidopsis and the result is shown in Figure 8. *NRB4-GFP* complemented the EMS alleles with some variation when the response to BTH in terms of fresh weight was considered (Figure 8A). This variation was representative of the transgenic lines obtained regardless of the background. A version of NRB4 containing the first 670 AA was also able to complement the mutations in some lines (Figure 8A), while the version of NRB4 containing only the first 112 AA did not. In all of these lines, GFP was not detectable by means of confocal microscopy or western blot (Figure S10C). However, since there was a detectable function, we also transformed the wild type Col-0 study the effects of *NRB4* over-expression. In this experiment, *35S:NPR1* was included as a control, since it is more sensitive to SA (Cao et al. 1998). The transgenic lines that overexpressed both versions (1335 and 670 AA) of *NRB4* had enhanced SA response in terms of fresh weight after BTH applications (Figure 8B). We additionally complemented the wild type restriction of *Pto* growth after SA or BTH application using these lines (Figure 8C). Note that the over-expression of *NRB4* did not produce a strong defense response under control conditions. But when SA or BTH was applied, there was an enhanced response to SA (Figure 8D). Therefore, the effect of *NRB4* was specific and limited to SA response.

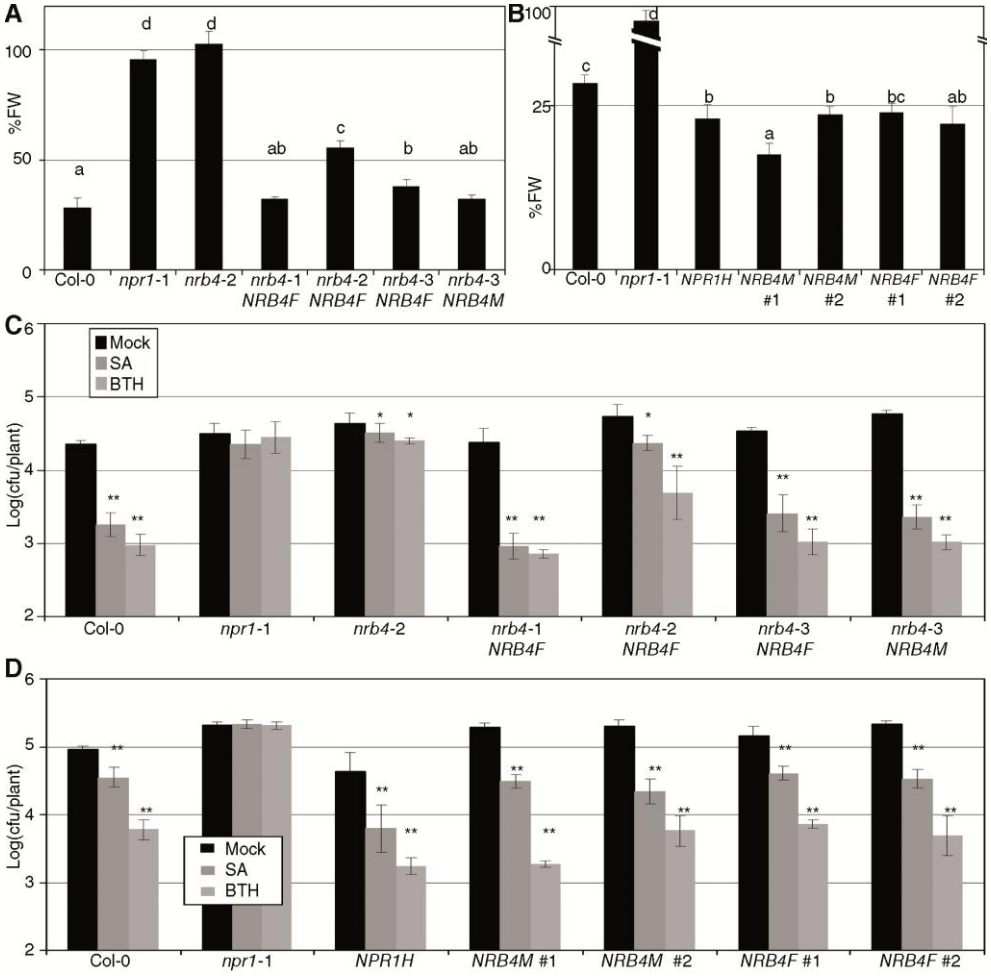


Figure 8.-Phenotypes of transgenic lines. A Transgenic plants homozygous for the construct *35S: NRB4-GFP* (*NRB4F*) or for the equivalent construct with only the first 670 AA (*NRB4M*), were obtained in the mutant alleles. The panel shows the response to BTH in fresh weight of the mentioned lines tested as in Figure 1A. **B** The constructs described in A were transformed in Col-0, and their response to BTH in fresh weight recorded. The number indicates an independent line. **C** Response of the transgenic lines described in A to SA and BTH in growth curves, as described in Figure 2A. **D** Idem for the transgenic lines described in B.

Discussion

A role for *Mediator* in SA response

While *Mediator* is a complex that interacts with RNA Pol II, mutations in specific subunits of this complex typically impact specific phenotypes, rather than general transcription (reviewed by Taatjes 2010). This observation has lead to the hypothesis that the *Mediator* complex performs both general and specific roles to regulate gene expression (Taatjes 2010). In plants, the *Mediator* complex is emerging as a crucial step in transcriptional regulation in response to specific signals (reviewed by Kidd et al. 2011). The identification of the *Mediator* complex has been done with biochemistry and genetics. The immunopurification of MED6 in Arabidopsis led to the identification of nineteen *Mediator* subunits, NRB4 among them (Backstrom et al. 2007). A null mutation in *SWP/MED14* produces sterile plants with reduced growth, small leaves, and an increase in endoreplication (Autran et al. 2002), similar to our observations with *nrb4*-4. The main differences were the size of the cells; in *nrb4*-4 the size of cells was similar to wild type (Figure S4), and in *35S:NRB4*, the plants were macroscopically similar to wild type. In the case of *SWP*, both the knock out and the overexpressor produced plants and cells of smaller size than the wild type.

Mutations in *MED21* (Dhawan et al. 2009), *PFT1/MED25*, and *SETH10/MED8*, (Kidd et al. 2009) affect disease resistance against necrotrophic pathogens to different degrees. Specifically, null homozygous mutants in *MED21* are lethal at the stage of embryo, and RNAi plants with low levels of *MED21* are more susceptible to necrotrophic pathogens (Dhawan et al. 2009). *PFT1* (*PHYTOCROME and FLOWERING TIME 1*) was first described as a gene required for the shade avoidance response and flowering (Cerdan and Chory 2003). Once PFT1 was identified as a *Mediator* subunit (Backstrom et al. 2007), a screen for similar phenotypes in the rest of subunits identified a mutant in *SETH10/MED8* as required for both wild type flowering time and resistance to necrotrophic pathogens (Kidd et al. 2009). Following this logic, we tested the available T-DNAs insertions for a phenotype in SA response, but found no additional *Mediator* subunit with a measurable phenotype (Table S1).

It is striking that, on one hand, mutations in three subunits of *Mediator* express defense phenotypes in response to necrotrophic pathogens that are related to MeJA response, while on the other hand, only one subunit, *NRB4*, has a phenotype related to SA-dependent defense responses. A plausible explanation would be that *NRB4* is a negative regulator of MeJA, and its removal would increase MeJA response. Then, this increase in MeJA signaling could be observed as loss in SA signaling, since both signals crosstalk negatively (Robert-Seilaniantz et al. 2011). But there is no specific phenotype related to MeJA in the *nrb4* EMS alleles (MeJA plates, MeJA induced resistance, *P. cucumerina* infection, and growth of *Pto(cor-)*, Figure S1). In any case, there are four subunits of *Mediator* in plants involved in biotic stress. Mutants in *MED25* are more sensitive to salt stress (Elfving et al. 2011), so there is clearly an over-representation of stress phenotypes in the described mutations of *Mediator* subunits.

Functions in stress response are also present -although not exclusively- in *MED15*. *MED15* in Drosophila was identified in a mosaic screening where the effect of the mutation was limited to the wings (Terriente-Felix et al. 2010). Homozygous null mutations were lethal at embryogenesis, and the weak point mutations found were lethal at later stages (Terriente-Felix et al. 2010). *MDT-15* is the ortholog of *MED15* in *C. elegans*, and the knock down by RNAi of this gene produced multiple deleterious effects (reduced life span, sterility, etc., Taubert et al. 2006). A reduction in functional MDT-15 protein leads to animals being hypersensitive to xenobiotics, thus affecting selectively stress response related to ingestion (Taubert et al. 2008). *GAL11* is the ortholog of *MED15* in yeast, and the deletion of this gene is not lethal, but is essential for growth on nonfermentable carbon sources, for sporulation, and for mating (Mylin et al. 1991, and references herein). The deletion of *GAL11* renders yeast more sensitive to cycloheximide (Shahi et al. 2010). Using this phenotype, we introduced *NRB4* in yeast Δ*gal11*, but it did not complement the growth in cycloheximide (Figure S6E). *NRB4* may not be the correct *MED15* ortholog, since an *in silico* analysis predicts that there are three *MED15s* in Arabidopsis (Mathur et al. 2011). The existence of more than one ortholog is not new in the *Mediator* complex in plants (Kidd et al. 2011) or other organisms (Bourbon et al. 2004). If *NRB4* is one of three *MED15* subunits of Arabidopsis, then its role in SA response is non-redundant, since the two null alleles of *NRB4* did not respond to SA.

How specific is NRB4?

Mediator is a complex required for the normal transcription of genes. In a high throughput screening of any process, there could be a moment when elements of the general transcriptional machinery would start to appear. Our data regarding *NRB4* do not fit this concept, but point towards a specific role for *NRB4* in SA response. First, the three hypomorhic EMS alleles do not have any noticeable phenotype besides response to SA. It is true that the first selection was done with BTH, but in the case of *npr1*, different alleles diverge in their response to MeJA (Dobón et al. 2011). Second, the phenotypes of *nrb4*-4 and *nrb4*-5, although dramatic, did not resemble mutants generally impaired in signaling (e.g. hormones and light). Third, the transcriptomes of both *nrb4*-2 and *nrb4*-4 were not indicative of perturbation of any specific process compared to untreated wild type plants (Figure S7). Fourth, *NRB4* has not been found in other screenings, and some are done *en masse*, like screenings in plates for lack of hormone response. It is not a small protein (1335 AA), and it has a glutamine rich region. EMS is the most frequent mutagen used in Arabidopsis, and its effect in glutamine is introducing stop codons (two possible stop codons and one silent mutation, Martinez-Zapater and Salinas 1998). Fifth, the overexpression of *NRB4* did not produce any additional phenotypes but an increase in response to SA (Figure 8). The specificity of *NRB4* should be localized in the KIX domain, since the three missense alleles were localized there. It is the more conserved domain, and half of the protein can be deleted without major loss of function (Figure 8).

A model of SA response

We have shown that *NRB4* is necessary for SA response, and the pivotal role of *NPR1* in this signaling has been abundantly reported (Dong 2004). In spite of the importance of these genes in response to SA, we did not detect any interaction between the genes or their proteins. The F1s between the mutant alleles were wild type (Figure 1A), and no protein-protein interaction was detected in yeast or *in planta*. The over-expression of *NPR1* in an *nrb4* mutant did not restore the response to SA (Figures 3B and S2C). Such an effect could have occurred if the corresponding proteins worked together. As a consequence, with the necessary precautions for the evaluation of negative results, it seems that NRB4 and NPR1 act in different points of the SA

signaling, which also explains the phenotypes of the double mutants, both with strong and weak alleles (Figure 3A and S2A). A version of NPR1 tagged with GFP was able to respond to BTH applications and concentrated in the nucleus, both in an *nrb4* and in a wild type background (Figure 3), but did not rescue altered response to SA. Therefore, NRB4 does not play any role in the stability of NPR1 (i.e. it does not act as chaperone), nor in its traffic towards the nucleus (e.g. in *atgsnor1*-3 plants there is no change in NPR1-GFP upon SA treatment, Tada et al. 2008). Thus, the concentration of NPR1 in the nucleus is NRB4 independent, and NRB4 functions downstream of NPR1.

As mentioned in the introduction, there are several genes that act downstream of NPR1. Among the genes found to be relevant in the SA response, there are several DNA repair (Song et al. 2011) and chromatin remodeling (Wang et al. 2010) genes. Since these proteins play a role in forming complex relevant for transcription (Durrant et al. 2007), perhaps NRB4 is required for the proper function of these proteins.

Alternatively, it is plausible that NRB4 interacts with an SA receptor. In yeast, GAL11/MED15 is necessary for the expression of genes required for growth in galactose media (Suzuki et al. 1988). But other functions include the use of fatty acids (see below) and the regulation of multidrug resistance (MDR). Thus, Δ*gal11* yeast does not grow in media with small amounts of ethidium bromide (Mylin et al. 1991) or cycloheximide (Shahi et al. 2010), while the wild type grows unaffected. This pathway is the same used by *Candida glabrata* to pump out of the cell ketoconazole (Thakur et al. 2008). The mechanism is that Pdr1p and Pdr3p are xenobiotic nuclear receptors that bind GAL11 (specifically in the KIX domain) in a xenobiotic-dependent manner (Thakur et al. 2008). This is not a unique case, since in *C. elegans*, NHR-49 binds MDT-15/MED15 also in the KIX domain (Taubert et al. 2006). In this and other organisms, MED15 regulates the metabolism of fatty acids, with a proposed model that NHR-49 and other nuclear receptors are binding a hormone-like small molecule(s) present in the food (Taubert et al. 2006).

Although none of the previous examples involves a SA receptor, similar molecules have been found to participate in these pathways. Oaf1P is a yeast nuclear receptor that, upon binding fatty acids, interacts with GAL11, and activates the transcription of genes required for the use of fatty acids (Thakur et

al. 2009). A similar function is carried out by NHR-49 in *C. elegans* (Taubert et al. 2006), and by PPARα in vertebrates (Issemann and Green 1990). These three receptors bind fatty acids, but also bind nonsteroidal anti-inflammatory drugs (NSAIDs), such as salicylates. Therefore, the orthologs of MED15 interact with receptors in the KIX domain that bind salicylates and fatty acids. There is a strong representation of proteins related to lipids among the Arabidopsis defense mutants (*eds1*, *pad4*, *sag101*, Wiermer et al. 2005; *dir1*, Maldonado et al. 2002; *ssi2*, Kachroo et al. 2003; *sfd1*, Nandi et al. 2004; etc.), so it is plausible that this connection is maintained in Arabidopsis. There are no genes in Arabidopsis with significant homology to *Oaf1P*, *NHR*-49, or *PPARa* (Table S2), so if there is SA receptor interacting with NRB4 it has evolved divergently from the aforementioned receptors.

There is a striking difference in the phenotypes between the EMS mutations and the null mutations (Figure 5). The null mutants have a stronger phenotype in defense than the EMS alleles, and a severe phenotype in development. The phenotype in defense is even stronger than the *npr1* alleles so far described (Canet et al. 2010b). *Psm* CR299 grows a hundred fold in *nrb4*-4, while it does not grow in the rest of genotypes (Figure 5G). More strikingly, SA itself is upregulated in *npr1* and *nrb4* alleles, but in *nrb4*-4 the levels reach a maximum. Interestingly, this implies that the metabolism of SA is partially independent of NRB4 and NPR1. It also implies that SA itself does not affect *Pto* directly, since the levels of SA in *nrb4* clearly exceed the levels of SA in wild type plants (Figure 5H). This increased phenotype in the null mutants could be explained if the EMS alleles were not completely void of function. The point mutations in the KIX domain do not impair *nrb4*-1 metabolism of SA in plates (Figure 1B), or *nrb4*-2 to mount a proper SAR (Figure 2D). The *npr1* null alleles do not have the same phenotype as the *nrb4* null alleles, perhaps because the rest of *NPR1* paralogs are able to compensate for its loss in the SA response (Canet et al. 2010b). The strong phenotype of the null *nrb4* in development could be due to additional signals that are lost in this mutant. However, we have found so far no indications for such signals.

SA plays an important role in different plant processes besides disease resistance (Rivas-San Vicente and Plasencia 2011), and one possible explanation could be that the phenotypes of *nrb4* null alleles are only due to a lack of response to SA, therefore supporting the postulated essential role of SA in

normal plant development (Vanacker et al. 2001). Thus, *npr1*-1 and *nrb4*-4 express increased in the endoreplication of the nuclear DNA (Figure S6A), reflecting a role of SA in this process. The available mutants with less SA do not show any of the previous phenotypes (Vanacker et al. 2001), but several analysis show that these plants still have some SA (Rivas-San Vicente and Plasencia 2011), and plants that lack several SA biosynthetic genes are not viable (Garcion et al. 2008).

Materials and methods

Plant growth and inoculation

Arabidopsis thaliana (L.) *Arabidopsis thaliana* (L.) Heynh. was sown and grown as described (Canet et al. 2010a), in controlled environment rooms with days of 8 h at 21°C, 150 μmol m^{-2} s^{-1} and nights of 16 h at 19°C. The treatments, inoculations, and sampling started 30 minutes after the initiation of the artificial day to ensure reproducibility. The following genotypes were used: *npr1*-1 (Cao et al. 1997), *35S:NPR1-HBD* and *35S:NPR1-GFP* (Kinkema et al. 2000), *rpm1* (Grant et al. 1995), *rps2* (Mindrinos et al. 1994), *nho1* (Lu et al. 2001), *arr1*-3 *arr10*-5 *arr12*-1 (Argyros et al. 2008). *nrb4*-4 was SAIL_792_F02 and *nrb4*-5 was GABI_955_E02. The line in which we did not find any insertion was SALK_106110C. *Pseudomonas syringae pv. tomato* DC3000 (*Pto*) was grown, inoculated and measured as described (Tornero and Dangl 2001). Briefly, plants of 18 days were inoculated by spray with *Pto* at OD_{600}=0.1 with 0.02% Silwet L-77 (Crompton Europe Ltd, Evesham, UK). Three days later, the amount of colony forming units (cfu) per plant was quantified and represented in a logarithmic scale. When inoculations of older plants was measured, a sample of known surface was taken, and the resulting unit was Log(cfu/cm^2). Another strains used were *Pto(avrRpm1)* (Ritter and Dangl 1996), and *Pto(avrRpt2)* (Debener et al. 1991). *P. syringae* pv. *tabaci*, and pv *phaseolicola* NPS3121 were obtained from Dr. Jeff Dangl (UNC, Chapel Hill, NC, USA). *P. syringae* pv *maculicola* CR299 has been described by (Ritter and Dangl 1995). Systemic Acquired Resistance was performed as reported by Macho et al. 2010, inoculating leaves with *Pto(avrRpm1)* or a mock treatment using a blunt syringe. For all the experiments, at least three independent treatments were performed

(three independent sets of plants sown and treated on different dates). *Pto* was maintained as described (Ritter and Dangl 1996).

Chemical treatments

Primers and chemical products were purchased from SIGMA (St. Louis, MO, USA) unless otherwise is stated. Benzothiadiazole (BTH, CGA 245704), in the form of commercial product (Bion® 50 WG, a gift from Syngenta Agro S.A. Spain) was prepared in water for each treatment and applied with a household sprayer. The response to BTH in terms of fresh weight was done as reported (Canet et al. 2010a). Briefly, plants were treated with mock or 350 μM BTH four times along three weeks. Then, the fresh weight of the plants was recorded and expressed as the ratio between BTH and mock treated plants in percentage. 100 μM methyl jasmonate (MeJA, Duchefa, Haarlem, The Netherlands) was applied by spray in 0.1% DMSO and 0.02% Silwet L-77. Dexamethasone was applied at 2 μM, diluted in water from a stock of 20 mM in EtOH. SA (in the form of sodium salicylate) was applied at 500 μM. For the treatments with cytokinins, trans-zeatin at 5 μM was used to imbibe pieces of wool rock (from a local gardening shop). Seeds were shown directly in the wool rock, and additional water was added to compensate for evaporation.

SA in plates and *in planta*

Arabidopsis seeds were surface-sterilized for 10 min in 70% ethanol and for 10 min in commercial bleach. Then, five washes were done with distilled water and the seeds were distributed on agar plates. The medium contains 0.5x Murashige and Skoog salts (Duchefa BV, Haarlem, the Netherlands), 0.6% (w/v) Phyto Agar (Duchefa), 2% (w/v) sucrose, with 0, 400 or 500 μM SA (final concentration). The result was evaluated 14 days after transferring to growing conditions. The chlorophyll was extracted with ethanol for 2 hours at 65 C, and quantified as described by (Frye et al. 2001). The measurements consisted in three samples per treatment and genotype of 10 plants. For the measurement of SA *in planta*, three samples of c. 100 mg were frozen in liquid nitrogen. SA extraction was performed as described by Huang et al. 2005 and Defraia et al. 2008.

Expression *in planta* and microscope

NRB4 was cloned in pDONR221 (Invitrogen, Barcelona, Spain) from RT-PCR, and then transferred to pMDC43 (Curtis and Grossniklaus 2003; *GFP-NRB4*) and pB7FWG2 (Karimi et al. 2002; *NRB4-GFP*) for expression *in planta*. *N. benthamiana* leaf tissue was mounted in water under a coverslip 4 days after infiltration with *Agrobacterium tumefaciens* containing the constructs. The Arabidopsis plants containing NPR1-GFP had three weeks at the time of the pictures. The imaging was conducted with a Leica TCS SL confocal laser scanning microscope (Leica, Heidelberg, Germany) using an HCX PL APO CS 40X/1.25 oil objective to study the subcellular localization of the fluorescence-tagged proteins. Green fluorescent protein was visualized by 488-nm excitation with an Ar laser, and its emissions were examined with a band-pass filter for 500 to 530 nm. The primers used are included as Supplemental Table S3. The SEM pictures were taken with a JSM-5410 scanning electron microscope (JEOL, Tokyo, Japan) in the Electron Microscopy Service (Universidad Politécnica de Valencia, Spain).

Western blot and RT-qPCR

Immunodetection of PR1 protein was carried out as described (Wang et al. 2005), using an Amersham ECL Plus Western Blotting Detection Reagents (GE HealthCare, Little Chalfont, UK). The second antibody was a 1:25000 dilution of Anti-Rabbit IgG HRP Conjugate (Promega, Madison, USA). Chemiluminescent signals were detected using a LA-3000 Luminescent Image Analyzer (Fujifilm Life Science, Stamford, CT, USA).

Total RNA was extracted with Trizol (Invitrogen), following the manufacturer's instructions. cDNA was synthesized with RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Madrid, Spain), and the quantitative PCR performed with LuminoCt Sybr Green qPCR Ready Mix (SIGMA) in a 7000 RT-PCR Systems machine (Applied Biosystems, Madrid, Spain), following the manufacturer's instructions. For each measurement, three biological replicates were done. The obtained values were referred to the geometric average of three reference genes (At3G18780, At1G49240, and At5G60390), as described (Vandesompele et al. 2002), and normalized, being the value of Col-0 in mock equal to one. The list of primers used is provided in Supplemental Table S3.

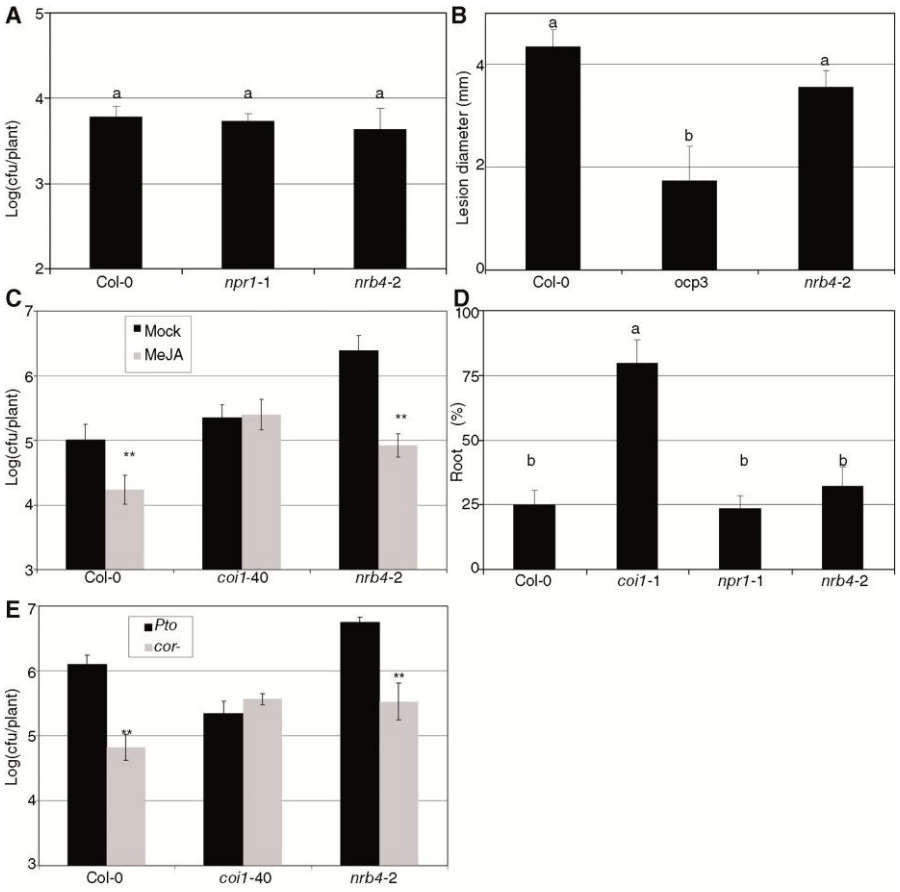
Microarrays and Software used

RNA was isolated as described above and purified with "RNeasy Mini Kit" (Qiagen, Valencia, CA, USA). Array hybridization to an Arabidopsis GeneChip ATH1 (Affymetrix, Santa Clara, CA, USA) was performed following the manufacturer's recommendation. The hybridization was carried out in the "Sección de Chips de DNA-S.C.S.I.E.", University of Valencia (Valencia, Spain). The original hybridization data files were submitted to the European Bioinformatics Institute (EMBL-EBI) ArrayExpress repository and the Accession number E-MEXP-3602 was assigned to this experiment. The analysis of the microarrays was accomplished with Robin 1.1.7 (Lohse et al. 2010). Then, the following software was used: Sample Angler (http://142.150.214.117), AtCAST (Sasaki et al. 2011), Cluster 3.0 (de Hoon et al. 2004), and JavaTreeView (Saldanha 2004). For the statistic analysis, we used Excel 2003 (Microsoft, Redmond, WA, USA) and Statgraphics 5.1 (Statpoint Technologies, Inc., Warrenton, VA, USA). The data analyzed corresponded with the following experiments: *eds1,* E-MEXP-546; *NahG*, E-GEOD-5727; *npr1*-1, E-GEOD-5745; *sid2*, and BTH, E-GEOD-9955; *PsES*, E-GEOD-5685; Mildew, E-GEOD-431; *ARR21,* GSE5699; and *ARR22*, GSE5698.

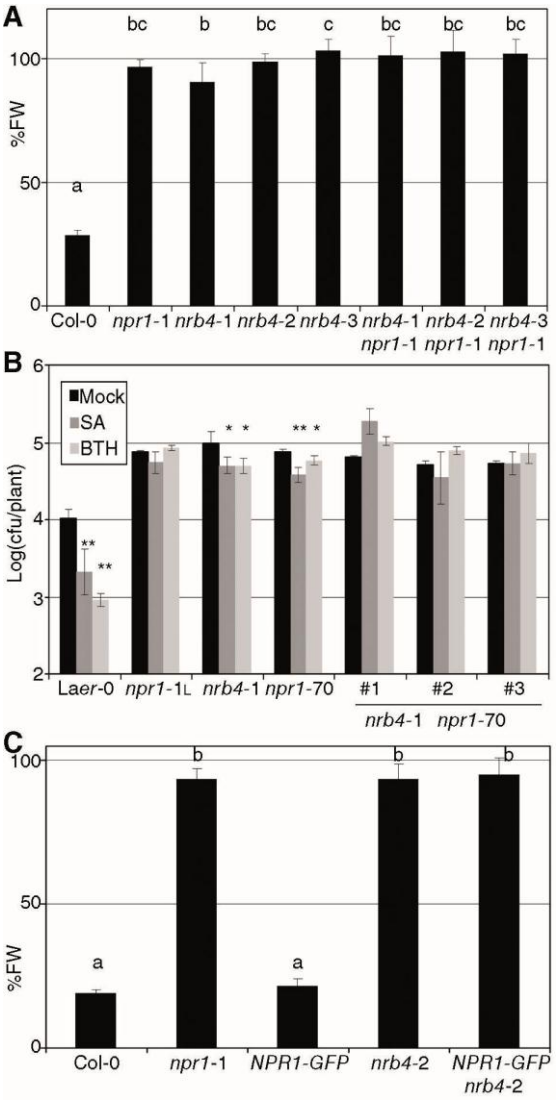
Acknowledgments

This work was supported by the "Ministerio de Economía y Competitividad" (MINECO) of Spain (grant BIO201018896 to PT, a JAE-CSIC Fellowship to JVC and a FPI-MINECO to AD) and "Generalitat Valenciana" of Spain (grant ACOMP/2012/105 to PT). We appreciate the opinions and generous help of Drs. Jeff Dangl and Pablo Vera about the manuscript. The authors declare that they have no conflicts of interest.

Supplemental information



Supplemental Figure 1. Additional phenotypes of *nrb4* in defense. A Responses to *Pto (hrpC-),* a strain that lacks virulence in Arabidopsis (Deng et al. 1998), inoculated and measured as indicated in Figure 2A. **B** Inoculations with *Plectosphaerella cucumerina*. *P. cucumerina* was provided by Brigitte Mauch-Mani (University of Neuchatel, Switzerland), and used as described (Ton and Mauch-Mani 2004). **C** Response to MeJA induced resistance. MeJA was applied by spray at 100 µM in 0.1% DMSO and 0.02% Silwet L-77 one day before *Pto* inoculation. **D** Responses to MeJA in the length of roots. Plants were grown in Johnson's media (Johnson et al. 1957) with 1 mM KH_2PO_4, with or without 50 µM MeJA (Duchefa). The length of the roots was measured with ImageJ software (MIH, Bethesda, MD, USA). **E** Responses to coronatine. *Pto(cfa⁻)* a strain that lacks coronatine (Mittal and Davis 1995) was inoculated, along *Pto*, as indicated in Figure 2A.



Supplemental Figure 2. Epistasis of *NRB4* with *NPR1*. **A** Three double mutants *nrb4 npr1*-1 and their controls were tested as in Figure 1A. **B** The same lines of Figure 3A were tested for its response to SA and BTH upon *Pto* inoculation. **C** *35S:NPR1-GFP* (*NPR1-GFP*) in a *nrb4*-2 *npr1*-1 background, along its controls, was tested for its response to BTH. The overexpression of *NPR1*, even if it was localized to the nucleus (Figure 3F), did not complement the mutation *nrb4*-2.

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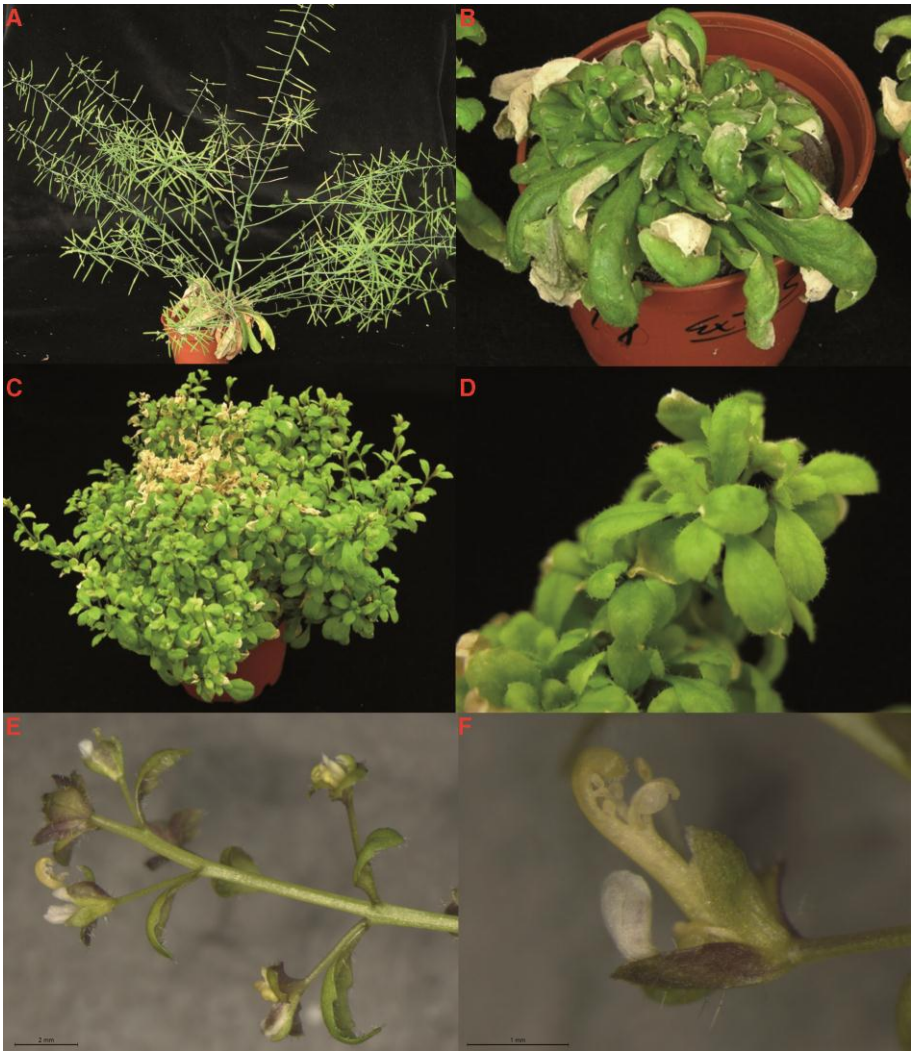
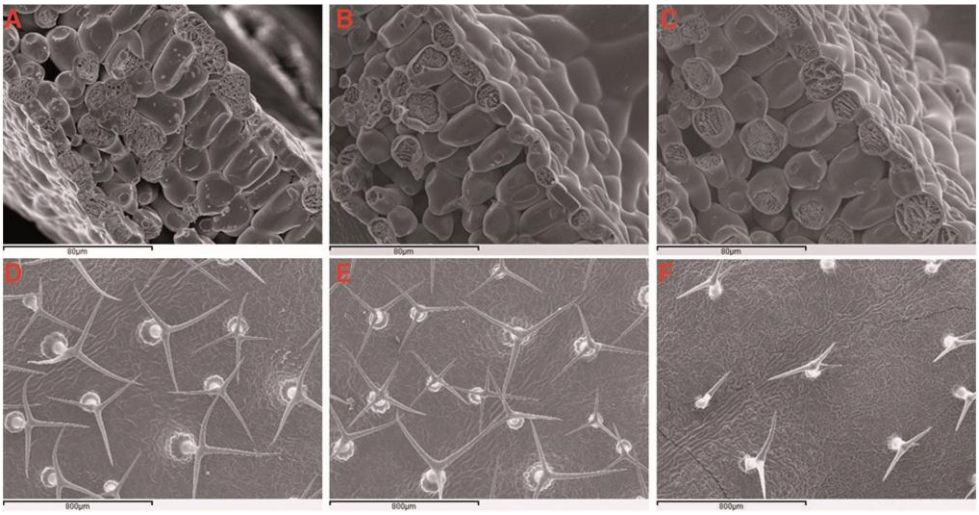
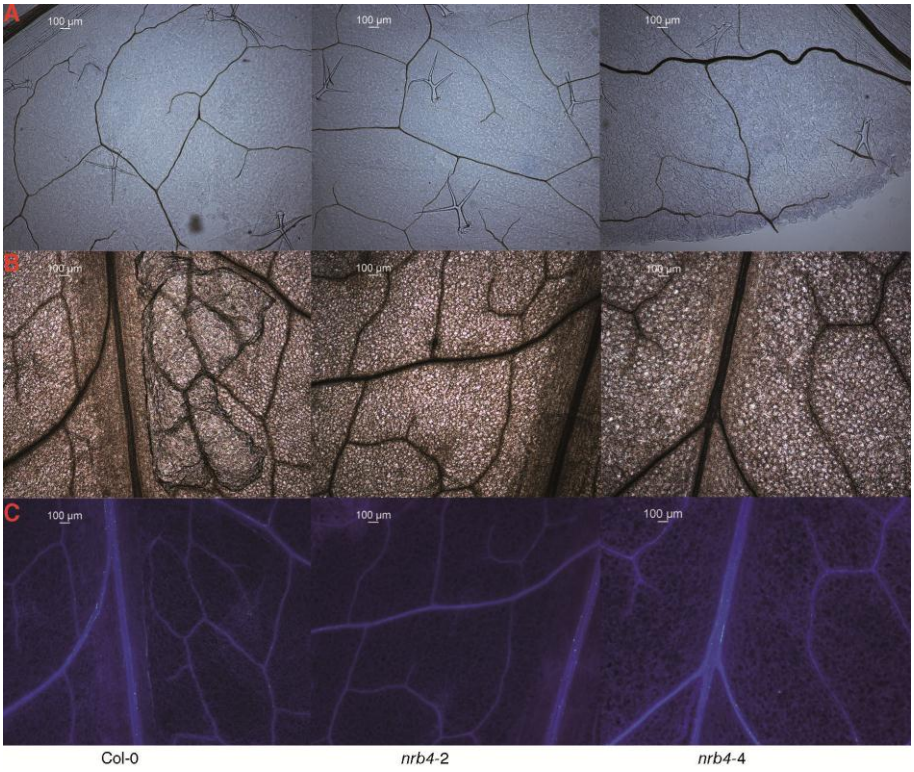


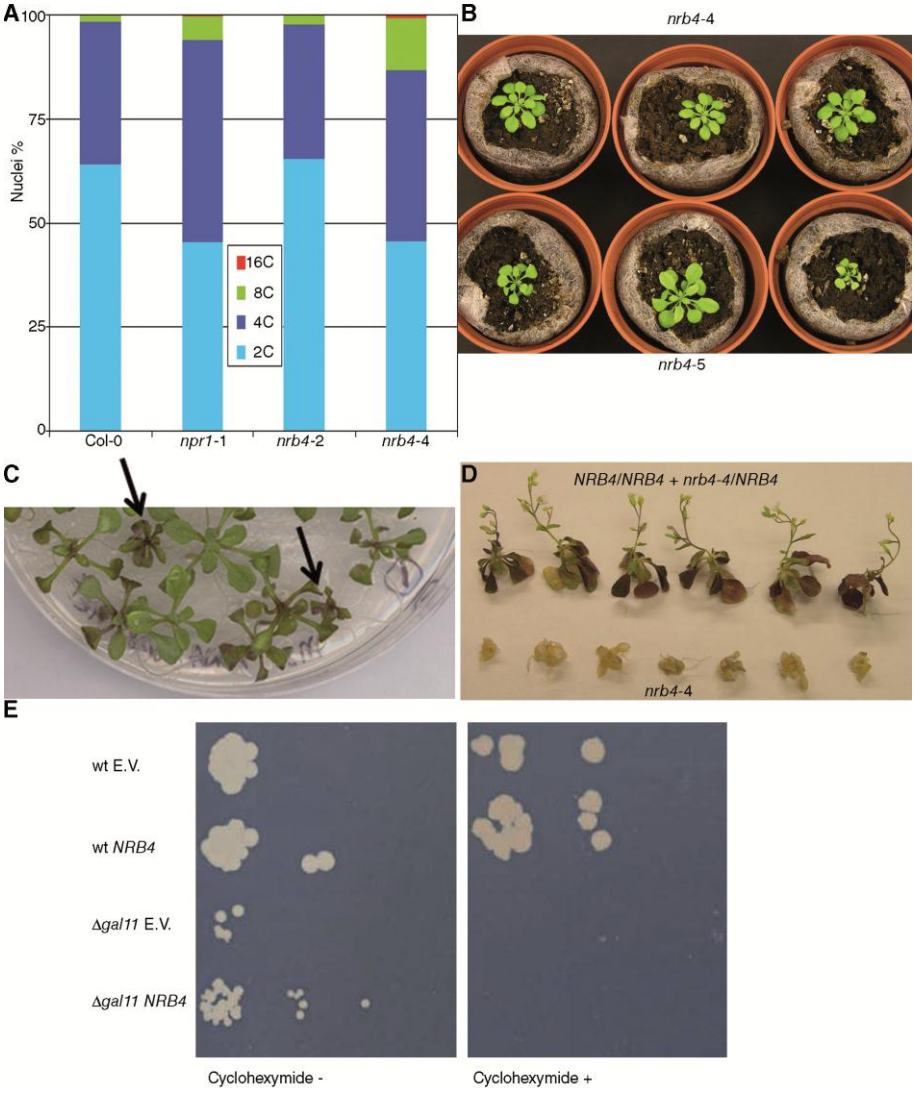
Supplemental Figure 3. Additional pictures of *nrb4*-4. A Wild type plant, growing seven weeks in short day and six weeks in long day with no treatment or inoculation. **B** *nrb4*-4 plant of the same age, growing in the same conditions. **C** *nrb4*-4 plant growing seven weeks in short day and eleven weeks in long day, as in A. **D** Detail of the plant in C, note the absence of flowers. **E** *nrb4*-4 plant with flowers. **F** Detail of the plant in E. As a reference, the pots have a diameter of 6 cm.



Supplemental Figure 4. Additional pictures of Cryo-SEM. A Section of Col-0. **B** Section of *nrb4*-2. **C** Section of *nrb4*-4. **D** Surface of a leaf from Col-0. **E** Idem from *nrb4*-2. **F** Idem from *nrb4*-4. The length of the bar in A, B, and C is 80 µm, and in D, E, and F is 800 µm. The leaves were samples as in Figure 5 (five weeks for Col-0 and *nrb4*-2 and seven weeks for *nrb4*-4).



Supplemental Figure 5. Stainings of *nrb4.* **A** Trypan blue stains, unveiling cell death and membrane damage in Col-0, *nrb4*-2, and *nrb4*-4. **B** Aniline blue stains under visible light. **C** The same Aniline blue stains under ultraviolet light, which detects callose depositions. Trypan Blue and Aniline Blue staining were performed as described (Tornero et al. 2002b; Conrath et al. 1989, respectively). No differences were observed with these stains.



Supplemental Figure 6. Characterization of *nrb4* null alleles. A DNA content. Nuclei from the indicated genotypes were extracted, stained with DAPI, and the relative amount of DNA measured with a CyFlow Ploidy Analyzer (Partec GmbH, Münster Germany) following the manufacturer's instructions. At least 5000 nuclei were counted in each measurement, and the same result was obtained in three independent experiments. **B** Phenotypes of *nrb4*-5 in comparison to *nrb4*-4. **C** Phenotypes of a T2 segregating family of *nb4*-4/*NRB4* in MS plates. The arrows point to *nrb4*-4 homozygous plants (later genotyped by PCR) Picture taken at two weeks of growing. **D** Plants selected in C, were transferred to MS plates with 500 µM

SA, and the picture was taken two weeks after the transfer. **E** Lack of complementation in yeast. Empty vector (E.V.) pAG423 (Alberti et al. 2007) and NRB4 cloned in pAG423 were introduced in wild type (wt) and *Δgal11*. The different strains were grown in liquid and then plated in SD His- plates with or without cyclohexymide (0.2µg/ml). The wt and *Δgal11* strains were obtained from EUROSCARF (Ref. Y00000 and Y01742, respectively).

A *Locus Id.*	*Identifier*	*Annotation*	*nrb4_2 - Col_0*	*nrb4_4 - Col_0*
Genes induced in *nrb4*-2				
AT1G52360	259638_at	coatomer protein complex, subunit beta 2 (beta prime), putative	1,27	0,75
Genes repressed in *nrb4*-2				
AT3G30720	256940_at	unknown protein	-1,87	-1,77
AT3G49400	252314_at	transducin family protein / WD-40 repeat family protein	-1,75	-1,74
AT1G14870	262832_s_at	[AT1G14870, Identical to Uncharacterized protein At1g14870 [Arabidopsis Thaliana] (GB:Q9LQU4)	-1,98	1,29
AT3G22231	256766_at	PCC1 (PATHOGEN AND CIRCADIAN CONTROLLED 1)	-1,93	-0,58
AT2G14560	265837_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G33840.1)	-1,84	0,81
AT3G22240	256617_at	unknown protein	-1,44	-0,21
AT1G19960	261221_at	similar to transmembrane receptor [Arabidopsis thaliana] (TAIR:AT2G32140.1)	-1,35	-0,79
AT5G03350	250942_at	legume lectin family protein	-1,24	-0,66
Genes induced in *nrb4*-4				
AT1G47400	261684_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G47395.1)	-0,06	5,50
AT3G56980	251677_at	BHLH039/ORG3 (OBP3-RESPONSIVE GENE 3); DNA binding / transcription factor	-0,06	4,21
AT1G23020	264751_at	ATFRO3/FRO3 (FERRIC REDUCTION OXIDASE 3); ferric-chelate reductase	0,29	3,98
AT5G59320	247717_at	LTP3 (LIPID TRANSFER PROTEIN 3); lipid binding	-0,03	3,75
AT5G59310	247718_at	LTP4 (LIPID TRANSFER PROTEIN 4); lipid binding	-0,13	3,55
AT1G10970	260462_at	ZIP4 (ZINC TRANSPORTER 4 PRECURSOR); cation transmembrane transporter	0,29	3,40
AT3G05690	258890_at	ATHAP2B/HAP2B/UNE8 (HEME ACTIVATOR PROTEIN (YEAST) HOMOLOG 2B); transcription factor	-0,06	3,01

AT5G05250	250828_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT3G56360.1)	-0,35	2,86
AT5G53450	248270_at	ORG1 (OBP3-RESPONSIVE GENE 1); kinase	-0,14	2,80
AT1G76960	264958_at	unknown protein	-0,12	2,79
AT2G14610	266385_at	PR1 (PATHOGENESIS-RELATED GENE 1)	-0,62	2,72
AT3G57260	251625_at	BGL2 (PATHOGENESIS-RELATED PROTEIN 2); glucan 1,3-beta-glucosidase/ hydrolase	-0,89	2,59
AT2G43510	260551_at	ATTI1 (ARABIDOPSIS THALIANA TRYPSIN INHIBITOR PROTEIN 1)	0,14	2,53
AT3G14020	258198_at	CCAAT-binding transcription factor (CBF-B/NF-YA) family protein	0,28	2,51
AT1G73260	260101_at	trypsin and protease inhibitor family protein / Kunitz family protein	-0,05	2,41
AT4G01480	255587_at	ATPPA5 (ARABIDOPSIS THALIANA PYROPHOSPHORYLASE 5); inorganic diphosphatase/ pyrophosphatase	0,18	2,37
AT5G10140	250476_at	FLC (FLOWERING LOCUS C); transcription factor	0,31	2,34
AT2G43570	260568_at	chitinase, putative	-0,19	2,32
AT5G50915	248460_at	basic helix-loop-helix (bHLH) family protein	-0,12	2,29
AT3G22600	256933_at	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	-0,32	2,29
AT4G21830	254385_s_at	[AT4G21830, methionine sulfoxide reductase domain-containing protein / SeIR domain-containing protein]	0,03	2,27
AT1G12030	257421_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G62420.1)	0,02	2,23
AT5G13740	250248_at	ZIF1 (ZINC INDUCED FACILITATOR 1); carbohydrate transmembrane transporter/ sugar:hydrogen ion symporter	-0,16	2,20
AT2G18660	266070_at	EXLB3 (EXPANSIN-LIKE B3 PRECURSOR)	-0,58	2,14
AT3G47640	252427_at	basic helix-loop-helix (bHLH) family protein	-0,18	2,12
AT1G26770	261266_at	ATEXPA10 (ARABIDOPSIS THALIANA EXPANSIN A10)	-0,16	2,10
AT5G39520	249454_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT5G39530.1)	-0,04	2,09
AT1G20380	255940_at	prolyl oligopeptidase, putative / prolyl endopeptidase, putative / post-proline cleaving enzyme, putative	0,00	2,07
AT5G50400	248499_at	ATPAP27/PAP27 (purple acid phosphatase 27); acid phosphatase/ protein serine/threonine phosphatase	0,23	2,06
AT1G60960	259723_at	IRT3 (Iron regulated transporter 3); cation transmembrane transporter/ metal ion transmembrane transporter	0,33	2,03
AT4G13920	254741_s_at	[AT4G13920, disease resistance family protein / LRR family protein]	0,02	2,00
AT5G13320	250286_at	PBS3 (AVRPPHB SUSCEPTIBLE 3)	-0,01	1,94
AT5G52760	248322_at	heavy-metal-associated domain-containing protein	-0,46	1,91

AT5G46230	248889_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G09310.1)	-0,15	1,89
AT1G65500	264635_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G65486.1)	-0,07	1,89
AT4G04830	255302_at	methionine sulfoxide reductase domain-containing protein / SeIR domain-containing protein	0,20	1,86
AT1G33960	260116_at	AIG1 (AVRRPT2-INDUCED GENE 1); GTP binding	-0,02	1,83
AT3G17790	258158_at	ATACP5 (acid phosphatase 5); acid phosphatase/ protein serine/threonine phosphatase	-0,21	1,80
AT4G16370	245296_at	ATOPT3 (OLIGOPEPTIDE TRANSPORTER); oligopeptide transporter	-0,12	1,75
AT1G54160	263158_at	CCAAT-binding transcription factor (CBF-B/NF-YA) family protein	-0,01	1,71
AT3G05400	259133_at	sugar transporter, putative	0,13	1,70
AT3G47480	252417_at	calcium-binding EF hand family protein	-0,57	1,65
AT3G18250	257061_at	contains domain PROKAR_LIPOPROTEIN (PS51257)	-0,17	1,60
AT1G72830	262378_at	HAP2C (Heme activator protein (yeast) homolog 2C); transcription factor	0,16	1,59
AT1G62290	264741_at	aspartyl protease family protein	0,42	1,55
AT5G64510	247293_at	similar to unnamed protein product [Vitis vinifera] (GB:CAO49799.1)	-0,06	1,54
AT2G29350	266292_at	SAG13 (Senescence-associated gene 13); oxidoreductase	0,00	1,51
AT5G24530	249754_at	oxidoreductase, 2OG-Fe(II) oxygenase family protein	-0,79	1,50
AT1G20350	255941_at	ATTIM17-1 (Arabidopsis thaliana translocase inner membrane subunit 17-1)	-0,02	1,49
AT5G67330	247001_at	ATNRAMP4 (Arabidopsis natural resistance-associated macrophage (NRAMP) protein 4)	-0,06	1,47
AT5G06190	250722_at	unknown protein	-0,24	1,46
AT5G39670	249417_at	calcium-binding EF hand family protein	-0,18	1,42
AT2G24190	266015_at	short-chain dehydrogenase/reductase (SDR) family protein	0,00	1,40
AT1G74710	262177_at	ICS1 (ISOCHORISMATE SYNTHASE1); isochorismate synthase	-0,03	1,40
AT3G08860	258983_at	alanine--glyoxylate aminotransferase, putative / beta-alanine-pyruvate aminotransferase, putative / AGT, putative	-0,08	1,39
AT5G03170	250933_at	FLA11 (fasciclin-like arabinogalactan-protein 11)	0,29	1,37
AT1G64980	262868_at	similar to unnamed protein product [Vitis vinifera] (GB:CAO62125.1)	-0,06	1,37
AT4G37990	252984_at	ELI3-2 (ELICITOR-ACTIVATED GENE 3)	-0,37	1,35
AT1G17190	262516_at	ATGSTU26 (Arabidopsis thaliana Glutathione S-transferase (class tau) 26); glutathione transferase	0,27	1,35
AT4G23990	254185_at	ATCSLG3 (Cellulose synthase-like G3); transferase/ transferase, transferring glycosyl groups	0,10	1,35

AT5G09980	250455_at	PROPEP4 (Elicitor peptide 4 precursor)	-0,15	1,35
AT5G50200	248551_at	WR3 (WOUND-RESPONSIVE 3); nitrate transmembrane transporter	0,27	1,33
AT3G48740	252327_at	nodulin MtN3 family protein	-0,08	1,33
AT3G56950	251661_at	SIP2;1 (SMALL AND BASIC INTRINSIC PROTEIN 2); transporter	0,11	1,31
AT2G37540	267169_at	short-chain dehydrogenase/reductase (SDR) family protein	0,02	1,29
AT3G03990	258811_at	esterase/lipase/thioesterase family protein	-0,04	1,23
AT1G62422	260635_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G12020.1)	-0,14	1,22
AT3G08690	258678_at	UBC11 (ubiquitin-conjugating enzyme 11); ubiquitin-protein ligase	0,05	1,21
AT2G31390	263250_at	pfkB-type carbohydrate kinase family protein	-0,07	1,19
AT5G06750	250644_at	protein phosphatase 2C family protein / PP2C family protein	-0,36	1,19
AT2G30770	267567_at	CYP71A13 (CYTOCHROME P450, FAMILY 71, SUBFAMILY A, POLYPEPTIDE 13)	-0,13	1,19
AT5G60660	247586_at	PIP2;4/PIP2F (plasma membrane intrinsic protein 2;4); water channel	0,12	1,18
AT2G13290	265366_at	glycosyl transferase family 17 protein	-0,10	1,18
AT1G10660	257477_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT5G62960.1)	0,22	1,18
AT3G29810	245228_at	COBL2 (COBRA-LIKE PROTEIN 2 PRECURSOR)	0,04	1,18
AT1G05310	264573_at	pectinesterase family protein	0,25	1,17
AT1G06620	262616_at	2-oxoglutarate-dependent dioxygenase, putative	-0,14	1,16
AT2G11810	263391_at	MGDC (monogalactosyldiacylglycerol synthase type C); 1,2-diacylglycerol 3-beta-galactosyltransferase	0,06	1,16
AT5G65480	247194_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT4G38060.2)	0,06	1,16
AT4G23690	254226_at	disease resistance-responsive family protein / dirigent family protein	0,09	1,15
AT1G14340	261487_at	RNA recognition motif (RRM)-containing protein	0,29	1,14
AT4G39030	252921_at	EDS5 (ENHANCED DISEASE SUSCEPTIBILITY 5); antiporter/ transporter	0,00	1,13
AT1G17560	260683_at	HLL (HUELLENLOS); structural constituent of ribosome	0,36	1,13
AT2G18120	257456_at	SRS4 (SHI-RELATED SEQUENCE 4)	0,02	1,12
AT4G28740	253765_at	similar to LPA1 (LOW PSII ACCUMULATION1), binding [Arabidopsis thaliana] (TAIR:AT1G02910.1)	0,00	1,10
AT1G43790	260867_at	similar to hydroxyproline-rich glycoprotein family protein [Arabidopsis thaliana] (TAIR:AT5G48920.1)	0,10	1,10
AT2G17280	264907_at	phosphoglycerate/bisphosphoglycerate mutase family protein	0,24	1,10
AT5G18290	250025_at	SIP1;2 (SMALL AND BASIC INTRINSIC PROTEIN1B)	0,09	1,10

AT1G74410	260231_at	zinc finger (C3HC4-type RING finger) family protein	0,01	1,09
AT3G55470	251790_at	C2 domain-containing protein	0,00	1,09
AT5G05950	250707_at	MEE60 (maternal effect embryo arrest 60)	0,50	1,09
AT3G53230	251975_at	cell division cycle protein 48, putative / CDC48, putative	-0,23	1,09
AT5G08335	246009_at	ATSTE14B (PRENYLCYSTEINE ALPHA-CARBOXYL METHYLTRANSFERASE)	0,02	1,07
AT4G15610	245317_at	integral membrane family protein	0,26	1,07
AT4G01610	255590_at	cathepsin B-like cysteine protease, putative	0,00	1,07
AT3G23570	258108_at	dienelactone hydrolase family protein	-0,14	1,06
AT1G77370	246384_at	glutaredoxin, putative	0,08	1,06
AT1G36380	260129_at	electron carrier/ ubiquinol-cytochrome-c reductase	-0,01	1,05
AT1G26390	261020_at	FAD-binding domain-containing protein	0,20	1,05
AT3G56360	251704_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT5G05250.1)	-0,18	1,04
AT2G24040	266566_at	hydrophobic protein, putative / low temperature and salt responsive protein, putative	-0,14	1,04
AT1G13470	259385_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G13520.1)	-0,16	1,03
AT5G24430	249730_at	calcium-dependent protein kinase, putative / CDPK, putative	-0,05	1,03
AT3G44320	252677_at	NIT3 (NITRILASE 3)	0,10	1,02
AT4G01897	255554_at	similar to unnamed protein product [Vitis vinifera] (GB:CAO40169.1)	0,14	1,01
AT4G20110	254500_at	vacuolar sorting receptor, putative	0,03	1,01
Genes repressed in *nrb4*-4				
AT1G67865	260012_at	unknown protein	-0,26	-4,94
AT3G16670	258419_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT3G16660.1)	-0,09	-4,40
AT1G29395	259789_at	COR414-TM1 (cold regulated 414 thylakoid membrane 1)	0,07	-3,92
AT3G46780	252441_at	PTAC16 (PLASTID TRANSCRIPTIONALLY ACTIVE18); binding / catalytic	0,20	-3,82
AT1G09350	264511_at	ATGOLS3 (ARABIDOPSIS THALIANA GALACTINOL SYNTHASE 3)	-0,03	-3,65
AT5G01600	251109_at	ATFER1 (FERRETIN 1); ferric iron binding	0,00	-3,29
AT2G21650	263549_at	MEE3 (maternal effect embryo arrest 3); DNA binding / transcription factor	0,13	-2,99
AT2G38530	266415_at	LTP2 (LIPID TRANSFER PROTEIN 2); lipid binding	-0,29	-2,92
AT2G36885	263840_at	similar to unnamed protein product [Vitis vinifera] (GB:CAO63025.1)	0,34	-2,65
AT1G06080	260957_at	ADS1 (DELTA 9 DESATURASE 1); oxidoreductase	-0,23	-2,63
AT4G26530	253971_at	fructose-bisphosphate aldolase, putative	0,35	-2,63

AT5G51720	248377_at	similar to Os07g0467200 [Oryza sativa (japonica cultivar-group)] (GB:NP_001059590.1)	0,06	-2,63
AT1G16410	262717_s_at	[AT1G16410, CYP79F1 (SUPERSHOOT 1)]	0,00	-2,58
AT4G23800	254233_at	high mobility group (HMG1/2) family protein	0,17	-2,54
AT2G40300	263831_at	ATFER4 (FERRITIN 4); ferric iron binding	-0,04	-2,53
AT5G18600	249996_at	glutaredoxin family protein	0,17	-2,48
AT1G08560	264802_at	SYP111 (syntaxin 111); SNAP receptor	-0,06	-2,44
AT5G02760	251017_at	protein phosphatase 2C family protein / PP2C family protein	-0,29	-2,41
AT1G03130	263114_at	PSAD-2 (photosystem I subunit D-2)	0,07	-2,36
AT1G64370	259765_at	unknown protein	-0,12	-2,36
AT1G67860	260004_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G67865.1)	-0,02	-2,34
AT3G13310	257654_at	DNAJ heat shock N-terminal domain-containing protein	-0,12	-2,31
AT2G21140	264007_at	ATPRP2 (PROLINE-RICH PROTEIN 2)	0,15	-2,28
AT5G62280	247474_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT2G45360.1)	-0,17	-2,27
AT5G10430	250437_at	AGP4 (ARABINOGALACTAN-PROTEIN 4)	0,39	-2,24
AT5G33370	246687_at	GDSL-motif lipase/hydrolase family protein	0,00	-2,22
AT3G45160	252612_at	contains domain PROKAR_LIPOPROTEIN (PS51257)	0,06	-2,21
AT4G33270	253340_s_at	[AT4G33270, CDC20.1; signal transducer];[AT4G33260, CDC20.2; signal transducer]	-0,10	-2,18
AT1G78970	264100_at	LUP1 (LUPEOL SYNTHASE 1); lupeol synthase	0,20	-2,18
AT1G68790	260031_at	LINC3 (LITTLE NUCLEI3)	-0,05	-2,18
AT1G15340	262600_at	MBD10 (methyl-CpG-binding domain 10); DNA binding	0,05	-2,18
AT1G67870	260007_at	glycine-rich protein	0,24	-2,16
AT4G08950	255064_at	phosphate-responsive protein, putative (EXO)	-0,25	-2,12
AT1G20620	259544_at	CAT3 (CATALASE 3); catalase	-0,16	-2,12
AT3G23170	257925_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT4G14450.1)	-0,41	-2,08
AT5G38940	249477_s_at	[AT5G38940, manganese ion binding];[AT5G38930, germin-like protein, putative]	0,02	-2,07
AT3G44970	252629_at	cytochrome P450 family protein	-0,36	-2,03
AT5G23020	249867_at	IMS2/MAM-L/MAM3 (METHYLTHIOALKYMALATE SYNTHASE-LIKE)	0,05	-2,02
AT3G16530	257206_at	legume lectin family protein	0,23	-2,02
AT2G42840	263979_at	PDF1 (PROTODERMAL FACTOR 1)	0,12	-1,95
AT2G47880	266516_at	glutaredoxin family protein	0,50	-1,95

AT3G02120	258859_at	hydroxyproline-rich glycoprotein family protein	0,21	-1,94
AT5G22500	249895_at	acyl CoA reductase, putative / male-sterility protein, putative	-0,09	-1,94
AT1G58270	256021_at	ZW9	-0,12	-1,92
AT1G09750	264672_at	chloroplast nucleoid DNA-binding protein-related	0,32	-1,90
AT1G62500	265117_at	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	0,28	-1,89
AT1G68600	262232_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G25480.1)	0,29	-1,89
AT3G02640	258480_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT5G16250.1)	-0,03	-1,86
AT2G18510	265930_at	EMB2444 (EMBRYO DEFECTIVE 2444); RNA binding	0,20	-1,85
AT5G47500	248807_at	pectinesterase family protein	0,09	-1,84
AT1G02730	262109_at	ATCSLD5 (CELLULOSE SYNTHASE-LIKE D5); 1,4-beta-D-xylan synthase/ cellulose synthase	-0,09	-1,83
AT1G06830	260831_at	glutaredoxin family protein	0,18	-1,83
AT5G58310	247814_at	hydrolase, alpha/beta fold family protein	0,37	-1,83
AT1G71030	259751_at	ATMYBL2 (Arabidopsis myb-like 2); DNA binding / transcription factor	0,19	-1,83
AT3G62950	251196_at	glutaredoxin family protein	0,25	-1,79
AT3G27260	257146_at	GTE8 (GLOBAL TRANSCRIPTION FACTOR GROUP E8); DNA binding	0,24	-1,78
AT5G05960	250764_at	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	0,11	-1,77
AT3G30720	256940_at	unknown protein	-1,87	-1,77
AT1G29270	260883_at	similar to transcription regulator [Arabidopsis thaliana] (TAIR:AT2G40435.1)	-0,19	-1,76
AT4G02770	255457_at	PSAD-1 (photosystem I subunit D-1)	0,07	-1,75
AT3G49400	252314_at	transducin family protein / WD-40 repeat family protein	-1,75	-1,74
AT3G23890	256864_at	TOPII (TOPOISOMERASE II); ATP binding / DNA binding / DNA topoisomerase (ATP-hydrolyzing)	-0,01	-1,73
AT2G38040	266099_at	CAC3 (acetyl co-enzyme A carboxylase carboxyltransferase alpha subunit); acetyl-CoA carboxylase	0,00	-1,67
AT5G28640	246103_at	AN3 (ANGUSITFOLIA3)	-0,16	-1,64
AT4G32280	253423_at	IAA29 (indoleacetic acid-induced protein 29); transcription factor	-0,17	-1,63
AT5G23420	249836_at	HMGB6 (High mobility group B 6); transcription factor	0,15	-1,63
AT5G44400	249046_at	FAD-binding domain-containing protein	-0,01	-1,63
AT3G51720	252077_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT2G38370.1)	0,18	-1,62
AT1G12270	259519_at	stress-inducible protein, putative	-0,07	-1,62

AT1G75780	262978_at	TUB1 (tubulin beta-1 chain); structural molecule	-0,03	-1,61
AT4G25730	254079_at	FtsJ-like methyltransferase family protein	0,01	-1,61
AT2G47510	248461_s_at	[AT2G47510, FUM1 (FUMARASE 1); fumarate hydratase];[AT5G50950, fumarate hydratase]	-0,01	-1,61
AT1G68650	262277_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G25520.1)	-0,11	-1,60
AT3G61210	251360_at	embryo-abundant protein-related	0,01	-1,57
AT1G28400	261500_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT2G33850.1)	-0,02	-1,56
AT3G05600	258895_at	epoxide hydrolase, putative	0,15	-1,55
AT4G17030	245463_at	ATEXLB1 (ARABIDOPSIS THALIANA EXPANSIN-LIKE B1)	0,04	-1,55
AT5G38840	249538_at	forkhead-associated domain-containing protein / FHA domain-containing protein	0,24	-1,53
AT1G10640	261834_at	polygalacturonase, putative / pectinase, putative	-0,12	-1,53
AT4G35800	253133_at	NRPB1 (RNA POLYMERASE II LARGE SUBUNIT); DNA binding / DNA-directed RNA polymerase	0,12	-1,53
AT4G27300	253911_at	S-locus protein kinase, putative	0,15	-1,52
AT4G29060	253758_at	EMB2726 (EMBRYO DEFECTIVE 2726); translation elongation factor	0,14	-1,52
AT5G55660	248070_at	GTP binding / RNA binding	0,04	-1,51
AT4G01800	255540_at	preprotein translocase secA subunit, putative	0,18	-1,50
AT1G07360	261082_at	zinc finger (CCCH-type) family protein / RNA recognition motif (RRM)-containing protein	0,14	-1,50
AT5G18620	249997_at	CHR17 (CHROMATIN REMODELING FACTOR17); DNA-dependent ATPase	0,08	-1,47
AT5G35480	249726_at	unknown protein	0,15	-1,47
AT4G37750	253010_at	ANT (AINTEGUMENTA); DNA binding / transcription factor	0,06	-1,47
AT3G18960	256918_s_at	[AT3G18960, transcriptional factor B3 family protein];[AT4G01580, transcriptional factor B3 family protein]	-0,06	-1,47
AT5G59130	247760_at	subtilase family protein	-0,01	-1,46
AT1G06720	260824_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G42440.1)	0,07	-1,46
AT1G73960	260374_at	TAF2 (TBP-ASSOCIATED FACTOR 2); metallopeptidase/ zinc ion binding	-0,09	-1,46
AT4G02800	255460_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G30050.1)	0,02	-1,45
AT5G36710	249659_s_at	[AT5G36710, similar to unknown protein [Arabidopsis thaliana] (TAIR:AT5G36800.1)	0,16	-1,44
AT5G65770	247157_at	LINC4 (LITTLE NUCLEI4)	0,10	-1,42
AT1G78070	262164_at	WD-40 repeat family protein	0,12	-1,42
AT1G18870	261428_at	ICS2; isochorismate synthase	0,03	-1,42
AT1G74250	260251_at	DNAJ heat shock N-terminal domain-containing protein	-0,02	-1,42

AT3G54400	251899_at	aspartyl protease family protein	-0,17	-1,41
AT1G75250	256503_at	myb family transcription factor	0,38	-1,41
AT1G79150	264118_at	binding	0,00	-1,40
AT3G07050	258545_at	GTP-binding family protein	0,15	-1,40
AT3G13470	256983_at	chaperonin, putative	-0,04	-1,40
AT1G57820	245828_at	ORTH2/VIM1 (VARIANT IN METHYLATION 1)	0,21	-1,39
AT5G16730	246476_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT3G02930.1)	0,03	-1,39
AT3G62300	251256_at	agenet domain-containing protein	0,02	-1,39
AT1G64330	259794_at	myosin heavy chain-related	0,02	-1,39
AT1G18370	261660_at	HIK (HINKEL); microtubule motor	0,24	-1,38
AT5G63320	247370_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT2G22795.1)	0,10	-1,38
AT3G04290	258589_at	ATLTL1/LTL1 (LI-TOLERANT LIPASE 1); carboxylesterase	0,11	-1,38
AT3G23030	257766_at	IAA2 (indoleacetic acid-induced protein 2); transcription factor	0,05	-1,37
AT4G16750	245445_at	DRE-binding transcription factor, putative	-0,25	-1,37
AT1G01610	259430_at	ATGPAT4/GPAT4 (GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE 4)	0,50	-1,37
AT5G66540	247046_at	similar to hypothetical protein OsI_036143 [Oryza sativa (indica cultivar-group)]	0,14	-1,36
AT4G30110	253657_at	HMA2 (Heavy metal ATPase 2); cadmium-transporting ATPase	0,16	-1,36
AT3G51280	252148_at	male sterility MS5, putative	-0,27	-1,35
AT5G61660	247541_at	glycine-rich protein	-0,07	-1,35
AT2G37080	265464_at	myosin heavy chain-related	-0,06	-1,35
AT1G19485	260660_at	AT hook motif-containing protein	-0,68	-1,34
AT5G22880	249916_at	H2B/HTB2 (HISTONE H2B); DNA binding	0,00	-1,33
AT2G32100	265724_at	ATOFP16/OFP16 (Arabidopsis thaliana ovate family protein 16)	0,16	-1,33
AT5G13630	250243_at	GUN5 (GENOMES UNCOUPLED 5)	0,05	-1,33
AT1G06670	262636_at	NIH (NUCLEAR DEIH-BOXHELICASE)	0,21	-1,32
AT5G22580	249894_at	Identical to Uncharacterized protein At5g22580 [Arabidopsis Thaliana] (GB:Q9FK81)	-0,10	-1,32
AT3G59010	251509_at	pectinesterase family protein	0,16	-1,32
AT5G28290	245859_at	ATNEK3; kinase	-0,22	-1,31
AT3G50240	252215_at	KICP-02; microtubule motor	-0,02	-1,30
AT1G78170	260081_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G22250.1)	0,31	-1,30
AT3G04630	258796_at	WDL1 (WVD2-LIKE 1)	0,00	-1,29

AT1G09770	264709_at	ATCDC5 (ARABIDOPSIS THALIANA HOMOLOG OF CDC5); DNA binding / transcription factor	0,17	-1,29
AT5G57340	247903_at	similar to hypothetical protein MtrDRAFT_AC155282g59v2	-0,25	-1,29
AT5G57760	247878_at	unknown protein	-0,15	-1,29
AT5G55920	248036_at	nucleolar protein, putative	0,21	-1,28
AT5G66230	247134_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT3G51230.1)	0,15	-1,28
AT3G49160	252300_at	pyruvate kinase family protein	-0,08	-1,28
AT2G34300	267040_at	dehydration-responsive protein-related	-0,18	-1,28
AT2G03150	266727_at	EMB1579 (EMBRYO DEFECTIVE 1579); binding	0,25	-1,28
AT5G58040	247841_at	ATFIP1[V] (ARABIDOPSIS HOMOLOG OF YEAST FIP1 [V]); RNA binding / protein binding	0,22	-1,28
AT1G79150	264131_at	binding	0,10	-1,27
AT1G78460	263126_at	SOUL heme-binding family protein	0,16	-1,27
AT2G34357	267044_at	binding	0,23	-1,27
AT5G25090	246920_at	plastocyanin-like domain-containing protein	-0,05	-1,27
AT4G37740	253065_at	AtGRF2 (GROWTHREGULATING FACTOR 2)	-0,11	-1,26
AT4G11190	254907_at	disease resistance-responsive family protein / dirigent family protein	-0,16	-1,26
AT2G43680	260610_at	IQD14; calmodulin binding	0,27	-1,26
AT5G49930	248583_at	EMB1441 (EMBRYO DEFECTIVE 1441); nucleic acid binding	0,17	-1,25
AT2G25060	264377_at	plastocyanin-like domain-containing protein	-0,04	-1,25
AT2G43650	260585_at	EMB2777 (EMBRYO DEFECTIVE 2777)	0,02	-1,25
AT4G29030	253753_at	glycine-rich protein	-0,06	-1,24
AT5G08000	250565_at	E13L3 (GLUCAN ENDO-1,3-BETA-GLUCOSIDASE-LIKE PROTEIN 3)	-0,12	-1,24
AT3G23730	257203_at	xyloglucan:xyloglucosyl transferase, putative / xyloglucan endotransglycosylase, putative	0,03	-1,24
AT4G08390	255142_at	SAPX; L-ascorbate peroxidase	-0,09	-1,24
AT5G25060	246924_at	RNA recognition motif (RRM)-containing protein	0,12	-1,23
AT4G26760	253947_at	microtubule associated protein (MAP65/ASE1) family protein	0,04	-1,23
AT5G16250	246505_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT3G02640.1)	0,07	-1,23
AT1G03910	265092_at	similar to unnamed protein product [Vitis vinifera] (GB:CAO62957.1)	0,16	-1,22
AT1G28060	259582_at	small nuclear ribonucleoprotein family protein / snRNP family protein	0,15	-1,22
AT2G21440	263766_at	RNA recognition motif (RRM)-containing protein	0,09	-1,22

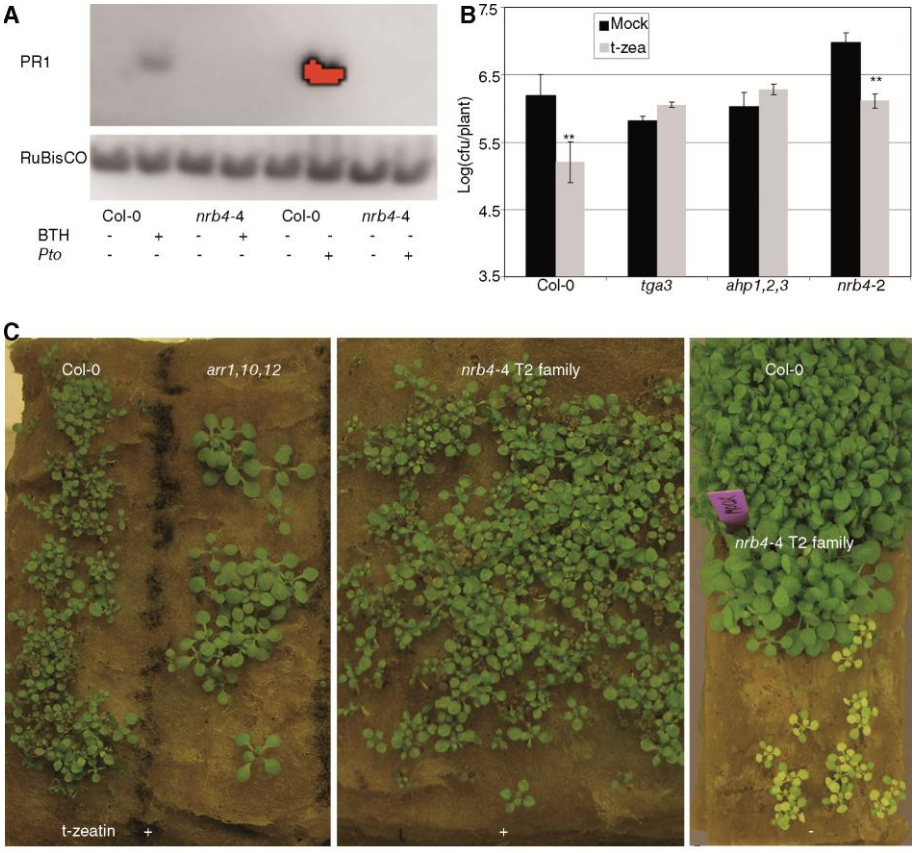
AT2G24050	266567_at	MIF4G domain-containing protein / MA3 domain-containing protein	0,08	-1,22
AT4G03100	255410_at	rac GTPase activating protein, putative	0,01	-1,21
AT1G72440	260425_at	EDA25 (embryo sac development arrest 25); binding	0,07	-1,21
AT1G50660	261853_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT3G20350.1)	-0,04	-1,21
AT5G04140	245701_at	GLS1/GLU1/GLUS (FERREDOXIN-DEPENDENT GLUTAMATE SYNTHASE 1); glutamate synthase (ferredoxin)	0,16	-1,21
AT5G01870	251065_at	lipid transfer protein, putative	-0,03	-1,20
AT2G13820	265656_at	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	-0,08	-1,20
AT4G28250	253815_at	ATEXPB3 (ARABIDOPSIS THALIANA EXPANSIN B3)	-0,22	-1,20
AT3G03710	259344_at	RIF10 (RESISTANT TO INHIBITION WITH FSM 10); 3'-5'-exoribonuclease/ RNA binding / nucleic acid binding	0,07	-1,20
AT4G31880	253497_at	binding	0,20	-1,20
AT4G03210	255433_at	XTH9 (XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 9); hydrolase, acting on glycosyl bonds	-0,02	-1,20
AT4G31840	253480_at	plastocyanin-like domain-containing protein	0,11	-1,20
AT2G39850	245088_at	subtilase	0,26	-1,19
AT2G18220	265326_at	Identical to Nucleolar complex protein 2 homolog [Arabidopsis Thaliana] (GB:Q9ZPV5)	0,12	-1,18
AT3G56040	251741_at	similar to hypothetical protein OsJ_018104 [Oryza sativa (japonica cultivar-group)]	-0,03	-1,18
AT5G57990	247838_at	UBP23 (UBIQUITIN-SPECIFIC PROTEASE 23); ubiquitin-specific protease	0,16	-1,18
AT3G19650	257073_at	cyclin-related	0,10	-1,18
AT1G15940	261844_at	binding	0,01	-1,18
AT4G11420	254873_at	EIF3A (eukaryotic translation initiation factor 3A)	0,13	-1,17
AT3G60390	251374_at	HAT3 (homeobox-leucine zipper protein 3); transcription factor	0,09	-1,17
AT2G35880	263957_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT4G32330.3)	-0,19	-1,17
AT2G29210	266283_at	splicing factor PWI domain-containing protein	0,21	-1,17
AT1G53910	262197_at	RAP2.12; DNA binding / transcription factor	-0,12	-1,17
AT4G14900	245398_at	hydroxyproline-rich glycoprotein family protein	-0,14	-1,17
AT4G28080	253849_at	binding	0,20	-1,16
AT5G40850	249325_at	UPM1 (UROPHORPHYRIN METHYLASE 1); uroporphyrin-III C-methyltransferase	0,52	-1,16
AT1G06230	260787_at	GTE4 (GLOBAL TRANSCRIPTION FACTOR GROUP E 4); DNA binding	0,08	-1,16
AT1G11600	262819_at	CYP77B1 (cytochrome P450, family 77, subfamily B, polypeptide 1); oxygen binding	-0,19	-1,15

AT1G14610	260780_at	TWN2 (TWIN 2); ATP binding / aminoacyl-tRNA ligase	-0,01	-1,15
AT1G14710	262849_at	hydroxyproline-rich glycoprotein family protein	0,12	-1,15
AT5G05210	250825_at	nucleolar matrix protein-related	0,23	-1,15
AT1G14900	262840_at	HMGA (HIGH MOBILITY GROUP A); DNA binding	-0,02	-1,15
AT5G65390	247189_at	AGP7 (Arabinogalactan protein 7)	0,08	-1,15
AT5G02540	251013_at	short-chain dehydrogenase/reductase (SDR) family protein	-0,02	-1,15
AT1G79000	264101_at	HAC1 (P300/CBP ACETYLTRANSFERASE-RELATED PROTEIN 2 GENE)	-0,06	-1,15
AT4G31500	253534_at	CYP83B1 (CYTOCHROME P450 MONOOXYGENASE 83B1); oxygen binding	-0,07	-1,15
AT1G72390	260449_at	similar to unnamed protein product [Vitis vinifera] (GB:CAO45587.1)	0,13	-1,15
AT1G67040	255851_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT5G26910.2)	-0,34	-1,14
AT3G01780	259190_at	TPLATE; binding	-0,22	-1,13
AT2G45470	251395_at	FLA8 (Arabinogalactan protein 8)	0,16	-1,13
AT3G57660	251593_at	NRPA1 (nuclear RNA polymerase A 1); DNA binding / DNA-directed RNA polymerase	0,17	-1,12
AT3G43600	246330_at	AAO2 (ALDEHYDE OXIDASE 2)	0,22	-1,12
AT3G22790	258341_at	kinase interacting family protein	-0,13	-1,12
AT1G10990	260472_at	unknown protein	-0,02	-1,12
AT5G64570	247266_at	XYL4 (beta-xylosidase 4); hydrolase, hydrolyzing O-glycosyl compounds	-0,12	-1,11
AT1G13030	262777_at	sphere organelles protein-related	-0,13	-1,11
AT4G04770	255305_at	ATABC1 (ARABIDOPSIS THALIANA NUCLEOSOME ASSEMBLY PROTEIN 1)	-0,01	-1,11
AT4G17330	245415_at	ATG2484-1 (Arabidopsis thaliana G2484-1 protein); RNA binding	0,15	-1,10
AT3G46370	252520_at	leucine-rich repeat protein kinase, putative	0,03	-1,10
AT4G36980	246285_at	similar to unnamed protein product [Vitis vinifera] (GB:CAO71849.1); contains domain PTHR13161 (PTHR13161)	0,13	-1,10
AT1G76690	259875_s_at	[AT1G76690, OPR2 (12-oxophytodienoate reductase 2); 12-oxophytodienoate reductase];[AT1G76680, OPR1]	-0,14	-1,10
AT5G62000	247468_at	ARF2 (AUXIN RESPONSE FACTOR 2); transcription factor	0,01	-1,09
AT1G16210	262710_at	similar to unknown [Picea sitchensis] (GB:ABK22525.1)	0,10	-1,09
AT4G02400	255501_at	similar to U3 ribonucleoprotein (Utp) family protein [Arabidopsis thaliana] (TAIR:AT5G08600.1)	0,13	-1,08
AT3G25900	258075_at	ATHMT-1/HMT-1; homocysteine S-methyltransferase	0,26	-1,08
AT3G11964	256661_at	S1 RNA-binding domain-containing protein	0,20	-1,08
AT1G64360	259766_at	unknown protein	0,25	-1,07

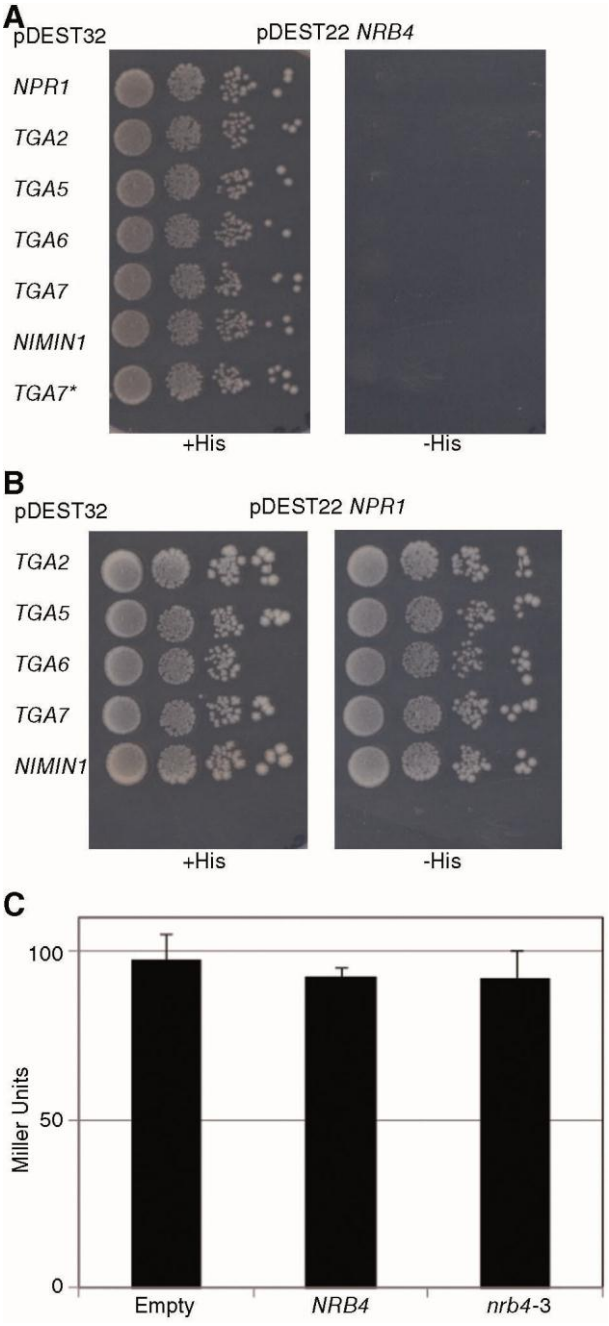
AT5G10010	250461_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT5G64910.1)	0,25	-1,06
AT3G04730	258797_at	IAA16 (indoleacetic acid-induced protein 16); transcription factor	-0,08	-1,05
AT5G06110	250711_at	DNAJ heat shock N-terminal domain-containing protein / cell division protein-related	-0,16	-1,05
AT1G06360	259391_s_at	[AT1G06360, fatty acid desaturase family protein];[AT1G06350, fatty acid desaturase family protein]	0,04	-1,04
AT5G66030	247082_at	ATGRIP/GRIP; protein binding	0,07	-1,04
AT1G53590	260987_at	NTMC2T6.1/NTMC2TYPE6.1	0,02	-1,04
AT4G02060	255513_at	PRL (PROLIFERA); ATP binding / DNA binding / DNA-dependent ATPase	0,12	-1,04
AT1G15780	259488_at	protein binding / transcription cofactor	0,09	-1,04
AT1G76810	259872_at	eukaryotic translation initiation factor 2 family protein / eIF-2 family protein	0,00	-1,04
AT2G38370	267054_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT3G51720.1)	-0,12	-1,03
AT5G20820	246000_at	auxin-responsive protein-related	0,05	-1,03
AT5G13840	250228_at	WD-40 repeat family protein	-0,01	-1,03
AT1G28420	261494_at	HB-1 (homeobox-1); transcription factor	-0,04	-1,03
AT1G78060	262181_at	glycosyl hydrolase family 3 protein	0,03	-1,03
AT3G18170	258143_at	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT3G18180.1)	-0,11	-1,03
AT5G16780	246447_at	SART-1 family protein	0,05	-1,02
AT1G65860	261913_at	flavin-containing monooxygenase family protein / FMO family protein	0,05	-1,02
AT3G13000	257547_at	transcription factor	0,01	-1,02
AT3G02550	258487_at	LBD41 (LOB DOMAIN-CONTAINING PROTEIN 41)	0,29	-1,02
AT1G15000	260739_at	SCPL50 (serine carboxypeptidase-like 50); serine carboxypeptidase	0,09	-1,02
AT5G01370	251113_at	unknown protein	0,02	-1,01
AT2G36910	263865_at	ATPGP1 (ARABIDOPSIS THALIANA P GLYCOPROTEIN1); calmodulin binding	0,12	-1,01
AT1G01600	259429_at	CYP86A4 (cytochrome P450, family 86, subfamily A, polypeptide 4); oxygen binding	0,12	-1,01
AT4G32420	253429_at	peptidyl-prolyl cis-trans isomerase cyclophilin-type family protein	0,15	-1,01
AT1G64050	262331_at	similar to unnamed protein product [Vitis vinifera] (GB:CAO65897.1)	0,18	-1,00
AT2G36050	263953_at	ATOFP15/OFP15 (Arabidopsis thaliana ovate family protein 15)	-0,16	-1,00
AT4G25100	254098_at	FSD1 (FE SUPEROXIDE DISMUTASE 1); iron superoxide dismutase	0,16	-1,00
AT1G18090	256077_at	exonuclease, putative	-0,12	-1,00

B bin	name	p-value
Main groups of genes altered in *nrb4*_2		
3	minor CHO metabolism	1,53E-03
6	gluconeogenese/ glyoxylate cycle	1,93E-02
12	N-metabolism	2,04E-03
17	hormone metabolism	2,98E-06
20	stress	3,49E-03
28	DNA	1,76E-02
30	signalling	3,95E-05
Main groups of genes altered in *nrb4*_4		
1	PS	3,78E-02
2	major CHO metabolism	2,74E-02
7	OPP	2,16E-02
9	mitochondrial electron transport / ATP synthesis	0,00E+00
10	cell wall	1,47E-02
18	Co-factor and vitamine metabolism	5,31E-04
19	tetrapyrrole synthesis	3,97E-04
21	redox	1,39E-04
24	Biodegradation of Xenobiotics	5,18E-06
27	RNA	1,68E-25
28	DNA	3,95E-10
30	signalling	2,04E-06
31	cell	4,07E-14
34	transport	0,00E+00

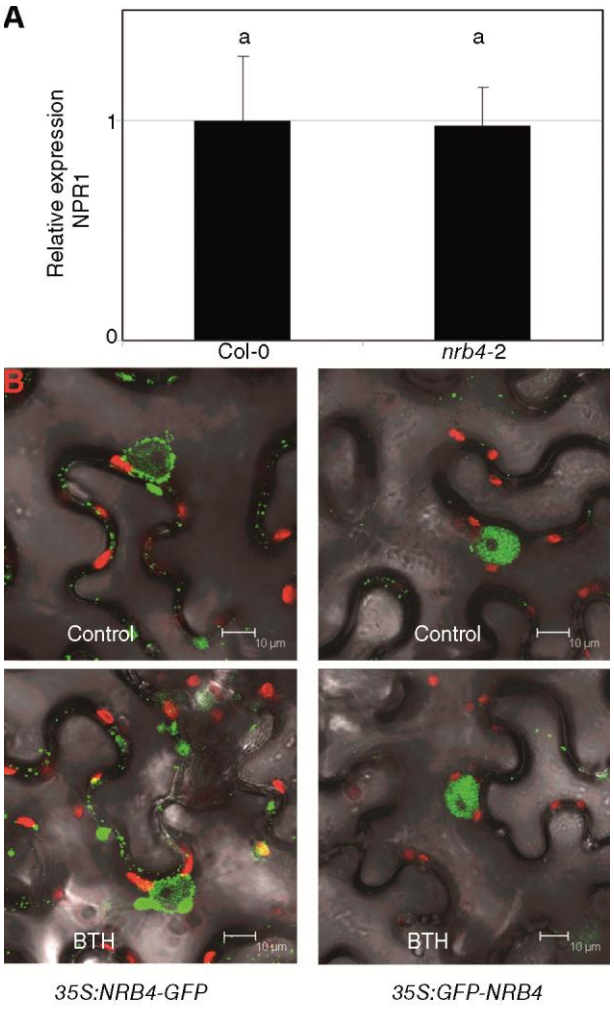
Supplemental Figure 7. Transcriptomic analysis. A List of genes statistically up or down regulated in *nrb4*-2 and *nrb4*-4 compared with Col-0. The first column describes the gene, the second the Affymetrix identifier, the third a brief annotation, the fourth the log2 of the ratio (expression in *nrb4*-2/expression in Col-0), and the fifth the log2 of the ratio (expression in *nrb4*-4/expression in Col-0). The genes mentioned in the text as examples, were marked in red. The two genes that were induced in both alleles are marked in yellow. *NRB4* was detected as down regulated in *nrb4*-4, marked in blue. **B** List of main groups of genes statistically overrepresented in the genes up or down regulated in *nrb4*-2 and *nrb4*-4 compared with Col-0.



Supplemental Figure 8. Phenotypes from the transcriptomic analysis. A PR1 Western blot of Col-0 and *nrb4*-4 three days after mock or a *Pto* inoculation and one day after mock or 350 µM BTH treatment, as in Figure 2B. The same blot was probed with anti-RuBisCO for loading and transfer control. **B** Resistance induced by cytokinins. Trans-zeatin (t-zea) 1 µM or a mock solution was applied one day previous to the inoculation with *Pto*. *tga3* (Choi et al. 2010) and *ahp1 ahp2 ahp3* (abbreviated as *ahp1,2,3*, Hutchison et al. 2006) were included as controls. **C** *nrb4*-4 did not have a specific phenotype with cytokinins. The controls Col-0 and *arr1 arr10 arr12* growing in 5 µM trans-zeatin (left) are the same as in Figure 6B. A T2 family, segregating for *nrb4*-4 (middle) did not produce plants with a different perception to cytokinins. Col-0 and the same T2 family of *nrb4*-4 plants growing in control conditions (right). In the case of *nrb4*-4, the space shown at the bottom of the picture was cleared of wild type plants, to check if the *nrb4*-4 homozygous plants can grow in this media.


A
pDEST32
pDEST22 NRB4
NPR1
TGA2
TGA5
TGA6
TGA7
NIMIN1
TGA7*
+His
-His
B
pDEST32
pDEST22 NPR1
TGA2
TGA5
TGA6
TGA7
NIMIN1
+His
-His
C
100
50
0
Miller Units
Empty
NRB4
nrb4-3

Supplemental Figure 9. Yeast n-hybrid interactions. A Interactions between NRB4 and proteins that have a role in SA perception. The yeasts that had NRB4 and any of these proteins were able to grow in His+ plates, but not in His- plates. Therefore, there was not detectable interaction. TGA7* stands for an additional control with an empty pDEST22, since TGA7 was able to autoactivate the system with no 3AT (the His- plates contained 5 mM 3AT). **B** As a control, interactions between NPR1 and the rest of proteins that have a role in SA perception. **C** NRB4 did not alter the interaction between NPR1 and TGA2. *NRB4* was cloned in a third vector, and introduced in the first yeast of B. There was no statistical difference between introducing *NRB4*, respect *nrb4*-3, or to the empty vector. All the plates were Lys-, Trp-, Leu-, and 100 µM SA. Similar experiments with no SA produced the same results. Similarly, bimolecular fluorescence complementation between *NRB4* and *NPR1* or *TGA2* did not produce a positive result in *Nicotiana benthamiana.* The pictures were taken 3-5 days after growing at 28°C. *NRB4* was cloned in three different versions for detection of interactions in the yeast two hybrid system: a short version, from 1 to 112 aa, included the KIX domain; an intermediate version, from 1 to 670 aa, spanned half of the coding sequence; and a full version. The full version when fused to the GAL4 BD was autoactivated, even with mutated versions of *NRB4* that recreated the EMS mutations herein described. The three versions of the wild type protein fused with the GAL4 AD were tested for interaction in yeast with genes described in SA response. The experiment shown in this figure corresponds to the full version of NRB4.


A
a
a
Relative expression NPR1
1
0
Col-0
nrb4-2
B
Control
Control
BTH
BTH
10 µm
35S:NRB4-GFP
35S:GFP-NRB4
C
160KDa
105KDa
75KDa
50KDa
35KDa
30KDa
E. coli GFP-NRB4
nrb4-1 NBB4F
nrb4-2 NRB4F
nrb4-3 NRB4F
nrb4-3 NRB4M
NRB4 M#1
NRB4 F#1

Supplemental Figure 10. Expression of *NPR1* and *NBR4*. **A** *NPR1* was detectable in *nrb4*-2 at normal levels, measured by RT-qPCR as in Figure 7A and B. **B** The nuclear localization did not change by the application of 350 µM BTH; *35S:NRB4-GFP*(left) and *35S:GFP-NRB4* (right) were infiltrated in *N. benthamiana*. Then, a mock or a 350 µM BTH was applied one day previous to these pictures. The controls correspond to the Figure 7C and 7D, respectively. **C** In the stable transgenic lines, GFP was not detectable by western blot. A western blot with antibody raised against GFP (Roche, Madrid, Spain) was performed in different extracts. The first line of the western is a *GFP-NRB4* fusion expressed in *E. coli*, which shows partial processing. The rest of lines correspond to plant extracts from the same lines described in Figure 8. The arrows point the position of the weight markers.

Supplemental Table 1. Evaluation of phenotypes in T-DNA insertion lines.

AGI	NASC	T-DNA	Status	Position	Phenotype (% wt)
	Col-0				100
	npr1-1		Homoz		0
AT1G07950	N656591	SALK_065283C	Homoz	Exon	100
AT1G11760	N665553	SALK_023845C	Homoz	5'	100
AT1G11760	N678300	SALK_028490C	Homoz	5'	100
AT1G15780	N835429	SAIL_792_F02	Heteroz	Intron, *nrb4*-4	75
AT1G15780		GABI_955_E02	Heteroz	Intron, *nrb4*-5	75
AT1G16430	N870082	SAIL_9_E04	Heteroz	Exon	100
AT1G23230	N659417	SALK_060062C	Homoz	Exon	100
AT1G23230	N671536	SALK_074015C	Homoz	Exon	100
AT1G25540	N679089	SALK_129555C	Homoz	Exon	100
AT1G25540	N677751	SALK_059316C	Homoz	Exon	100

AT1G26665	No info				
AT1G29940	N876306	SAIL_726_H01	Heteroz	Exon	100
AT1G31360	N661000	SALK_087178C	Homoz	Exon	100
AT1G44910	N521070	SALK_021070 (AZ)	Heteroz	Exon	100
AT1G54250	N679260	SALK_151800C	Homoz	Exon	100
AT1G55080	N529118	SALK_029118	Heteroz	Exon	100
AT1G55325	N861503	SAIL_1169_H11	Homoz	Exon	100
AT1G60850	N655705	SALK_088247	Homoz	Exon	100
AT2G03070	N682656	SALK_092406C	Homoz	Exon	100
AT2G22370	N677657	SALK_027178C	Homoz	Intron	100
AT2G28230	No info				
AT2G29540	N507414	SALK_007414	Hetz	Exon	100
AT2G38250	N667374	SALK_133090C	Homoz	5'	100
AT2G48110	N671698	SALK_092499C	Homoz	5'	100
AT2G48110	N667838	SALK_015532C	Homoz	Exon	100
AT3G01435	No info				
AT3G04740	N521711	SALK_021711	Heteroz	Exon	100
AT3G09180	N512449	SALK_012449	Heteroz	Exon	100
AT3G10690	N506294	SALK_006294	Heteroz	3'	100
AT3G21350	N662531	SALK_055723C	Homoz	5'	100
AT3G21350	N656864	SALK_110696C	Homoz	5'	100
AT3G23590	N667150	SALK_119561C	Homoz	Exon	100
AT3G23590	N661810	SALK_022477C	Homoz	Exon	100
AT3G25940	N562311	SALK_062311	Heteroz	3'	100
AT3G52860	N685672	SALK_037570C	Homoz	5'	100
AT3G57660	N673273	SALK_116823C	Homoz	3'	100
AT3G57660	N673356	SALK_122465C	Homoz	3'	100
AT3G59600	No info				

AT4G00450	N678935	SALK_108241C	Homoz	Exon	100
AT4G04780	No info				
AT4G04920	N548091	SALK_048091	Heteroz	Intron	100
AT4G09070	N553156	SALK_053156 (AP)	Heteroz	3'	100
AT4G25210	N599954	SALK_099954	Heteroz	Exon	100
AT4G25210	N607213	SALK_107213	Heteroz	5'	100
AT4G25630	N682661	SALK_093373C	Homoz	Exon	100
AT5G02850	N622082	SALK_122082	Heteroz	Exon	100
AT5G02850	N683125	SALK_007367C	Homoz	5'	100
AT5G03220	N678464	SALK_049958C	Homoz	5'	100
AT5G03500	N676132	SALK_088220C	Homoz	Intron	100
AT5G12230	N657910	SALK_037435C	Homoz	5'	100
AT5G12230	N658182	SALK_034955C	Homoz	Intron	100
AT5G19480	No info				
AT5G19910	N682219	SALK_035522C	Homoz	Exon	100
AT5G20170	N663678	SALK_111977C	Homoz	Exon	100
AT5G28540	N675173	SALK_054493C	Homoz	3'	100
AT5G28540	N675862	SALK_079156C	Homoz	5'	100
AT5G41010	N549327	SALK_049327	Heteroz	Intron	100
AT5G41910	N663226	SALK_087920C	Homoz	5'	100
AT5G41910	N678994	SALK_115673C	Homoz	5'	100
AT5G42020	N659850	SALK_047956C	Homoz	Exon	100
AT5G42060	N669407	SALK_014079C	Homoz	5'	100
AT5G63480	N654793	SALK_095631C	Homoz	Intron	100
AT5G64680	N685462	SALK_023879	Homoz	5'	100
AT5G67240	N542641	SALK_042641	Heteroz	Exon	100

Supplemental Table 2. Lack of homologs in Arabidopsis for several nuclear receptors.

Name	Organism	AA	Max. E value	Identities	Positives
Pdr1p	Yeast	1068	7.7	37/143	58/143
PDR3p	Yeast	976	2.3	27/99	47/99
Oaf1p	Yeast	1047	0.38	24/90	46/90
PPARα	H. sapiens	468	2.3	13/42	24/42
NHR-49	C. elegans	501	0.41	18/62	29/62

The mentioned proteins were used to search in the Arabidopsis genome with BLASTP (TAIR10, www.arabidopsis.org), with the defaults settings. The column "AA" indicates the number of AA of the original protein. The "Max. E value" indicates the maximal E value obtained with BLASTP, while the "Identities" and "Positives" columns indicate the ratio of AA either identical or similar in the best stretch of homology.

Supplemental Table 3. Primers used.

Name	Sequence	Objective
5249817-NlaIII-F	TGAGCAGCAAGAAAGATGATG	nrb4 mapping
5249817-NlaIII-R	CTTAGCAGAGGTACGAGGATCA	nrb4 mapping
5346165-DdeI-F	CACCAAACACCACACTTCTCA	nrb4 mapping
5346165-DdeI-R	CATATCTTCAAAATCTTTGAGTTGG	nrb4 mapping
5377218-NlaIII-F	CTGGATTTTGGTCGAGTTAGC	nrb4 mapping
5377218-NlaIII-R	GTGGCAATAGAGGCACAAGT	nrb4 mapping
5393430-CauI-F	GAAGAGTGGTTGCAAGCGTA	nrb4 mapping
5393430-CauI-R	TTTTTGCGAGTCCACGTTC	nrb4 mapping
5406030-NlaIII-F	AGTTGGTCGGAGCTTTTCCT	nrb4 mapping

5406030-NlaIII-R	GATTCTCCACACCACCCACT	nrb4 mapping
5425793-SecI-F	AGAACGAGCTCGAACACGAA	nrb4 mapping
5425793-SecI-R	CTGAAACATTGAATCCCATTTG	nrb4 mapping
5440252-MseI-F	TGCTTTCAATAATCGTTGTGTT	nrb4 mapping
5440252-MseI-R	CACACCAAAACAAGCTTCTGC	nrb4 mapping
5455705-HinfI-F	GAATCTTGATGCTTGCTTGG	nrb4 mapping
5455705-HinfI-R	CCATGTCCGGGAAACTTATC	nrb4 mapping
5494532-RsaI-F	GTTGATCGGAAAGGAAAAGTAAAA	nrb4 mapping
5494532-RsaI-F	AAAAACGGATAACCAAACATGG	nrb4 mapping
F10B6.1-F	ATTATATTGTTCAACATCAACTGCACAT	nrb4 mapping
F10B6.1-R	TTTATCTCTTAAACAAGTTCGTAAACCAAC	nrb4 mapping
T16N11.1-F	AATAGATTAGAAATGAACAGGAGAATTGACT	nrb4 mapping
T16N11.1-R	TGGCATTTTAATAACATCCTCACC	nrb4 mapping
15780.1	TAACAAAAAATCCCAATCACGTGTG	NRB4 sequencing
15780.2	AACAATTGGAGGCCTTCTCTTCC	NRB4 sequencing
15780.3	AAATATTGCACGCCAACAAGCA	NRB4 sequencing
15780.4	AAGGCGTTCAATAGGCAGCTCA	NRB4 sequencing
15780.5	TATGCACAGGCCGAGGAAGC	NRB4 sequencing
15780.6	GCATCTGCGGATTTGTTTGG	NRB4 sequencing
15780.7	CAAGCCTCTGGTATCCATCAGC	NRB4 sequencing
15780.8	TCTGTTGGATGCCTGAGCTATTTG	NRB4 sequencing
15780.9	AATCTATGGATGTGCCATTATTAGCG	NRB4 sequencing
15780.10	TGCGCAGAATGGAAACACTAAA	NRB4 sequencing
15780.11	TTCCGGTGGGATTGGCTATT	NRB4 sequencing
15780.12	GAATGAAATCTACCAGAGAGTTGCA	NRB4 sequencing
15780.13	TGGTTTGGGACAGCAACGG	NRB4 sequencing
NRB4FP2-attb1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGGATAATAACAATTGGAGGCCT	NRB4 Clonning in pDONR221, C-terminal

NRB4RP1-attb2	GGGGACCACTTTGTACAAGAAAGCTG GGTCTATGGATGTGCCATTATTAGC	NRB4S Clonning in pDONR221
NRB4RP2-attb2	GGGGACCACTTTGTACAAGAAAGCTG GGTAAGCCACCTTATCTTTTAATGC	NRB4M Clonning in pDONR221
NRB4RP3-attb2	GGGGACCACTTTGTACAAGAAAGCTG GGTGGGAAGCTGCTACATATTTCTC	NRB4F Clonning in pDONR221
NRB4FP4-attb1	GGGGACAAGTTTGTACAAAAAAGCAG GCTTCATGGATAATAACAATTGGAGG	NRB4 Clonning in pDONR221, N-terminal
qRT NPR1.1	GAAGAATCGTTTCCCGAGTTCC	NPR1 RT-qPCR
qRT NPR1.2	CATCACCGGGTGTAAAGATAGCA	NPR1 RT-qPCR
qRT NRB4.3	TTGCCACCTGATTCTCGTCA	NRB4 RT-qPCR
qRT NRB4.4	CTCTGGTCCGGAAAATGGAA	NRB4 RT-qPCR

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Discusión general

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Un nuevo modelo para el estudio de la percepción del SA

La presente Tesis Doctoral aborda el análisis de la percepción del SA. Para ello, se utiliza como modelo principal de estudio la interacción entre Arabidopsis y *P. syringae*, uno de los biotrofos de uso más extendido en este campo. El SA es una hormona necesaria para una respuesta defensiva completa de la planta y su ruta de señalización es la vía mayoritaria para combatir a patógenos biotrofos (Vlot et al. 2009). Pese a la importancia de esta ruta, aún se desconocen importantes pasos de la misma. En concreto, solamente se ha descrito un gen necesario en su percepción (*NPR1*) y no se ha podido demostrar que sea su receptor (Dong 2004; Nawrath et al. 2005). Así pues, esta Tesis parte de la hipótesis de que deben existir otros componentes genéticos que participan en la percepción de esta hormona. No obstante, otros grupos de investigación habían llevado a cabo rastreos genéticos con la finalidad de obtener nuevos mutantes insensibles al SA, que no habían generado los frutos deseados. Por tanto, antes de poder abordar un posible rastreo genético a gran escala con mayores posibilidades de éxito, era necesario plantear un modelo biológico alternativo para el estudio de la percepción del SA en Arabidopsis (art. 1).

El enfoque tradicional para estudiar este proceso está basado en el análisis de los síntomas detectados en la planta, asociados a la infección por el patógeno. Este modelo fue descartado, principalmente, por haber sido utilizado con anterioridad por otros grupos y no haber generado los resultados deseados en este punto de la ruta del SA (Glazebrook et al. 1996). En contraposición, se ha propuesto un modelo basado en la reducción del peso fresco de la planta provocado por la inducción de resistencia. Esta inducción genera como efecto colateral un impacto negativo en la biomasa/productividad de las plantas (Heil 2002). Este fenómeno puede resumirse en la siguiente afirmación: cuanto más resistente es un individuo, menos adecuado es para competir en ausencia de un patógeno, al menos en el sistema experimental utilizado en este trabajo. En este modelo, para evitar los problemas de fitotoxicidad del SA cuando se aplica en dosis elevadas (van Leeuwen et al. 2007), se ha trabajado con uno de sus análogos químicos, el BTH (Lawton et al. 1996). De los resultados presentados en el primer artículo se concluye que la aplicación exógena de BTH da lugar a dos fenotipos en la planta: incrementa su resistencia frente a patógenos biotrofos y disminuye su biomasa. Este segundo fenotipo es la base de este nuevo modelo biológico para estudiar la percepción del SA. Tras los pasos de

optimización necesarios, este fenotipo es una herramienta que permite realizar, además de medidas reproducibles en genotipos concretos, un rastreo de mutantes basado en el efecto negativo de los tratamientos con BTH sobre el crecimiento de las plantas silvestres. De esta forma, aquellas plantas que son insensibles y fenocopian al mutante *npr1* pueden ser seleccionadas fácilmente, es decir, que los candidatos podrían ser elegidos *de visu*.

Sin embargo, era necesario validar este modelo experimental antes de ponerlo en práctica. En primer lugar, se confirmó que el fenotipo de pérdida de peso y el de inducción de resistencia/activación de defensas están relacionados. En segundo lugar, se concluyó que el ecotipo seleccionado (Col-0) no presenta una respuesta extrema al BTH. En tercer lugar, tras analizar una vasta colección de mutantes publicados, se dedujo que el modelo biológico propuesto es adecuado para estudiar la percepción del SA. De esta forma, en un rastreo genético en el que dicho modelo se utilizase, solamente se rescatarían nuevos alelos de *npr1* u otros genes aún no descritos.

Los resultados presentados en el primer artículo también permiten especular acerca de la interacción existente entre defensa y desarrollo en las plantas. Mantener el equilibrio entre ambos procesos es fundamental para las mismas, tanto para asegurar su supervivencia como para permitir una competencia adecuada con otras especies de plantas (Lopez et al. 2008). Además, ambos pocesos están inversamente relacionados: a mayor defensa, menor desarrollo, y viceversa (Heil 2002). Para intentar explicar este hecho se han propuesto diversas hipótesis: bien que la defensa es costosa (utiliza recursos que estaban destinados a producir biomasa), o bien que la defensa es tóxica (produce moléculas para detener al patógeno que también afectan a la planta). En la literatura se han descrito varios supresores de *npr1*. En concreto, el doble mutante *sni1 npr1*-1 es capaz de inducir la expresión de genes *PR* y la resistencia tras el tratamiento con SA (Li et al. 1999). Sin embargo, este doble mutante no actúa como un supresor para el fenotipo de pérdida de biomasa. Por tanto, la existencia de un genotipo capaz de producir defensas sin presentar una pérdida de biomasa es contradictoria con las dos hipótesis previamente mencionadas. Estos resultados permiten especular sobre una tercera hipótesis, que puede denominarse como "política de tierra quemada". Según esta idea, la planta activaría dos programas diferentes a la vez: la síntesis de defensas para combatir directamente al patógeno y la eliminación de nutrientes de la zona de infección

para limitar su disponibilidad para el patógeno. Este segundo programa podría llevarse a cabo mediante la regulación antagonista existente entre el SA y las auxinas (Wang et al. 2007a).

El modelo llevado a la práctica: el rastreo genético

En el segundo artículo se presentan los resultados del rastreo genético realizado. La aplicación del modelo anteriormente planteado ha permitido analizar unos 5,3 millones de semillas mutagenizadas, aumentando así las posibilidades de éxito del rastreo. El principal grupo de complementación obtenido ha sido *npr1*, confirmando la gran importancia del gen en la ruta. Los 43 nuevos alelos identificados representan una herramienta muy valiosa para el estudio de la función de esta proteína puesto que hasta la fecha solo había sido descrito un número pequeño. Estos alelos han sido utilizados para ampliar el conocimiento sobre la función de NPR1 en la percepción del SA (art. 2) y en la resistencia inducida por MeJA (art. 3). No obstante, también se han obtenido otros grupos adicionales, hecho que implicaría la existencia de otros genes aún no descritos implicados en la percepción del SA. Esto ha sido confirmado en el caso del segundo grupo de complementación identificado (*nrb4*, art. 4). El resto de candidatos seleccionados ha sido posteriormente descartado tras un estudio detenido, por la complejidad de análisis que presenta. Por tanto, este rastreo genético se puede dar por finalizado con los 43 alelos de *npr1* y los 3 alelos de *nrb4* identificados.

El BTH es un análogo perfecto del SA cualitativamente

El análisis de los 43 nuevos alelos de *npr1* (art. 2) muestra que presentan tanto insensibilidad al BTH como al SA. Esto confirma que en un rastreo genético basado en la pérdida de respuesta al BTH se obtienen mutantes en la ruta de percepción del SA, validándose así el modelo planteado. Además, se observa que no existen diferencias entre la percepción del SA y del BTH en los genotipos analizados. La posterior caracterización de los 3 alelos del segundo grupo de complementación (art. 4) reafirma la utilidad de este modelo y la no discriminación entre SA y BTH. Además, el uso de la línea transgénica *NPR1-HBD* permite concluir que es necesaria la localización nuclear de la proteína NPR1 para inducir la respuesta dependiente del BTH (art. 1), del mismo modo que sucede para la dependiente del SA (Kinkema et al. 2000). Así pues, del

conjunto de resultados presentados en la Tesis es posible concluir que el BTH actúa en la ruta de señalización del SA del mismo modo que la propia hormona.

El papel de los parálogos de *NPR1* en la percepción del SA

El estudio del tipo de mutaciones presentes en los alelos de *npr1* indica que han sido recuperadas preferentemente aquellas situadas en aminoácidos conservados entre los seis genes de la familia *NPR1*. Además se observa un sesgo en la distribución de las mutaciones que no corresponde con ninguno de los dominios descritos de la proteína y tampoco con las cisteínas descritas como fundamentales para la oligomerización de la proteína (Mou et al. 2003). Este sesgo corresponde a una zona de la proteína conservada en los parálogos descritos y que había sido previamente etiquetada como una región de represión autónoma de la transcripción (Rochon et al. 2006). El resultado de este rastreo avala la importancia de esta región en la funcionalidad de la proteína.

La comparación entre los resultados de este rastreo genético con otro de características similares (Tornero et al. 2002a) indicaría, con las reservas pertinentes ante este tipo de comparaciones, que en el actual se ha registrado un número relativamente bajo de mutantes. De hecho, puesto que existe un sesgo en la distribución de las mutaciones obtenidas y que los mutágenos empleados no presentan ninguna preferencia por una zona concreta de la proteína, se podría concluir que existen un determinado número de alelos que no habrían podido ser recuperados en el rastreo. Además, ninguno de los alelos obtenidos sería un mutante nulo evidente, puesto que no se han recuperado mutaciones que introduzcan un codón de parada al inicio de la proteína. Estas características del rastreo podrían explicarse mediante dos hipótesis: letalidad (solo se recuperan aquellas mutaciones que no alteran la función esencial de NPR1) o redundancia (existen otras proteínas que participan en la percepción del SA y por tanto el alelo nulo presenta un fenotipo similar al silvestre). La obtención a partir de los bancos de semillas de dos alelos nulos de *npr1* viables descarta la hipótesis de la letalidad. Estos alelos nulos presentan un fenotipo intermedio para el modelo biológico presentado (no hubieran sido seleccionados en el rastreo), hecho que respalda la existencia de una redundancia parcial en la percepción de la hormona. Sería parcial puesto que el fenotipo de los nulos es muy distante del silvestre y por la elevada proporción

de alelos de *npr1* recuperados del total de mutantes obtenidos en el rastreo. El fenotipo más fuerte de los alelos de *npr1* recuperados en el rastreo se debería a que estas mutaciones presentan un efecto negativo adicional en las otras proteínas que también participan en la ruta de señalización del SA.

Los resultados presentados en el segundo artículo respaldan la teoría de la redundancia parcial. Los cinco parálogos de *NPR1* descritos en Arabidopsis (*NPR2*, *NPR3*, *NPR4*, *BOP1* y *BOP2*) presentan una función medible en la percepción del SA, en un fondo genético nulo para *NPR1*. No obstante, existe un papel predominante de *NPR1* en esta ruta, hecho que no permite detectar un fenotipo en las mutaciones de estos parálogos en un fondo genético silvestre para *NPR1*. Los resultados también indican que la función secundaria de los parálogos en la percepción del SA es gradual, con un mayor peso para *NPR2*, seguido de *NPR3* y *NPR4*.

Resultados recientes postulan a *NPR3* y *NPR4* como receptores del SA (Fu et al. 2012). Tales resultados confirmarían el papel de estos parálogos en la percepción de esta hormona, aunque dicho papel sería menos secundario que el sugerido por los resultados presentados en esta Tesis. De hecho, el doble mutante *npr3 npr4*, silvestre frente al BTH en el presente trabajo (art. 1), ha sido descrito como incapaz de inducir una correcta respuesta defensiva mediada por SA (Fu et al. 2012). Estas diferencias podrían deberse a la distinta forma de medir los fenotipos, a falta de estudios más pormenorizados.

El mecanismo por el que estas proteínas npr1 mutadas podrían causar un efecto negativo en la funcionalidad de las proteínas de los parálogos que explique el fenotipo más fuerte de los alelos del rastreo respecto de los nulos aún no ha sido identificado. Entre las posibilidades planteadas en el segundo artículo, la idea de que estas proteínas mutadas puedan actuar reduciendo la disponibilidad de las otras proteínas implicadas en la ruta a través de sus interactores comunes cobra relevancia a tenor de los resultados presentados en la última figura del tercer artículo. Los TFs que forman la familia de *TGAs* serían unos buenos candidatos puesto que cumplen ambas condiciones. Por una parte, se ha demostrado tanto su interacción con NPR1 (Zhang et al. 1999) como con sus parálogos (Hepworth et al. 2005; Zhang et al. 2006), por lo que sí podrían actuar como puente entre la proteína mutada y las de los parálogos. Por otra parte, su papel en la ruta de señalización del SA ha quedado demostrado,

puesto que la activación transcripcional de un conjunto de genes *PR*, con cuyos promotores pueden interaccionar físicamente, es dependiente de su interacción con NPR1 (Després et al. 2000; Johnson et al. 2003). Además, pese a la redundancia funcional existente en los TGAs, un triple mutante defectivo en tres de los miembros de esta familia multigénica (*tga2 tga6 tga5*) tiene bloqueada la inducción de resistencia mediada por SA (Zhang et al. 2003) y presenta un fenotipo similar al de los alelos nulos de *npr1* en nuestro modelo (art. 1).

NPR1 no es necesario para la MIR

NPR1 es el principal gen necesario para la percepción del SA (Dong 2004). Esta proteína presenta una doble localización (nuclear y citosólica), pero su función en la regulación defensiva dependiente del SA tiene lugar en el núcleo (Kinkema et al. 2000). El SA presenta una relación principalmente de tipo antagonista con el JA, probablemente debido a que ambas hormonas participan en vías de señalización que combaten a patógenos con diferentes estilos de vida (Vlot et al. 2009). *NPR1* también ha sido implicado en el proceso de inhibición de la vía del JA, dependiente del SA. Para esta función se ha postulado que la forma citosólica de la proteína sería la más relevante (Spoel et al. 2003). *NPR1* también es un gen regulador clave en los dos tipos de resistencias sistémicas más estudiadas, SAR e ISR (Pieterse y van Loon 2004). Mientras que la correcta inducción de la SAR necesita de la vía de señalización del SA (Vlot et al. 2009), la ISR requiere de la ruta defensiva dependiente del JA y el ET (van der Ent et al. 2009). Esta inducción de resistencia en las plantas puede mimetizarse con la aplicación exógena de las propias hormonas defensivas o de análogos funcionales. Esto se puede observar en los resultados del primer y segundo artículo para el SA (y su análogo, el BTH) y en los del tercer artículo para el JA (en forma de MeJA) y el ET (mediante la aplicación de su precursor, el ACC). En el caso de la inducción de resistencia tras la aplicación de MeJA (también conocida como MIR), nuevamente se ha postulado al gen *NPR1* como relevante (Pieterse et al. 1998), aunque su función en este proceso aún no ha sido bien definida. No obstante, estudios posteriores han sugerido que la forma citosólica de la proteína estaría implicada en la regulación de las respuestas defensivas dependientes del JA (Glazebrook et al. 2003; Johansson et al. 2006; Ramirez et al. 2010). Estos estudios se han basado en las diferencias existentes entre dos de los mutantes descritos: *npr1*-1 y *npr1*-3 (Cao et al. 1997). Brevemente, npr1-3 es una proteína citosólica truncada (carente del dominio de

localización nuclear) pero que es funcional en la modulación de las respuestas dependientes del JA y por tanto presenta una respuesta MIR+ (como el control silvestre). En cambio, el alelo *npr1*-1, que había sido asumido como nulo por presentar una mutación en uno de los dominios de la proteína, presenta una respuesta MIR-. Los 43 nuevos alelos de *npr1* rescatados en el rastreo genético junto con los dos alelos nulos obtenidos de los bancos de semillas (art. 2) han sido utilizados para indagar en la función de NPR1 en la MIR (art. 3).

Los resultados del tercer artículo indican que la NPR1 citosólica no es la responsable de la MIR. Esto se observa, principalmente, en el hecho de que una NPR1 funcional anclada al citoplasma (*NPR1-HBD*) es incapaz de complementar la respuesta MIR- del alelo *npr1*-1. Además, el comportamiento silvestre de los alelos nulos de *npr1* en su respuesta al MeJA indican que *NPR1* no es necesario para la MIR. También se ha descartado que NPR1 actúe sobre la MIR mediante un aumento de la inhibición de la vía del JA provocado por el mayor nivel de SA existente en los mutantes *npr1* tras ser infectados (Zhang et al. 2010b), puesto que el fenotipo MIR- no es revertido en plantas con bajos niveles de SA. Por otra parte, los resultados del segundo artículo presentan una redundancia parcial en la percepción del SA entre *NPR1* y sus parálogos, por lo que se plantea la posibilidad de que dicha redundancia tenga lugar también en la MIR. Los resultados presentados indican que *NPR1* no participa de forma redundante con sus parálogos en esta respuesta defensiva.

El papel de los *BOPs* y los *TGAs* en la MIR

Estos mismos experimentos sí presentan a dos de los parálogos de *NPR1*, *BOP1* y *BOP2*, como actores relevantes en este proceso. Ambos genes presentan una redundancia funcional en la MIR, fenómeno que comparten también en sus funciones previamente descritas en desarrollo (Hepworth et al. 2005; McKim et al. 2008). Los experimentos de silenciamiento y de sobreexpresión de los *BOPs* respaldan su función en la MIR. Además sugieren que la aparición de los fenotipos asociados a desarrollo necesitan de un mayor descenso en los niveles de expresión de ambos genes respecto a los necesarios para el fenotipo en defensa. Asimismo, el umbral de sobreexpresión necesario para la aparición de fenotipos en desarrollo sería superior al necesario para inducir una mayor MIR.

Los BOPs pueden interaccionar físicamente con los TGAs (Hepworth et al. 2005), y esta familia multigénica también está implicada en la percepción del SA (Zhang et al. 2003). El triple mutante *tga2 tga6 tga5* también presenta suprimida la MIR, indicando un nuevo papel de esta familia en esta respuesta defensiva. La participación de los TGAs, también interactores de NPR1 (Zhang et al. 1999), en la respuesta al MeJA permite plantear una hipótesis para explicar porque la mayoría de los alelos de *npr1* rescatados en el rastreo son defectivos en MIR pese a que los alelos nulos son silvestres. Se plantea que los alelos de *npr1* MIR- actuarían interfiriendo en las interacciones normales entre BOPs y TGAs, originándose de esta manera el fenotipo en defensa. El hecho de que los niveles de expresión de los *BOPs* sean muy inferiores a los de *NPR1*, facilitaría esta posible interferencia negativa. Los resultados de los experimentos llevados a cabo en levaduras indican que los alelos de *npr1* pueden afectar a la interacción BOPs-TGAs, aunque no respaldan la teoría presentada en su totalidad.

Los resultados presentados en esta Tesis (art. 2 y 3) plantean que el conjunto de los parálogos de *NPR1* tiene un papel significativo en la defensa de las plantas, puesto que se han relacionado con las respuestas defensivas dependientes de las dos hormonas más relevantes en esta función (SA y JA). Como se discutirá más adelante, no se ha detectado una interacción directa entre *NPR1*, que era el único gen descrito como necesario para la percepción del SA, y *NRB4*, también necesario en dicho proceso. Esta falta de conexión directa concuerda con las diferencias existentes entre los fenotipos en desarrollo descritos para el doble *bop1 bop2* y los más drásticos presentes en los alelos nulos de *nrb4* (Art. 4).

NRB4, necesario en la percepción del SA y esencial para la planta

Los resultados del cuarto artículo presentan un nuevo gen necesario para la percepción del SA, *NRB4*. Los tres alelos recuperados en el rastreo presentan fenotipos que muestran que la respuesta defensiva dependiente del SA está seriamente alterada en dichos mutantes. Con ligeras diferencias (serán abordadas más adelante), los tres alelos *nrb4* comparten con *npr1* todos los fenotipos estudiados relacionados con la defensa. *NRB4*, como se ha descrito para *NPR1* en los resultados del tercer artículo, tampoco está implicado en la MIR. Los tres alelos *nrb4*, nuevamente al igual que *npr1*, no presentan ningún

fenotipo destacable en desarrollo, siendo en su crecimiento indiferenciable del control silvestre.

Al tratarse de los únicos genes descritos como necesarios para la percepción del SA, era pertinente clarificar su posición en esta vía. Los resultados presentados indican que no existe interacción genética en F1 entre ambos mutantes y tampoco se ha podido demostrar interacción bioquímica ni en levadura ni en experimentos de expresión transitoria en *N. benthamiana*. Mediante el uso de la líneas transgénicas *NPR1-HBD* y *NPR1-GFP* (Kinkema et al. 2000) se ha determinado que la sobreexpresión y localización nuclear de NPR1 no puede revertir la insensibilidad al SA/BTH de *nrb4*. Además, también se ha comprobado que la movilización de NPR1 al núcleo, necesaria para su funcionalidad, no está alterada en *nrb4*. Estos resultados indican que NRB4 y NPR1 participan en diferentes puntos de la ruta de señalización del SA, como también lo indica la aditividad de fenotipos observada en los dobles mutantes obtenidos a partir de los alelos con respuestas más intermedias. Por tanto, todos los resultados apuntan a que NRB4 actuaría en la ruta después de NPR1 y que no afectaría a su relocalización nuclear.

Cada uno de los tres alelos *nrb4* del rastreo presenta una mutación puntual que implica el cambio de solo un aminoácido en la secuencia de la proteína. Estas mutaciones, a pesar de los fenotipos que presentan, no alterarían la posible estructura de la proteína ni comportarían un descenso en la expresión del gen. El número de alelos *nrb4* obtenidos en el rastreo es muy inferior a los recuperados de *npr1*. Esta carencia es más evidente si se tiene en cuenta el mayor tamaño de NRB4 (más del doble) y que no se pudieron recuperar los alelos nulos de *npr1* por la redundancia parcial existente. Hay dos fenómenos que explican el bajo número de alelos *nrb4*. Por una parte, las tres mutaciones se agrupan en un pequeño dominio de la proteína llamado KIX (Radhakrishnan et al. 1997) por lo que sería el dominio más sensible para detectar una alteración grave en la respuesta al SA. Además, los resultados de complementación indican que la segunda mitad de la proteína es prescindible para su funcionalidad. Por otra parte, existe una cuestión de letalidad que ha sido detectada a partir de los dos alelos nulos de *nrb4* obtenidos en heterocigosis de los bancos de semillas. Dichos alelos nulos, en los que el nivel de expresión del gen ha bajado hasta el límite de detección, se sitúan nuevamente en el dominio KIX, rompiéndolo.

Los alelos nulos de *nrb4* poseen un patrón de crecimiento claramente diferente al del control silvestre y son estériles. Asimismo, presentan insensibilidad al SA y al BTH. Sin embargo, el uso de patógenos más débiles indica que su susceptibilidad es mucho mayor que la de los otros alelos *nrb4* y la de *npr1*. La desregulación del nivel endógeno de SA es también mucho mayor en los *nrb4* nulos respecto a los otros mutantes. Por tanto, además de los fenotipos en desarrollo, los nulos *nrb4* presentan una pérdida total de la respuesta defensiva mediada por SA, frente a los mutantes puntuales que presentan algunos fenotipos más intermedios en dicha respuesta.

***NRB4* es *MED15*, una subunidad del *Mediator* implicada en la percepción del SA**

NRB4 ha sido etiquetado como el ortólogo de *MED15* en Arabidopsis debido a su coinmunoprecipitación con otras subunidades del complejo *Mediator* de esta planta (Backstrom et al. 2007). Este complejo multiproteico, conservado en todos los eucariotas analizados, actuaría como puente necesario entre la maquinaria transcripcional y el amplio conjunto de TFs (Kidd et al. 2009). De los tres módulos que forman la parte central del complejo, el módulo cola interaccionaría con los TFs y los módulos cabeza e intermedio se unirían a la RNA pol. II (Malik y Roeder 2005). La presencia de NRB4 en el *Mediator* concuerda con su situación después de NPR1 en la ruta de señalización del SA antes comentada. La respuesta defensiva inducible mediada por SA requiere de la modificación de los niveles de expresión de una gran cantidad de genes. Existen muchos TFs cuya actuación en la regulación de dicha respuesta ha sido demostrada, como los *TGAs* (Jakoby et al. 2002) y los *WRKYs* (Eulgem et al. 2000). Pero dichos TFs no interaccionan directamente con la RNA pol. II. NRB4/MED15 se sitúa en el módulo cola del *Mediator* (Taatjes 2010) y su interacción con un determinado subconjunto de TFs resultaría imprescindible para la inducción de una respuesta dependiente del SA completa. Por otra parte, la localización nuclear detectada para NRB4 coincide con la descrita para el *Mediator*.

Puesto que el complejo *Mediator* es necesario para la regulación global de la transcripción (Boube et al. 2002), la especificidad de *NRB4* en el control de la respuesta mediada por SA podría ponerse en duda. Sin embargo, se han descrito funciones específicas para muchas de las subunidades del complejo en

procesos fisiológicos concretos (Kidd et al. 2011). Además, estas subunidades podrían ejercer su funcion independientemente del resto del complejo *Mediator*. En el caso de la regulación defensiva, se han descrito tres subunidades implicadas en la respuesta frente a patógenos necrotrofos (principalmente mediada por JA): *MED21* (Dhawan et al. 2009), *PFT1/MED25* y *SETH10/MED8* (Kidd et al. 2009). Por tanto, es lógico que exista alguna subunidad implicada en la respuesta a biotrofos (principalmente mediada por SA). Los resultados presentados en el cuarto artículo confirman la función específica de *NRB4* en la respuesta mediada por SA. Básicamente, los fenotipos de los alelos *nrb4* del rastreo se ciñen a esta función. Incluso la grave alteración del desarrollo en los alelos nulos podría explicarse por la pérdida completa de la percepción del SA (se discutirá más adelante). De hecho, el análisis del transcriptoma de uno de los alelos nulos no identifica ningún otro proceso fisiológico específico alterado además de la defensa y el SA. Por otra parte, la sobreexpresión de la proteína permite tanto complementar los fenotipos de los alelos *nrb4* como incrementar la sensibilidad al SA en el control silvestre, pero no produce ningún fenotipo adicional. Pese a su gran tamaño, *NRB4* no ha sido detectado en ninguno de los muchos rastreos genéticos a gran escala realizados en Arabidopsis. Aunque es posible que *NRB4* sea uno de los tres ortólogos de *MED15* existentes en Arabidopsis (Mathur et al. 2011), su función en la ruta del SA no sería redundante. Además, aplicando las mismas condiciones del rastreo en los mutantes disponibles para el resto de subunidades del *Mediator*, no se ha podido detectar ninguna otra implicada en este proceso.

¿Cómo funciona NRB4 en la vía del SA? ¿Por qué es esencial?

Se han descrito varios ortólogos de *MED15* que pueden interaccionar mediante su dominio KIX con determinados receptores nucleares. Es el caso de *GAL11* y el receptor Oaf1P en levadura (Thakur et al. 2009) o de *MDT-15* y el receptor NHR-49 en *Caenorhabditis elegans* (Taubert et al. 2006). Además, estos receptores también pueden unir salicilatos. Estos datos sugieren la posibilidad de que en Arabidopsis NRB4 pudiera interaccionar con el receptor del SA. Esta teoría no puede ser contrastada por el momento porque no se ha descrito dicho receptor ni encontrado ningún gen en Arabidopsis con el suficiente grado de similitud con estos receptores de otras especies como para ser considerado un buen candidato.

En la Figura 6 de la Introducción se presenta a *MED25* como una subunidad capaz de regular múltiples procesos en Arabidopsis. Su interacción con diferentes TFs permite a la planta regular procesos fisiológicos distintos. En el caso de *NRB4/MED15* se plantea un modelo similar. Los TFs implicados en la respuesta mediada por SA interaccionarían con el dominio KIX de NRB4. Mutaciones puntuales en dicho dominio bloquearían bastantes o todas las respuestas defensivas mediadas por el SA, de forma total o parcial. Esto explicaría las pequeñas diferencias existentes entre los tres alelos recuperados en el rastreo (la correcta inducción de la SAR en *nrb4*-2 o la tolerancia de *nrb4*-1 a crecer en medios suplementados con SA). NRB4, al igual que otras subunidades, también podría realizar parte de su función de forma independiente del resto del complejo.

Los resultados obtenidos con los alelos nulos indican que *NRB4* es esencial para la planta. La explicación más sencilla es que dicha subunidad presente otras funciones, además de las relacionadas con el SA, que al ser suprimidas dan lugar a una planta inviable. No obstante, el análisis de los transcriptomas no apunta a la supresión de ninguna otra señalización o función. Además, los alelos nulos presentan fenotipos más severos en defensa, pero ningún otro fenotipo destacable salvo el evidente en desarrollo.

El SA es importante para muchos otros procesos de la planta distintos de la defensa, muchos de ellos relacionados con su crecimiento y desarrollo (Rivas-San Vicente y Plasencia 2011). De hecho, se ha propuesto que esta hormona puede ser esencial para el desarrollo normal de la planta (Vanacker et al. 2001). Por otra parte, si bien existen mutantes con bajos niveles de SA, en ninguno de ellos el nivel es cero. Uno de los casos más extremos es el del doble mutante *ics1 ics2*, que tiene bloqueada la vía principal de síntesis de la hormona (Garcion et al. 2008). Esta planta, que aún posee cierto nivel de SA residual, es de menor tamaño, de pigmentación amarillenta y sobrevive únicamente en cultivo *in vitro*. Por tanto, se podría especular que una planta sin SA no sería viable. Dicho de otra manera: el SA sería esencial para la planta. Así pues, el hecho de que *NRB4* sea esencial para la planta podría explicarse por el papel clave del SA en su correcto desarrollo.

conclusiones

conclusiones

Conclusiones

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- El análisis de la reducción del peso fresco de la planta provocada por la inducción de resistencia tras la aplicación del BTH constituye un modelo biológico válido y eficaz para el estudio de la percepción del SA en Arabidopsis.

- Con el modelo anterior, se ha llevado a cabo un rastreo genético en busca de mutaciones que afecten a la percepción del SA. Del análisis de los alelos de *npr1* obtenidos se concluye que las mutaciones se agrupan en la zona carboxilo terminal de la proteína. Del análisis de los alelos nulos de *npr1* se concluye que los cinco parálogos de *NPR1* descritos en Arabidopsis presentan una función secundaria en la percepción del SA. Esta función solamente puede ser detectada en un fondo genético nulo para *NPR1* debido al papel predominante de este gen.

- *NPR1* no es necesario para la resistencia inducida por MeJA en un fondo silvestre, pero sí participan en esta respuesta defensiva sus parálogos *BOP1* y *BOP2* y la familia multigénica de factores de transcripción *TGAs*.

- *NRB4* es el segundo gen necesario para la percepción del SA obtenido en el rastreo. Forma parte del complejo *Mediator* y es esencial para la planta. Mecanísticamente, NRB4 ejerce su función después de que NPR1 se localice en el núcleo.

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Artículo 5

Quantitative genetic analysis of salicylic acid perception in Arabidopsis

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Este artículo ha sido publicado en la revista:

Planta (2011) 33, 1911-1922

Abstract

Salicylic acid (SA) is a phytohormone required for a full resistance against some pathogens in Arabidopsis, and *NPR1* (*Non-Expresser of Pathogenesis Related Genes 1*) is the only gene with a strong effect on resistance induced by SA which has been described. There can be additional components of SA perception that escape the traditional approach of mutagenesis. An alternative to that approach is searching in the natural variation of Arabidopsis. Different methods of analyzing the variation between ecotypes have been tried and it has been found that measuring the growth of a virulent isolate of *Pseudomonas syringae* after the exogenous application of SA is the most effective one. Two ecotypes, Edi-0 and Stw-0, have been crossed, and their F2 has been studied. There are two significant Quantitative Trait Loci (QTLs) in this population, and there is one QTL in each one of the existing mapping populations Col-4 x L*aer*-0 and L*aer*-0 x No-0. They have different characteristics: while one QTL is only detectable at low concentrations of SA, the other acts after the point of crosstalk with methyl jasmonate signalling. Three of the QTLs have candidates described in SA perception as *NPR1*, its interactors, and a calmodulin binding protein.

Keywords: Arabidopsis, Defence, Natural variation, Salicylic acid.

Introduction

Salicylic acid (SA, for a review, see Vlot et al. 2009) is a hormone with an impact on several areas of plant biology such as the induction of flowering (Rhoads and McIntosh 1992), and it is required for resistance against microbes, especially virulent pathogens like *Pseudomonas spp*. (Katagiri et al. 2002). *Arabidopsis thaliana* (Arabidopsis) is a well studied plant, and most of the knowledge on SA has been developed with Arabidopsis.

There are two biosynthetic genes (*EDS5*, Nawrath et al. 2002 and *SID2,* Wildermuth et al. 2001) regulated by SA itself and by other genes (e.g. EDS1, Wiermer et al. 2005), although mutations in these genes do not completely eliminate SA. For a severe depletion in the levels of SA, the transgenic plant *NahG* has to be used (Lawton et al. 1995). This plant overexpresses a salicylate

hydroxylase from *Pseudomonas putida* thus converting SA to catechol (You et al. 1991). Regarding SA perception, NPR1 is the only protein that is known to be necessary for signal transduction (Pieterse and van Loon 2004), although there is evidence pointing to a SA-dependent, NPR1-independent resistance (Desveaux et al. 2004).

In order to define the components of SA signalling, a number of mutant screenings have been performed, but more *NPR1* alleles are the only result reported so far (Cao et al. 1994; Delaney et al. 1995; Glazebrook et al. 1996; Shah et al. 1997). The biochemical study of NPR1 has produced a number of proteins, although none of them is as relevant as NPR1 itself in terms of mutant phenotype of the corresponding gene. Thus, NPR1 interacts with proteins that regulate (NIMINs, Weigel et al. 2001) or carry on the signalling (TGAs, Zhang et al. 1999). In non inductive conditions NPR1 is reported to be located mainly in the cytoplasm. Upon SA perception, it is monomerized by a thioredoxin and migrates to the nucleus where it activates the genes that lead to resistance (Tada et al. 2008). This activation requires NPR1 degradation via proteosome (Spoel et al. 2009). An alternative model has been recently proposed where –at least in tobacco- NPR1 is always in the nucleus and is sensitive to SA, thus activating its function (Maier et al. 2011). SA has a negative crosstalk with at least methyl jasmonate (MeJA, Genoud and Metraux 1999). Interestingly, applications of MeJA are capable of triggering a small resistance, and this resistance is dependent on NPR1 in its cytosolic form (Spoel et al. 2003). Other approaches like transcriptomics (Pylatuik and Fobert 2005) and metabolomics (Hien Dao et al. 2009) have rendered a wealth of data, but have not produced any candidate different from the ones already described.

Our ongoing interest in SA perception has led us to inquire about it in the reported mutants in defence (Canet et al. 2010a) as well as in new screenings (Canet et al. 2010b). A resource that has not been used extensively to study SA is the natural variation of Arabidopsis. Arabidopsis is an almost perfect tool for natural variation studies (Alonso-Blanco and Koorneef 2000) since it has hundreds of ecotypes (also known as accessions or land races), and a good number of mapping populations (www.arabidopsis.org). The recombinant inbreed lines (RILs) constitute an important tool in natural variation. In this resource, the F2 plants from selected parents are taken from generation to generation until F8. The result is that the level of heterozygosity is negligible,

allowing for the detection of quantitative trait loci (QTLs) with relatively few lines. Another advantage is that RILs are a stable resource so they have to be genotyped only once. Once a QTL is detected, the usual approach is to construct lines derived from RILs that share all the genome except that in the interval where the QTL is predicted to be. These *ad hoc* tools are called near isogenic lines (NILs, Alonso-Blanco et al. 2006).

There is extensive work dealing with the natural variation of the response of Arabidopsis to pathogens, like *Hyaloperonospora spp.* (Adam and Somerville 1996) or *Pseudomonas spp* (Perchepied et al. 2006). There is also work done that deals with the glucosinolate synthesis as a response to exogenous SA (Kliebenstein et al. 2002), and with the transcriptomic response to exogenous SA (van Leeuwen et al. 2007).

We have tried different approaches to best capture the natural variation of Arabidopsis in response to SA. The best option is to treat the plants directly with SA and then to inoculate them with virulent bacteria. With this method, we have found differences between ecotypes that give rise to four different QTLs. Two of them come from an *ad hoc* F2 population, and the other two from different RILs. The positions of the last two have been confirmed with NILs, thus allowing speculation about their different position in SA signalling on the basis of their phenotypes.

Materials and methods

Plant growth and inoculation

Arabidopsis thaliana (L.) Heynh. was sown and grown as described (Canet et al. 2010a), in phytochambers with days of 8 h at 21°C, 150 μmol m^{-2} s^{-1} and nights of 16 h at 19°C. All the ecotypes, mutants, and mapping populations were obtained from NASC (www.arabidopsis.info). When the population Edi-0 x Stw-0 was used, plants were grown as usual and after two weeks vernalized for six weeks at 4º C. After this treatment, plants were grown in normal conditions. The treatments, inoculations, and sampling started 30 minutes after the initiation of the artificial day to ensure reproducibility. *Pseudomonas syringae pv. tomato* DC3000 (*Pto*) containing an empty pVSP61 was obtained from Dr.

Dangl (University of North Carolina at Chapel Hill, NC, USA) and maintained as described (Ritter and Dangl 1996). The bacteria were grown, inoculated and measured as described (Tornero and Dangl 2001). Briefly, plants of 14 days were inoculated by spray with *Pto* at OD_{600}=0.1 with 0.02% Silwet L-77 (Crompton Europe Ltd, Evesham, UK). Three days later, the amount of colony forming units (cfu) per plant was quantified.

Chemical treatments

For measuring the effect in *Pto* growth, water, SA (in the form of sodium salicylate, S3007 Sigma, St Louis, MO, USA), 10 g/L fosetyl-al 80 (Aliette; Rhone-Poulenc, Lyon, France), and 35 or 350 μM BTH were applied by spray. Fosetyl was applied four days previous to pathogen inoculation, SA and BTH one day. SA was applied at 100 μM unless other concentration is stated. 100 μM methyl jasmonate (Sigma) was applied by spray one day previous to pathogen inoculation. For the measurement of SA *in planta*, three samples of 100 mg were frozen in liquid nitrogen. SA measurements were performed with the biosensor *Acinetobacter sp. ADPWH_lux* (Huang et al. 2005). For *in vitro* culture, plants were grown in MS media (Duchefa, Haarlem, The Netherlands) with 500 μM SA, and the growth of the plants evaluated every few days for three weeks (data not shown).

QTL mapping

Plants of Arabidopsis were treated with either mock or 100 μM SA as described above. The logarithm of cfu per plant and the genotype of each line were used as input for the program WinQTLCart 2.5 (Wang et al. 2007b), that calculates the probability that a QTL is linked to a particular region of the genome. The populations analyzed were: Col-0 x Nd-1, 98 lines (Deslandes et al. 1998); Col-gl1 x Kas-1, 115 lines (Wilson et al. 2001); Cvi-1 x La*er*-2, 50 lines (Alonso-Blanco et al. 1998b); La*er*-0 x Sha-0, 114 lines (Clerkx et al. 2004); Bay-0 x Sha-0, 162 lines (Loudet et al. 2002); Col-4 x La*er*-0, 85 lines (Lister and Dean 1993); and La*er*-0 x No-0, 135 lines (Magliano et al. 2005). In the case of Edi-0 x Stw-0, 266 F2s and their parental were genotyped with iPLEX® in the CEGEN (Spanish National Genotyping Centre, www.cegen.org), with 24 markers (www.naturalvariation.org). Two additional SSLP markers were added to the map to complete the Chromosome II (Supplemental Fig. S1). The program

GGT 2.0 (van Berloo 2008) was used for the selection of RILs and the STAIRs lines.

Benzothiadiazole (BTH) treatment and fresh weight determination

BTH (CGA 245704), in the form of a commercial product (Bion® 50 WG, a gift from Syngenta Agro S.A. Spain) was prepared in water for each treatment and applied with a household sprayer. The BTH treatments were done as described in (Canet et al. 2010a). Briefly, plants were treated with either mock or 350 μM BTH four times during two weeks, starting when the plants were one week old. Then, the fresh weight of each genotype was recorded in both treatments and expressed as percentage of the control fresh weight.

Statistical analysis

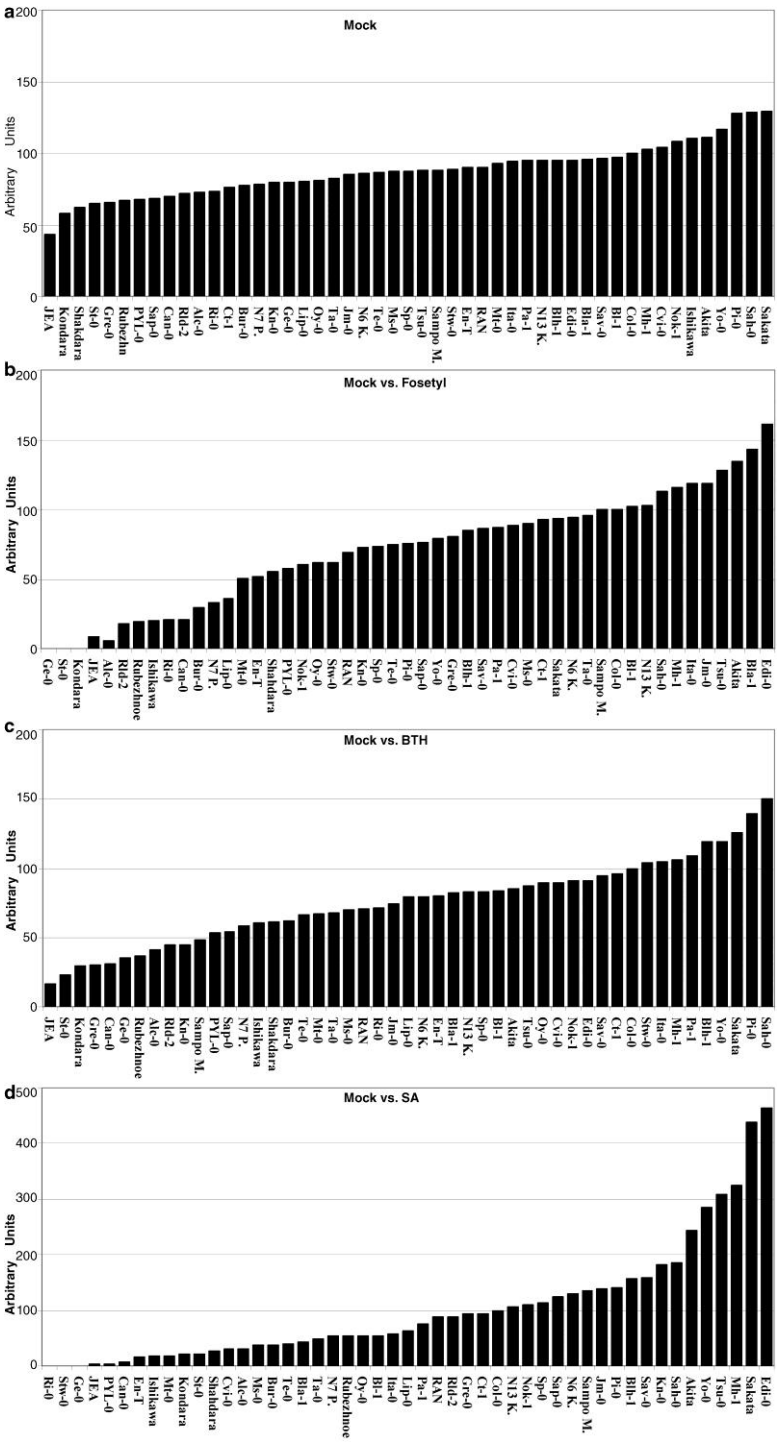
Figure 1 shows the average of three independent experiments to show which treatment is the most informative, and which ecotypes are in the extremes. Due to the number of ecotypes, the error bars are not shown. To compare the 49 ecotypes in a single figure, the value of Col-0 in mock was assigned a value of 100, and the rest of ecotypes were expressed in relation to Col-0. Thus, the value of a given ecotype would be the Log(cfu/plant) of the ecotype divided the Log(cfu/plant) of Col-0, expressed in percentage. To represent the resistance induced by the chemicals, in the last three panels the arbitrary value of 100 assigned to Col-0 represents the resistance induced by the chemicals. Thus, the value of a given ecotype would be the ratio of the (Log(cfu/treated plant) minus Log(cfu/mock plant)) of the ecotype divided the (Log(cfu/treated plant) minus Log(cfu/mock plant)) of Col-0, expressed in percentage. In Figures 4 and 5, a t-student was performed, considering populations of equal variance, a single tailed test, and alpha equals 0.05. For the QTL mapping (Fig. 2, 3 and 7), two independent experiments were done, producing essentially the same result. The rest of experiments are done independently three times, and when all three experiments show the same information, one of them is shown. Each experiment consists in at least three measures, each measure with at least five plants sampled. The average and the standard deviation of the three measures are shown in the figures; two averages are statistically different if the error bars are not overlapping.

Results

Searching for natural variation in SA response among the ecotypes

We are interested in finding the genetic steps involved in salicylic acid (SA) perception. A system was proposed where no pathogen is involved, but this framework does not produce any relevant output in terms of natural variation (Canet et al. 2010a). For this reason, we have looked for the best conditions to maximize the differences among ecotypes by using chemicals that induce resistance through SA perception. As a representation of the natural variation of Arabidopsis, all measures were done in a nuclear core collection which maximizes the variation with a minimal number of genotypes (McKhann et al. 2004). Figure 1 shows the growth of *Pseudomonas syringae pv. tomato DC3000* (*Pto,* a virulent pathogen in most Arabidopsis ecotypes) upon different treatments. Three pots of each ecotype were treated with chemicals that induce resistance through SA perception, namely Fosetyl, (Molina et al. 1998), benzothiadiazole (BTH, an analogue of SA, (Lawton et al. 1996), and SA itself (Nawrath et al. 2005), in addition to a mock-treated one. The four pots were then inoculated with *Pto*, and the logarithmic growth of the pathogen was measured in three independent experiments. In order to compare the 49 ecotypes in a single figure, the value of Col-0 in mock was assigned an arbitrary value of 100, and the rest of ecotypes were expressed in relation to Col-0 (Fig. 1a).

Figure 1. Natural variation among the ecotypes of Arabidopsis in response to induced resistance. Chemicals that induce resistance through salicylic acid (SA) perception were applied to a collection of ecotypes (McKhann et al. 2004). One day later, *Pseudomonas syringae pv. tomato DC3000* (*Pto*) was inoculated and its growth measured three days later in a logarithmic scale. For each genotype and treatment, three samples, with 5 plants per sample were taken. In order to represent in a single figure all the ecotypes tested, the average value of three independent experiments were expressed in arbitrary units relative to Col-0 being 100. **a** Mock treatment. **b** 10 g/L fosetyl. **c** 350 µM Benzothiadiazole (BTH). **d** 100 µM SA. In the case of the chemical treatments (**b-d**), the values correspond to the induced resistance (mock treated minus chemical treated) of each ecotype related to the same value (mock treated minus chemical treated) in Col-0. Rubezhnoe stands for Rubezhnoe-1, N7 P. for N7 Pinguba, N6 K. for N6 Karelian, Sampo M. for Sampo Mountain, and N13 K. for N13 Konchezero.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Edi-0

This way, growth curves done in different days can be compared, since Col-0 was included in all experiments. To represent the resistance induced by the chemicals, in the last three panels the arbitrary value of 100 assigned to Col-0 represents the resistance induced by the chemicals, that is, the growth of *Pto* under mock treatment minus the growth of *Pto* under chemical treatment. Thus, a value of 50 indicates that an ecotype responds to a chemical inducing half of the resistance that Col-0 does. Correspondingly, a value of 200 implies that an ecotype responds twice as strongly as Col-0, again in relative terms. Note that we consider relative induction (mock minus chemical) in order to minimize the variation due to basal resistance and to maximize the variation due to the treatment. Figure 1b represents the response to Fosetyl; Fig. 1c shows the response to BTH, and Fig. 1d the response to SA. It is important to notice that these chemicals are used in concentrations that are not comparable: BTH is used at maximum concentration (350 μM, Lawton et al. 1996) while SA is used at a concentration close to the minimum dose that we can detect (100 μM, see below). From Fig. 1 it is clear that the treatment with 100 μM SA maximizes the natural variation of Arabidopsis with respect to the other treatments. As a consequence, this treatment was used to find and evaluate possible QTLs.

The ecotypes Edi-0 and Stw-0 were chosen as parentals for establishing a mapping population due to their extreme phenotype regarding SA. Other ecotypes that are frequently used in research or that are parentals of RILs were also tested (data not shown). Figure 2a shows the ecotypes that were found to be relevant for the rest of this work (see below). *NPR1* is the only known gene required for SA perception, and so *npr1*-1 is included as a negative control (Dong 2004).

Searching for natural variation in SA response among the mapping populations

The cross between Edi-0 and Stw-0 was done and 266 F3s and the parental ecotypes challenged with *Pto* after the application of a treatment of 100 μM SA or a mock treatment (the measurement is destructive so the F2 is measured through 6 F3 individuals). Similarly, we tested all the RILs available at the beginning of this work (seven, see Materials and methods). The Edi-0 x Stw-0

F2 produced the strongest variation (Fig. 2b, standard deviation of 87 vs. values of 73 to 62 in the rest of RILs of Fig. 2b and 2c).

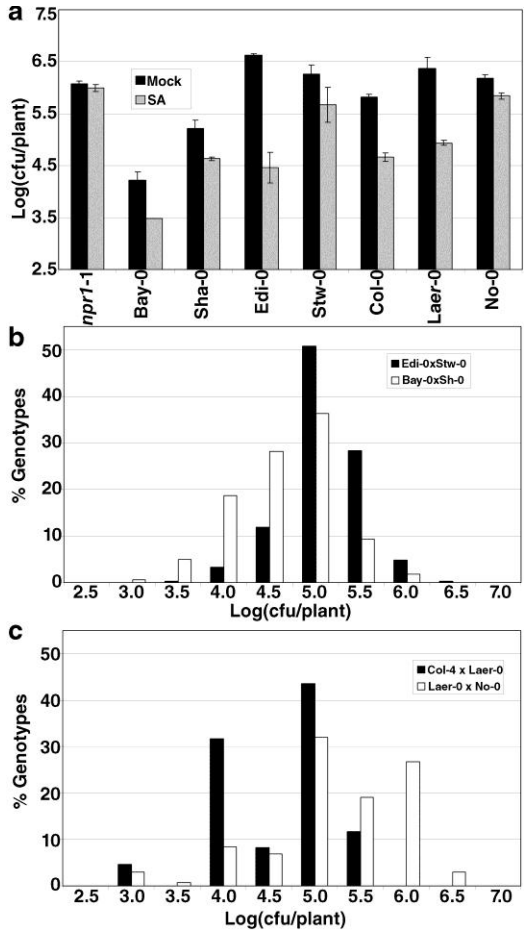


Figure 2. Variation among some ecotypes and their progeny in response to SA. **a** Growth of *Pto* in selected ecotypes after a mock or 100 µM SA treatment, as described in Fig. 1. In the "Y" axis, logarithm of colony forming units per plant. This experiment was done three times with similar results. **b** Frequency distribution of the genotypes tested with SA. F2s from a cross Edi-0 x Stw-0 and RILs from Bay-0 x Sha-0 (Loudet et al. 2002). In the "Y" axis, percentage of genotypes for a given value of Log(cfu/plant), while in the "X" axis, Log(cfu/plant) after SA treatment for a given percentage of genotypes. The number indicates the maximal value (intervals of 0.5 Logs). **c** idem with the RILs Col-4 x La*er*-0 and La*er*-0 x No-0.

It is similar in shape to the one produced by the RIL Bay-0 x Sha-0 (Fig. 2b, Loudet et al. 2002). The distribution is quite different from the ones produced by the RILs Col-4 x L*aer*-0 (Lister and Dean 1993) and L*aer*-0 x No-0 (Magliano et al. 2005, both in Fig. 2c). While the populations in Fig. 2b show a bell-shaped distribution according to several QTLs, Fig. 2c shows two populations with a distribution far from normal, indicating that there are few and/or strong QTLs producing the variation in the phenotype.

In order to find the QTLs that explain this variation, the Edi-0 x Stw-0 F2 was genotyped with 26 markers (see Methods and Supplemental Fig. S1), and a QTL mapping approach was done with WinQTLCart (Wang et al. 2007b). The threshold of the logs of the odds (LOD) was calculated at 0.05 significance with 1,000 permutations (Churchill and Doerge 1994). While there is no significant QTL with the mock treatment, there are two significant QTLs with the SA treatment, SAQ1 and SAQ2 (Fig. 3a). SAQ1 is located between 53.2 and 91.7 cM on Chr I (2 LOD support interval in all QTLs described). It explains 9% of the variation, and the Stw-0 allele has a slightly dominant effect (0.12). On the other hand, SAQ2 is located between 0 and 17.3 cM of Chr IV, explains 8% of the variation, and the Edi-0 allele has a dominant effect (0.19).

Out of the seven RILs tested, only three produced significant QTLs in the response to SA. In the RIL Bay-0 x Sha-0, there is a single QTL that co-localizes with one QTL in mock treatment (Fig. 3b). Since the QTLs in response to *Pto* in this population have been already reported (Perchepied et al. 2006) and the QTL of SA response is likely to be one of them, we did not pursue it. The RILs Col-4 x L*aer*-0 (Fig. 3c) and L*aer*-0 x No-0 (Fig. 3d) have one single QTL with the SA treatment: SAQ3 and SAQ4, respectively. SAQ3 is seated between 14.8 and 22 cM of Chr. III and explains 18% of the variation produced by the response to SA, while SAQ4 is between 66.6 and 89.4 cM of Chr V and explains 15% of the variation. Regarding Col-4, there is no difference in SA perception with respect to Col-0 (data not shown), and that is the reason why Col-0 is shown in Fig. 2a.

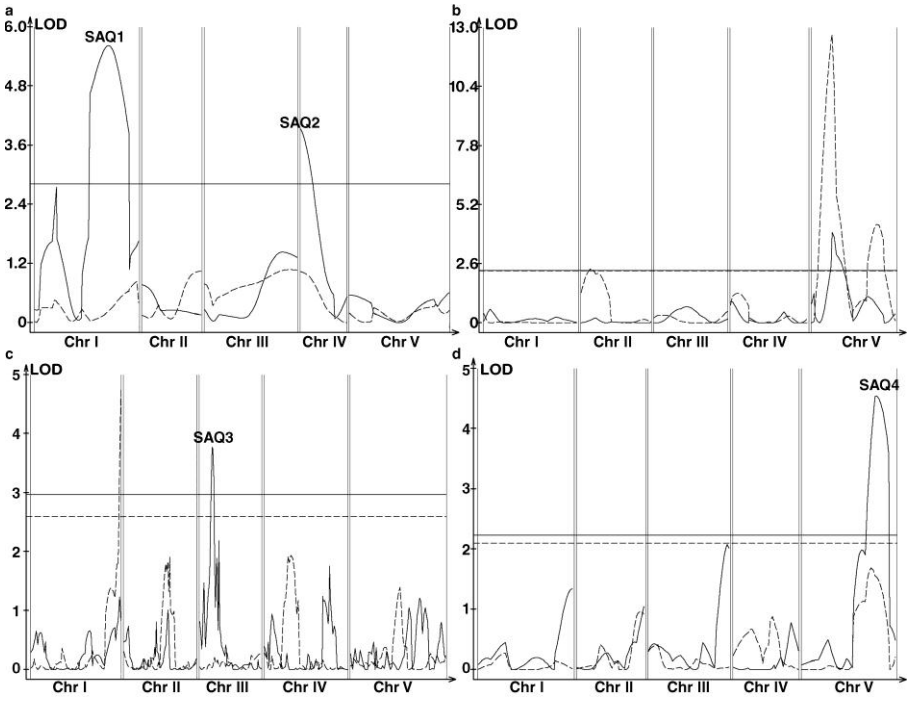


Figure 3. QTLs among some ecotypes in response to mock and SA pretreatment and *Pto* growth. Plants were treated with either mock or 100 µM SA, as described in Fig. 1. The output shown is the likelihood of a QTL (in logarithm of odds; LOD, in the Y axis) in a particular region of the genome (X axis). The horizontal line shows the threshold of significance. The continuous lines show the QTLs for SA treatment, and the dotted line the QTLs for mock treatment. The Chromosomes of Arabidopsis are delimited by vertical lines and named with roman numbers. The populations analyzed were **a** F3s of Edi-0 x Stw-0. **b** RILs of Bay-0 x Sha-0. **c** RILs of Col-4 x La*er*-0. **d** RILs of La*er*-0 x No-0.

Establishing near isogenic lines

These four QTLs pinpoint four different regions of the genome, so we continued with the characterization of all of them. For Edi-0 x Stw-0, a RIL is being established, (note that each generation requires six weeks of vernalization). For Col-4 x L*aer*-0, we took advantage of the Stepped Aligned Inbred Recombinant Strains (STAIRs) lines (Koumproglou et al. 2002) already constructed between Col-0 and L*aer*-0. After a careful selection, the result is a

collection of lines that form a stepwise introgression of L*aer*-0 in Col-0. The lines N9459 and N9464 (hereafter P59 and P64, respectively) were chosen, since they diverge in a small region that includes SAQ3 but share most of the genome (see Supplemental Fig. S2). Therefore, comparing these two lines implies "mendelizing" SAQ3 and there is no need of the original parents.

For L*aer*-0 x No-0, a conventional construction of NILs was carried out. The RIL174 was selected due to its content in No-0 (see Supplemental Fig. S3) and crossed with No-0, and the F1 was crossed again with No-0. Out of the resulting F2, the NIL N15 was selected, since it is heterozygous for SAQ4, but No-0 for most of the genome. Out of the progeny of N15, the line N15.15 was selected, which is homozygous L*aer*-0 for SAQ4 but No-0 for most of the genome. On the other hand, the RIL132 was also selected (this time due to its content in L*aer*-0, see Supplemental Fig. S3) and crossed with L*aer*-0. Out of the resulting F2, the NIL N297 was kept, since it is heterozygous for SAQ4. From its progeny, the line N297.46 was selected (L*aer*-0 in all the genome, except No-0 for the region containing SAQ4). Thus, by comparing No-0 vs. N15.15 and L*aer*-0 vs. N297.46, we reduce the variation to the region containing SAQ4 (see Supplemental Fig. S3), and the characterization and mapping of the QTL is more accurate. By selecting N15 and N297 and analyzing their progeny, we have effectively constructed two mapping populations, thus mendelizing SAQ4.

Characterization of the QTLs in SA perception

Once all the corresponding NILs had been established, we sought to confirm the QTLs by checking the different behaviour between the NILs. Edi-0 and Stw-0 were included in these analyses, even though if they are not NILs, to document the difference between genotypes. In the case of L*aer*-0 x No-0, we kept the two sets of introgression lines (L*aer*-0 vs. N297.46 and No-0 vs. N15.15).

The first thing was to confirm if the NILs have indeed a measurable difference with respect to SA. Treatments with less than 50 μM of SA are not detectable in our hands, and even this concentration sometimes does not induce resistance in Col-0 (data not shown). When more than 500 μM of SA was applied, we got experiments with a strong resistance, but with some plants suffering from the phytotoxicity of SA (data not shown). Therefore, several concentrations of SA

(from 50 μM to 500 μM), along with a mock treatment, were applied to plants before a *Pto* inoculation (Fig. 4a). The amount of growth in *Pto* reflects the response to SA. Edi-0 vs. Stw-0 and No-0 vs. N15.15 respond differentially to all four concentrations, L*aer*-0 vs. N297.46 to three, and P59 and P64 to two concentrations. In all cases there is a differential behaviour with 100 μM, which is the concentration used for the mapping population, thus validating the QTLs. A similar experiment with plants grown in media containing SA did not produce any visible difference between the genotypes (data not shown).

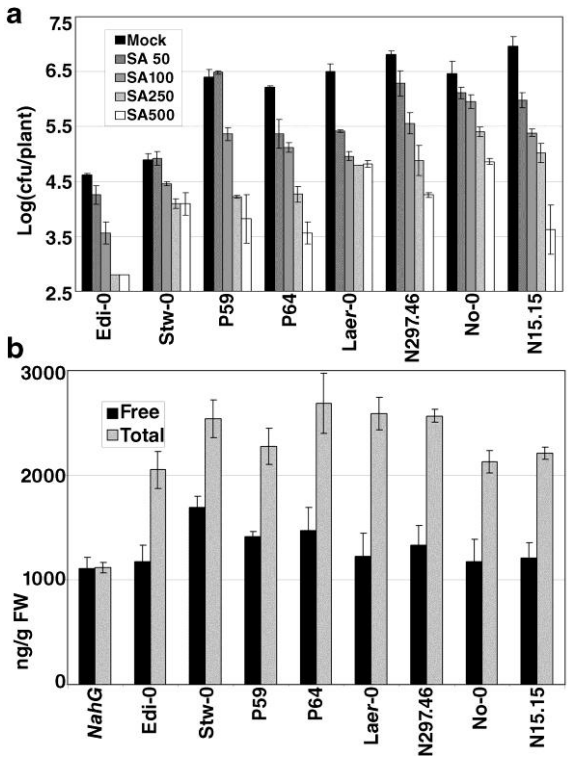


Figure 4. Differential response to SA in selected genotypes. a Growth of *Pto* in some genotypes after a mock or different SA treatments (50, 100, 250 and 500 μM), as described in Fig. 1. **b** Accumulation of SA after 100 μM SA treatment. Plants were treated as in Fig. 1, and one day after the treatment, their concentration of SA, both "Free" and "Total" was measured. *NahG* is included as a control, taking into account the SA that remains on the surface of the plant. There is a significant difference between Edi-0 and Stw-0 in both free and total SA, but not between the rests of pairs (t-student, $P < 0.05$). These experiments were done three times with similar results.

Once the QTLs had been confirmed, the next step was to characterize their role in SA perception. The QTLs could make a difference in any number of the multiple steps required for SA to produce a measurable effect. We reasoned that the QTLs could be mechanistically located in the penetration, stability, accumulation or degradation of the exogenous SA, in short, in any step that could affect the amount of SA that reaches the cell. There is no difference in SA concentration in mock conditions (data not shown), and Fig. 4b shows SA contents measured one day after spraying the plants with 100 μM SA. An important part of SA is stored as glucoside (Nawrath et al. 2005), and therefore both free and total SA (free plus glucoside conjugated) were measured. *NahG* was included as a control, since *NahG* plants degrade the cytosolic SA (Lawton et al. 1995) and therefore its presence takes into account the SA that remains in the outside of the cell (Niederl et al. 1998), thus establishing a basal line. The result of Fig. 4b is that in terms of SA concentration one day after the SA treatment, there are no differences between the genotypes that define SAQ4. There is a small (but not significant) difference in the genotypes of SAQ3, and an unexpected difference in Edi-0 vs. Stw-0. In this pair, Edi-0 has less SA (both free and total) than Stw-0 at the time of measuring, and yet Edi-0 shows a stronger response to SA in terms of stopping the pathogen. Therefore, none of the QTLs present a strong difference in the accumulation of SA that could explain the difference in the growth of the pathogen.

An important question in SA perception is the difference between SA and benzothiadiazole (BTH). BTH is a chemical analogue of SA, its effect *in planta* being stronger than that of SA and without its phytotoxicity. Fig. 5a shows the result of the application of 35 μM BTH to plants before a *Pto* inoculation. The concentration used is 35 μM because it increases the window of detection for small changes between close genotypes, while lower amounts of BTH do not produce robust results (data not shown). While Edi-0 vs. Stw-0 do not show a different behaviour, the other three comparisons show a considerable difference in response to BTH. There is an alternative way of assessing the response to BTH: by measuring the difference in plant fresh weight after several treatments (Canet et al. 2010a). When this alternative measurement of response was used, the differences were reduced in SAQ3 and SAQ4. In the case of Edi-0 vs. Stw-0, the response is even opposite to SA. Therefore, Edi-0 vs. Stw-0 discriminate SA from BTH, and SAQ3 and SAQ4 do not discriminate between these two analogues. Regarding this way of measuring SA perception,

note that the BTH treatments used do not produce macroscopic cell death that could affect the fresh weight measurements (Canet et al. 2010a).

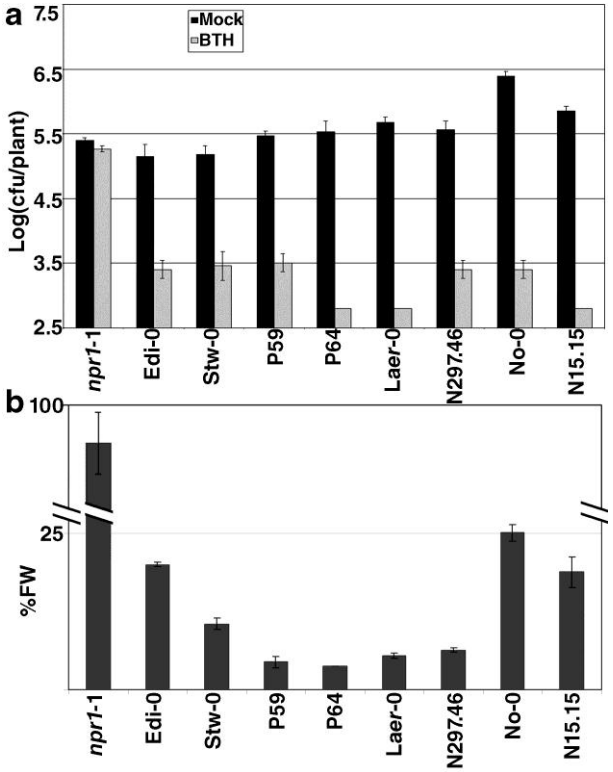


Figure 5. Differential response to BTH in selected genotypes. a Growth of *Pto* in some genotypes as described in Fig. 1, after a mock or 35 µM BTH treatment. In the BTH treatment, there is no significant difference between Edi-0 and Stw-0, while it is significant in each of the other pairs (t-student, $P < 0.05$). **b** Plants were treated with either mock or 350 µM BTH four times, their fresh weight (FW) recorded, and the ratio between the BTH and mock treated plants represented (average and SD of 15 plants in three groups of five). This experiment was done three times with similar results.

SA has a negative crosstalk with several hormones, among them MeJA (Genoud and Metraux 1999). If genotypes that have different SA perception show the opposite pattern for MeJA, it would imply that the QTL(s) are located in steps previous to (or even in) the point of crosstalk. Fig. 6 shows the result of treating plants with 100 μM MeJA prior to an inoculation with *Pto*. The differential response of the genotypes is significant (and opposite to the behaviour with SA) in SAQ3, but not in the rest of comparisons. In the case of Edi-0 vs. Stw-0 there is no difference between mock and treated plants, but in SAQ4 there is clear indication that the same trend seen with SA occurs with MeJA.

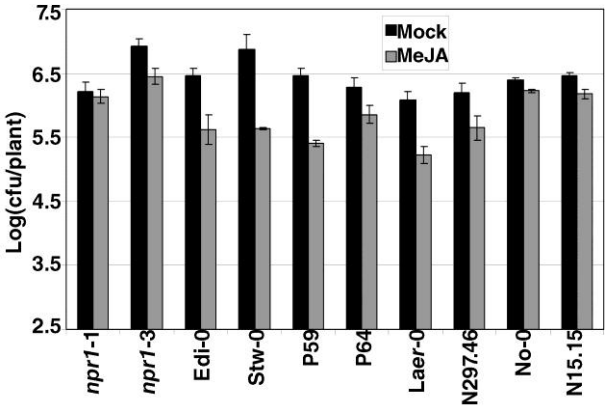


Figure 6. Differential response to methyl jasmonate in selected genotypes. Growth of *Pto* in some genotypes after a mock or 100 μM methyl jasmonate (abbreviated as "MeJA") treatment as described in Fig. 1. This experiment was done three times with similar results.

Fine mapping of the QTLs

The QTLs SAQ3 and SAQ4 were confirmed and further delimited with the creation of *ad hoc* populations. In the case of Col-0 vs. L*a*er-0, an F2 population between P59 x P64 was created and genotyped. Out of 288 plants, 11 were recombinants in the interval (see Supplemental Fig. S2). When SAQ3 was mapped with these lines, the actual position was shifted towards the telomere, between 8.1 and 12.2 cM (Fig. 7a), with the L*a*er-0 allele being dominant over Col-0.

In the case of L*aer*-0 vs. No-0, two mapping populations were constructed. Out of the progeny of NIL15 (heterozygous for the interval and No-0 for the rest of the genome, see Supplemental Fig. S3), 96 plants were genotyped and 35 selected due to their informative genotype. From the progeny of NIL297 (heterozygous for the interval and L*aer*-0 for the rest of the genome, see Supplemental Fig. S3), 96 plants were also genotyped, and 42 selected due to their informative genotype. When the descendants of NIL15 were analyzed, the previous QTL was divided into several (Fig. 7b, continuous line). The main QTL was also shifted towards the telomere, between 35 and 42.2 cM. The mapping of the descendants of NIL297 produced a QTL that overlaps with the main QTL produced by NIL15 (between 35 and 45 cM, Fig. 7b, discontinuous line), with the L*aer*-0 allele being dominant.

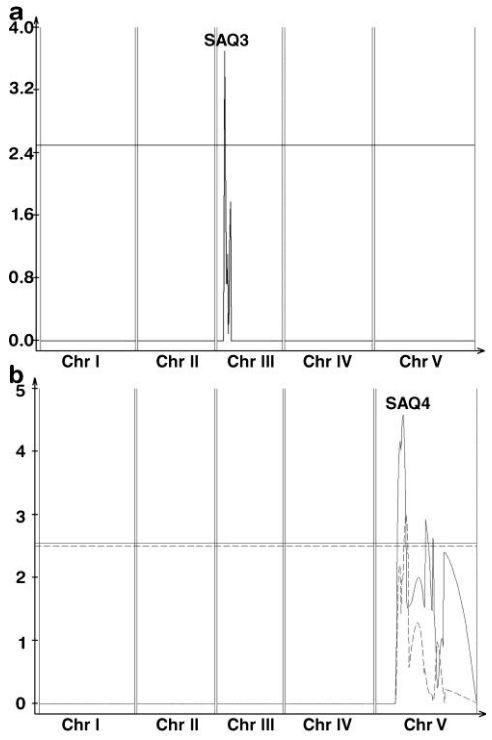


Figure 7. Refined QTLs mapping. a Col-0 x La*er*-0; eleven recombinants were selected from an F2 originated by P59 x P64. **b** La*er*-0 x No-0; 35 recombinants were selected from the line N15, heterozygous for the QTL (continuous line) and 42 recombinants from the line N297 (dotted line). In both cases, the plants were genotyped with markers described in Supplemental Fig. S2, and their QTLs mapped as described in Fig. 3 with 100 µM SA and *Pto*.

Discussion

Finding the best system for SA perception in natural variation

Since our goal is to describe SA perception by using different approaches, we have tried to explore the underused natural variation of Arabidopsis. There are works that describe differences between ecotypes in their response to *Pto* (e.g. Perchepied et al. 2006; Fan et al. 2008) corresponding to our mock inoculations (Fig. 1a). Other reports deal with SA, but from the perspective of either its relationship with MeJA (Genoud and Metraux 1999) or the complex transcriptomic networks that differ from ecotype to ecotype (Genoud and Metraux 1999). A simplified system to explore the artificial variation (mutagenized populations) of Arabidopsis has been proposed, but it does not reveal any relevant difference in the RILs tested (Canet et al. 2010a). Since the simplified system did not work for natural variation, we searched for chemicals that trigger resistance against *Pto* and worked through SA perception. As shown in Fig. 1, the best option to maximize variation is 100 μM of SA itself, at least out of the doses and treatments used. Thus, SA is used in a concentration close to the minimum that we can robustly detect (Fig. 4a). It is interesting to note that the LOD score obtained (Fig. 3) was lower in comparison with other biological systems (e.g. flowering, Alonso-Blanco et al. 1998b or Werner et al. 2005). Our low LODs could be caused by the strong variation that the pathogen produces, even if its growth is measured in logarithmic scale. Note that we did not use Systemic Acquired Resistance (SAR) as a system (that is, one first inoculation that induces defence and a second one to measure the resistance, Vlot et al. 2008a). Though this setup would have been likely to produce a strong variation among ecotypes, its application would have been difficult with more than a handful of genotypes at the same time.

Edi-0 versus Stw-0

The SA treatment defines the ecotypes that are most diverse. Out of them, Edi-0 and Stw-0 were chosen. These ecotypes were selected because they share the same level of basal resistance (mock treatment in Fig. 2a) and are quite different in their response to SA. The result is that the population defines two QTLs which are specific for SA response, SAQ1 and SAQ2 (Fig. 3a). The opposite effect is observed in Bay-0 vs. Sha-0. These two ecotypes differ approximately

in the same degree in the basal and SA-triggered resistance (Fig. 2a). Logically, the QTLs found are not specific of SA response, but in basal resistance (Fig. 3b).

The characterization of the F3s from the cross Edi-0 x Stw-0 unveiled a strong variation in the response to SA, even stronger than that of other mapping populations with only homozygous alleles due to their F8 state (Fig. 2b and c), so the variation is likely to increase when this population is taken through several generations (Alonso-Blanco et al. 2006).

The difference between ecotypes is maintained through all the concentrations of SA tested (Fig. 4a), so the differences are quite robust. When the concentration of SA is measured after an exogenous application, there is less SA in Edi-0 (Fig. 4b) even if it is the one that responds more to SA. This apparent contradiction could be explained by the fact that metabolism of SA is triggered by its perception. For example, the expression of *NPR1* is induced by SA and *npr1* alleles accumulate more SA, presumably because they are unable to trigger its degradation (Cao et al. 1997). In any case, the differences in SA accumulation do not explain any of the differences found between genotypes, so the four QTLs described herein are not due to differential intake or stability of SA (see below for a detailed account of SAQ3). Then, the logical conclusion is that the QTLs are located at some point between SA perception and the execution of the resistance.

The response to MeJA in Edi-0 vs. Stw-0 (Fig. 6) does not help to clarify the situation of the QTLs in the SA signal transduction. It could be due to differences among ecotypes besides the QTLs; while in other cases the comparisons are between NILs that share 90-94% of the genome (Supplemental Fig. S2 and S3), these are two wild type ecotypes that are quite divergent (McKhann et al. 2004). Therefore, the precise assessment of the differences in the MeJA signal transduction regarding SAQ1 and SAQ2 is postponed until a RIL is developed.

SA has several analogues that trigger resistance in plants, such as 2,6-dichloroisonicotinic acid (Uknes et al. 1992) and BTH (Lawton et al. 1996). We reasoned that it would be informative to compare the different genotypes with BTH (Fig. 5), and so it is in the case of Edi-0 vs. Stw-0: Fig. 5a shows that there

is no significant difference in the resistance triggered by BTH. It is possible that BTH triggers too much resistance and we are not able to detect the differences. But the reduction of plant fresh weight triggered by BTH (Fig. 5b) shows that Stw-0 perceives BTH better than Edi-0, while Edi-0 perceives SA better than Stw-0 (Fig. 2a). There is only one precedent to this discrimination between these two chemicals, since the transgenic *NahG* degrades SA (and therefore does not react to it) but not BTH (Lawton et al. 1996). Since the difference between Edi-0 and Stw-0 is not in the intake or metabolism of SA, and it differentiates between two close chemicals, the simplest explanation is that the difference between ecotypes is in the receptor of SA itself.

There is no *bona fide* receptor for SA yet, but NPR1 is the strongest candidate. Mutations in NPR1 disrupt SA perception and none of the reported alleles differentiate between SA and BTH (Canet et al. 2010b). From the two QTLs found in the population Edi-0 x Stw-0, SAQ1 maps to a region of Chromosome I that includes *NPR1*. NPR1 presents a fair amount of polymorphisms, with 4 protein variants in the 96 ecotypes studied, while the average of defence response is 2.78 out of 96 ecotypes, and the background of the genome is 5.38 (Bakker et al. 2008). Therefore, it is plausible that polymorphisms in NPR1 are responsible for SAQ1. Nevertheless, there are other 51 genes labelled with the keywords "defence", "salicylic", or "systemic acquired resistance" in the mapping interval defined by SAQ1, and while 37 of them are resistance genes (TAIR, www.arabidopsis.org), unlikely to be responsible for SAQ1, there are other plausible candidates.

Among the genes that could be responsible for SAQ2, there are nine labelled with the keywords previously mentioned. Among them, the most interesting one is NIMIN1b (At4G01895). NIMIN proteins interact with NPR1 *in vitro* and *in planta* (Weigel et al. 2001), and overexpression of *NIMIN1* phenocopies *npr1* plants (Weigel et al. 2005). Therefore, a model has been proposed where NIMIN proteins are repressors of NPR1 activity (Weigel et al. 2005). Then, a simple and elegant model could be true, where the two proteins that produce SAQ1 and SAQ2, namely NPR1 and NIMIN1-like, would interact biochemically.

Col-0 versus La*er*-0

The RIL Col-4 x L*aer*-0 was one of the first mapping populations available (Lister and Dean 1993) and it has been genotyped with a large set of markers (Alonso-Blanco et al. 1998a). Note that, regarding the response to SA, we found no differences between Col-0 and Col-4 (data not shown). Although the difference between Col-0 and L*aer*-0 in response to SA is not as strong as in other cases (Fig. 2a), the fact that these two ecotypes are the most used ones in Arabidopsis research granted a closer look. There is a strong transgression in the RIL population (Fig. 2c) which does not fit a normal distribution. This could be indicative of a small number of QTLs and indeed the result of mapping the population is a single QTL in Chromosome III (Fig. 3c). Taking advantage of the great work done with the STAIRs lines (Koumproglou et al. 2002), we were able to validate SAQ3 with newly generated lines from a different population, thus reducing the mapping interval (Fig. 7a).

Among the genes in this interval, there are no clear candidates labelled with the aforementioned keywords, and while there are QTLs of glucosinolates in response to SA described in the RIL Col-4 x L*aer*-0 (Kliebenstein et al. 2002), none of them are close to SAQ3.

SAQ3 shows the negative interaction between SA and MeJA (Fig. 6), which indicates that the QTL is located before the point of crosstalk. This role has been proposed to be fulfilled by NPR1 or WRKY70 (Spoel et al. 2003 and Li et al. 2004, respectively), so SAQ3 would be relevant in the steps previous to these proteins. We interpret that the differential responses to MeJA proves that SAQ3 does not only respond to exogenous SA, but also to endogenous, physiological levels of SA. In this line of argument, SAQ3 is quite dependent on the SA dose used (Fig. 4a). The other pairs of genotypes differ at almost all the SA doses used, but P59 and P64 show the greatest difference at SA 50 μM, some difference at 100 μM, and no difference at the other two concentrations. This result suggests a process with two affinities. Thus, SAQ3 would be a quantitative trait gene with high affinity for SA in a process with another gene(s) with low affinity for SA. Regarding SA, such systems have been proposed in the influx and efflux SA carriers (Chen et al. 2001), although the measures of SA in Fig. 4b contradict this possibility. Perhaps further experiments in more advanced introgression lines could confirm the tendency

of P59 having less total SA than P64. If this result were to be true, it would add more weight to the hypothesis of SAQ3 being an influx or efflux high affinity carrier. Alternatively, there could be a different process with two affinities that could explain the small difference in total SA but not that in free SA provoked by SAQ3. SAQ3 is detectable with high doses of BTH (Fig. 5). This fact could be due to the stability of BTH (Lawton et al. 1996), whereas SA is more readily metabolized, conjugated, or specifically transported (Nawrath et al. 2005).

La*er*-0 versus No-0

The ecotypes La*er*-0 and No-0 are strongly different in their response to SA (Fig. 2a), and, like in the previous Col-4 x La*er*-0 RIL, the mapping population is not normally distributed (Fig. 2c). And as before, there is a single QTL, SAQ4 (Fig. 3d). In this case, there are no NILs available, so we constructed the corresponding lines. Two different lines were pursued, in order to test the effect of SAQ4 both in La*er*-0 and No-0 background. The mapping population derived from N15 (heterozygous for SAQ4, No-0 in most of the genome) reveals a clear, strong QTL that confirms SAQ4 and other small QTLs (continuous line in Fig. 7b). On the other hand, the mapping population derived from N297 confirms the position of the main QTL but it is not as strong as the population derived from N15 (discontinuous line in Fig. 7b). An explanation could be that the background La*er*-0 is more responsive to SA even when SAQ4 is segregating (Fig. 4a and 5a), and this heightened response could partially mask the difference produced by the QTL. Thus, the difference between La*er*-0 and N297.46 is smaller than that between No-0 and N15.15 in several experiments (Fig. 4a and 5b). In the mapping interval defined in Fig. 7b, there is, among other candidates, a calmodulin-binding protein (CBP60g; At5G26920), a gene reported to participate in SA signalling (Wang et al. 2009).

SAQ4 is quite robust and is detectable under all the SA concentrations used (Fig. 4a). Although the pairs No-0 vs. N15.15 and La*er*-0 vs. N297.46 show significant differences, SAQ4 does not explain most of the difference in weight found between the ecotypes when BTH is applied (Fig. 5b, difference between La*er*-0 and No-0). This fact agrees with our previous report that no single QTL is significant in this population when the effect of the BTH on the weight of the plant is considered (Canet et al. 2010a).

The resistance triggered by MeJA in SAQ4 does not show negative crosstalk with SA (Fig. 6). The simplest explanation is that SAQ4 is located after NPR1 and/or WRKY70, since these are the candidates of this hormone interaction. But a closer look to Fig. 6 shows that the genomic region that includes SAQ4 reacts to MeJA as it does to SA. If indeed SAQ4 is responsible for both responses, it could imply that SAQ4 is at the last steps of the SA signal transduction, the execution of the resistance. The reason is that both signals, although antagonistic, reduce the growth of *Pto*. Therefore, it is plausible that the same effect is produced by the same genes, and hence that SAQ4 is located among those same genes.

Conclusions

To summarize (Fig. 8), the results of the genotypes and the candidate genes in the intervals aforementioned lead us to speculate that none of the QTLs are affecting SA stability or accumulation after exogenous application. SAQ3 could be a QTL in a high affinity process (like SA carriers or conjugation enzyme), while SAQ1 and SAQ2 could be polymorphisms in NPR1 and NIMIN1-like genes that would result in a difference in SA perception but not in BTH perception. Then, SAQ4 would be a polymorphism in the execution part of the defence. For example, a calmodulin binding protein that would sense the changes in cytosolic Ca^{2+} produced by the pathogen, integrating this information with the SA and MeJA signals. Of course these genes are named according to their homology or description, but there are alternatives both known and unknown for the mentioned genes. For example, there are receptor-like proteins in the intervals of three of the QTLs which could be the genes responsible of the observed differences.

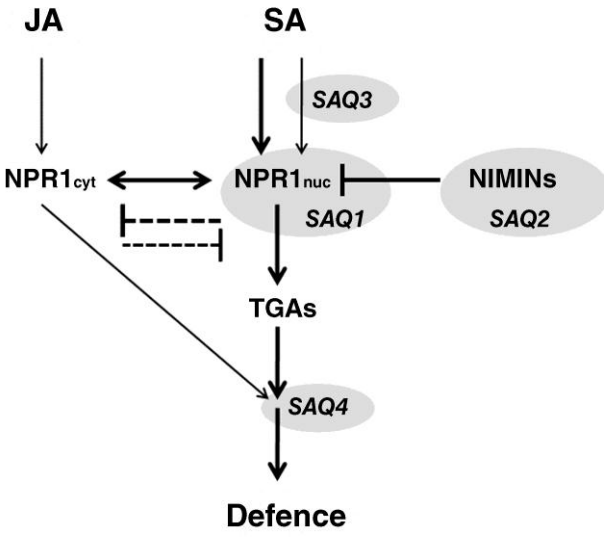


Figure 8. A putative model of the QTLs described in this work. SA is perceived by NPR1 in its nuclear localization. NIMINs proteins interact with NPR1, repressing its activity. TGAs also interact with NPR1, inducing the expression of genes that eventually will have an impact in the growth of *Pto*. There is a negative crosstalk between SA and methyl jasmonate, and this hormone also requires NPR1 (but in its cytosolic form) to trigger a small resistance against *Pto*. The ellipses point to the positions that may correspond to the QTLs described, on the basis of the previous experiments and the genes in the mapping intervals. See the text for references of the mentioned genes.

The defined QTLs are valuable in themselves and will also help to complement other approaches, such as the search for mutants lacking a response to SA (Canet et al. 2010b), or transcriptomics descriptions of the response to biotic stress (Bilgin et al. 2010).

Acknowledgments

This work was supported by the Ministerio de Ciencia e Innovación (MICINN) of Spain (grant BIO201018896 to PT, a JAE-CSIC Fellowship to JVC and a FPI-MICINN to AD). We appreciate the BTH provided by Syngenta and the genotyping by CEGEN (Fundación Genoma España).

Supporting information

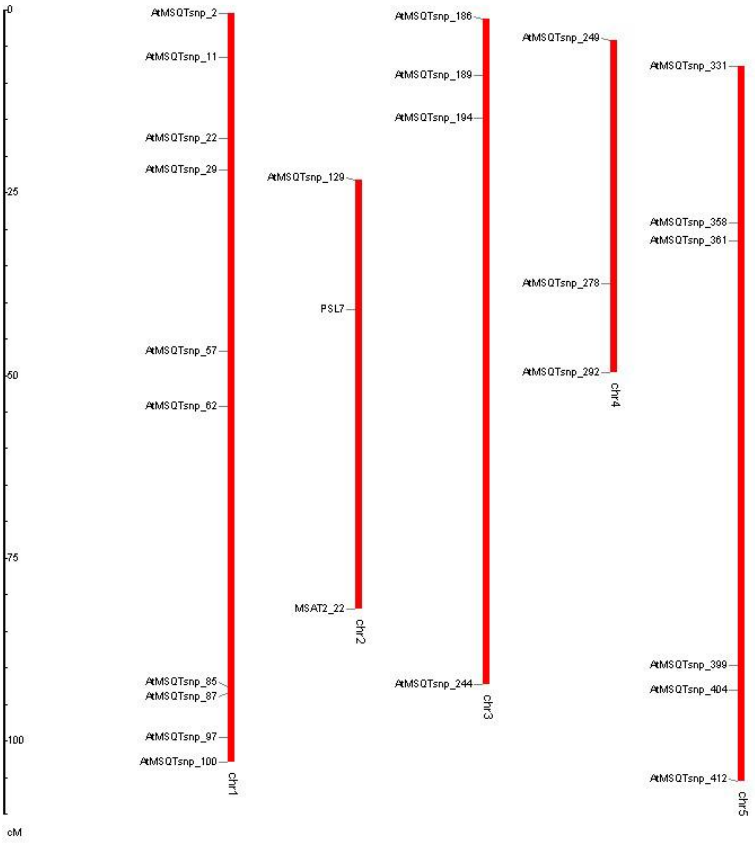


Figure S1. Markers used in the F2 between Edi-0 and Stw-0. The markers described in www.naturalvariation.org as polymorphic for Edi-0 vs. Stw-0 were used to genotype with iPLEX® in the CEGEN (Spanish National Genotyping Centre, www.cegen.org). Two additional SSLP markers were added to complete the chromosome II. These figures were done with the program GGT 2.0 (van Berloo 2008).

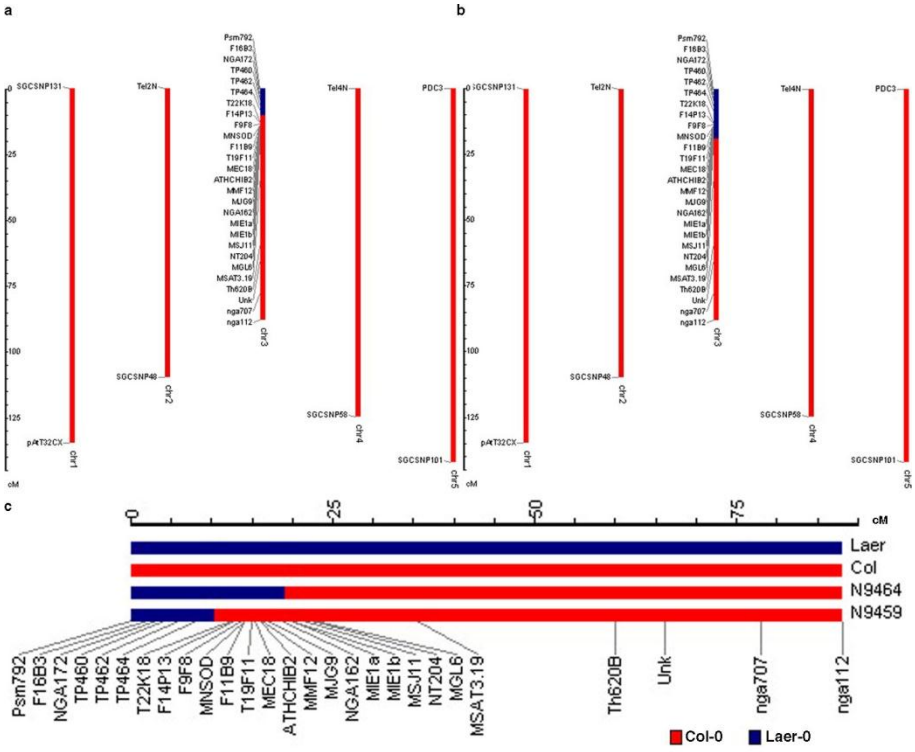
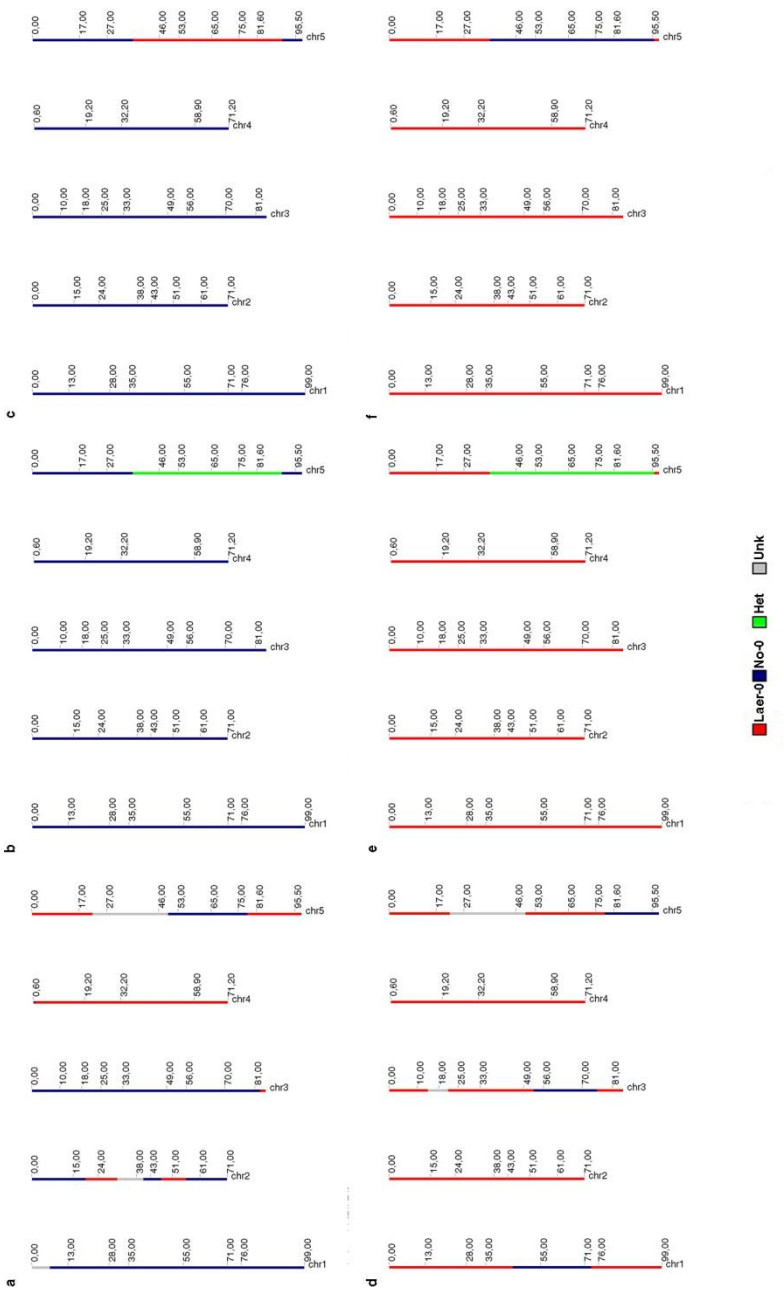


Figure S2. STAIRs lines used in this work. The following STAIRs (Koumproglou et al. 2002) lines were genotyped with the indicated markers: a N9459 (abbreviated in the main text as P59) and b N9464 (P64 in the main text). c detail of Chromosome III in both lines, along Col-0 and L*aer*-0. Note that our result differs slightly from what is available at www.arabidopsis.info.

Figure S3. RILs of La*er*-0 x No-0 selected and NIL generation. From the RIL L*aer*-0 x No-0 (Magliano et al. 2005), the RIL 174 (a) was crossed with No-0 twice, and from that population, the NIL N15 (b) was selected. From the progeny of N15, the NIL N15.15 (c) was selected. In the other hand, the RIL 132 (d) was crossed with L*aer*-0, and from that population, the NIL N297 (e) was selected. From the progeny of N297, the NIL N297.46 (f) was selected.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