UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Departamento de Biotecnología

"Función de OCP3 en la resistencia de Arabidopsis frente a hongos necrotrofos"

MEMORIA DE TESIS PRESENTADA POR

Javier García-Andrade Serrano

PARA OPTAR AL GRADO DE DOCTOR POR LA
UNIVERSIDAD POLITÉCNICA DE VALENCIA

DIRECTOR

Prof. Pablo Vera Vera

Valencia, Febrero 2016

El **Dr. Pablo Vera Vera**, Profesor de Investigación del CSIC, perteneciente al Instituto de Biología Molecular y Celular de Plantas (IBMCP, CSIC-Universidad Politécnica de Valencia) de Valencia.

CERTIFICA:

que el Ingeniero Agrónomo **Javier García-Andrade Serrano** ha realizado bajo su dirección en el Instituto de Biología Molecular y Celular de Plantas el trabajo que lleva por título “Función de OCP3 en la resistencia de Arabidopsis frente a hongos necrotrofos”, y autoriza su presentación para optar al grado de Doctor.

Y para que así conste, expido y firmo el presente certificado en Valencia, a 15 de Enero de 2016.

Dr. Pablo Vera Vera

A mi familia y a Fer

ÍNDICE

RESUMEN

Las plantas mutantes *ocp3* presentan una acelerada e intensificada deposición de calosa en respuesta a la infección producida por *Botrytis cinerea* o *Plectosphaerella cucumerina* así como una mayor resistencia. Tras el análisis fenotípico de una serie de dobles mutantes en el fondo *ocp3* observamos que tanto el incremento en la deposición de calosa como de la resistencia requieren de la calosa sintasa PMR4 y de la hormona ácido abscísico (ABA). En las plantas silvestres, tras la infección por alguno de estos hongos necrotrofos, se incrementa la síntesis de ABA, incremento éste que aún es mayor en las plantas *ocp3*. La mayor resistencia que se observa en las plantas *ocp3* también requiere de la correcta percepción del ácido jasmónico (JA) a través del receptor COI1. Sin embargo, el JA no es requerido para la síntesis y deposición de calosa basal. De esta forma se podría proponer un modelo en el que OCP3 ejerce un control específico para la deposición de calosa regulada por JA y que requiere, indispensablemente, de ABA.

La proteína OCP3, por homología de secuencia, se clasifica como un factor de transcripción nuclear miembro de la familia de los Homeobox. Sin embargo, encontramos que OCP3 se importa y acumula en los cloroplastos, y se colocaliza con proteínas que contienen motivos de repetición pentatricopeptidos e implicadas en la edición de RNA. Concretamente, OCP3 participa regulando el mecanismo de edición del transcrito *ndhB* que codifica una de las subunidades del complejo multiproteico NADPH deshidrogenasa (NDH) del cloroplasto. La ausencia de OCP3 se traduce en una deficiente edición de *ndhB;* hecho éste que conlleva un deterioro en la funcionalidad del complejo NDH y que por tanto acarrea un defecto en el flujo cíclico de electrones (CEF) alrededor del fotosistema I (PSI). También pudimos confirmar que mutantes que presentan alteraciones en la actividad del complejo NDH, como *crr2* y *crr21*, a su vez presentan un incremento en la resistencia frente a la infección por *P. cucumerina*, además de mostrar una mayor deposición de calosa. Por otra parte, observamos que la edición de los mRNA para otras subunidades del complejo NDH codificadas en el cloroplasto están, también, sujetas a una regulación

que se ve notablemente alterada en presencia de un estimulo patogénico. Al mismo tiempo, la estabilidad del complejo NDH también se ve comprometida tras un insulto patogénico. Todos estos resultados indicarían que el complejo NDH está sujeto a una sutil regulación en concordancia con las condiciones cambiantes del entorno.

El ABA es determinante en la activación de mecanismos de defensa frente a *P. cucumerina*. Por otra parte, patógenos hemibiotrofos como por ejemplo *Pseudomonas syringae* DC3000 también provocan incremento en la síntesis de ABA. Sin embargo, este incremento acarrea la supresión de las respuestas defensivas frente a estos patógenos, por lo que se le puede atribuir un papel dual. La regulación del papel dual de esta hormona, activador de unas defensas y represor de otras, indica la existencia de una regulación posterior a la síntesis de la hormona. En la presente Tesis Doctoral, se ha identificado a PYR1 como el miembro de la familia de los receptores de ABA PYR/PYL/RCAR que percibe esta hormona para desencadenar una respuesta a la infección por un patógeno. PYR1 amortigua la respuesta dependiente de ácido salicílico (SA) frente al ataque de un patógeno biotrofo. De esta forma ejerce un control positivo sobre las respuestas dependientes de JA requeridas para activar las defensas frente a patógenos necrotrofos. La pérdida de la percepción del ABA por PYR1 provoca una activación de los genes de respuesta a SA, una alteración de la remodelación de la cromatina y un incremento en la activación de las MAP quinasas tras el ataque de patógenos biotrofos. De esta forma, el ABA a través del receptor PYR1 y a través de un control epigenético, tendría un papel regulador de la inmunidad vegetal en función del estilo de vida del patógeno que infecte a la planta.

RESUM

Les plantes mutants *ocp3*, presenten una accelerada e intensificada deposició de calosa en resposta a la la infecció produïda per *Botrytis cinerea* o *Plectosphaerella cucumerina.* Darrere l'anàlisi d'una sèrie de dobles mutants amb *ocp3*, observem que tant l'increment en la deposició de calosa com de la resistència requereixen de la calosa sintasa PMR4, i de l'hormona àcid abscisic (ABA). Les plantes silvestres infectades amb algú d'aquests fongs necrotrofs, incrementen la síntesi de ABA, increment aquest que encara és major en les plantes *ocp3*. La major resitència que s'observa en les plantes *ocp3* també requereix de la correcta percepció de l'àcid jasmonic (JA) a través del receptor COI1. No obstant això, el JA no és requerit per a la síntesi i deposició de calosa basal. D'aquesta forma, es podria proposar un model en el qual OCP3 exerceix un control especific per a la deposició de calosa regulada pel JA i que requerix, indispensablement, de l'ABA.

La proteïna OCP3, per homologia de seqüència, es classifica com un factor de transcripció nuclear membre de la familia dels Homeobox. No obstant això, trobem que OCP3 s'importa i acumula en els cloroplasts, i es colocalitza amb proteïnes que contenen motius de repetició pentatricopeptids i implicades en l'edició de RNA. Concretament, OCP3 participa regulant el mecanisme d'edició del transcrit *ndhB* que codifica una de les subunitats del complex multiproteic NADPH deshidrogenasa (NDH) del cloroplast. L'absència d'OCP3 es tradueix en una deficient edició de *ndhB*; fet aquest que comporta una pèrdua de la funcionalitat del complex NDH i que per tant implica un defecte en el flux cíclic d'electrons (CEF) al voltant del fotosistema I (PSI). També vam poder confirmar que mutants que presenten alteracions en l'activitat del complex NDH, com *crr2* i *crr21*, al seu torn presenten un increment en la resistència enfront de la infecció per *P. cucumerina*, a més de mostrar una major deposició de calosa. D'altra banda, observem que l'edició dels mRNA per a altres subunitats del complex NDH codificades en el cloroplast estan, també, subjectes a una regulació que es veu notablement alterada en preséncia d'un estimul patogènic. Tots aquests resultats indicarien que el complex NDH està

subjecte a una sutil regulació en concordança amb les condicions canviants de l'entorn.

El ABA es determinant en l'activació de mecanismes de defensa enfront de *P. cucumerina*. D'altra banda, patògens hemibiotrofs com per exemple *Pseudomonas syringae* DC3000 també provoquen increment en la síntesi de ABA. No obstant això, aquest increment implica la supressió de les respostes defensives, per la qual cosa se li pot atribuir un paper dual. La regulació del paper dual d'aquesta hormona, activador d'unes defenses i repressor d'unes altres, indica l'existència d'una regulació posterior a la síntesi de l'hormona. A la present Tesis Doctoral, s'ha indentificat a PYR1 com el membre de la familia dels receptors de ABA PYR/PYL/RCAR que percep aquesta hormona per desencadenar una resposta a la infecció per un patogen. PYR1 esmorteix la resposta dependent d'acid salicílic (SA) enfront de l'atac d'un patogen biotrof. D'aquesta forma exerceix un control positiu sobre les respostes dependents de JA requerides per activar les defenses enfront de patògens necrotrofs. La pèrdua de la percepció del ABA per PYR1 provoca una activació de les MAP quinases després de l'atac de patògens biotrofs. Així donçs, el ABA a través del receptor PYR1 i a través d'un control epigenètic, tindria un paper regulador de la immunitat vegetal en funció de l'estil de vida del patogen que infecti a la planta.

SUMMARY

ocp3 mutant plants exhibit an accelerated and intensified callose deposition in response to infection by *Botrytis cinerea* or *Plectosphaerella cucumerina*. After phenotypic analysis of a series of double mutants in *ocp3* background, we observed that both, the increase of callose deposition and resistance, require PMR4 callose synthase and abscisic acid (ABA). In wild plants after infection with any of these necrotrophic fungi, ABA synthesis increases, this increase is even higher in *ocp3*. The greater resistance observed in *ocp3* plants also requires the perception of jasmonic acid (JA) through COI1 receptor. However, JA is not required for the synthesis and basal callose deposition. In this way it could propose a model in which OCP3 exerts a specific control for callose deposition regulated by JA and requires, indispensably, ABA.

The OCP3 protein, by sequence homology, is classified as a nuclear transcription factor family member of Homeobox. However, we found that OCP3 is imported and accumulated in the chloroplasts, and colocalized with pentatricopeptide repeat proteins involved in RNA editing. Specifically, OCP3 participates regulating *ndhB* transcript editing. *ndhB* encodes one subunit of the multiprotein chloroplast complex NADPH dehydrogenase (NDH). The absence of OCP3 results in a decay of *ndhB* editing; a fact which entails a reduction of the activity of NDH complex and therefore leads to a defect in the cyclic electron flow (CEF) around the photosystem I (PSI). We were also able to confirm that mutants having altered activity of NDH complex, as *crr2* and *crr21,* have an increased resistance to infection by *P. cucumerina*, and show an increased callose deposition. Moreover, we note that the editing of the mRNA for other subunits encoded in the chloroplast NDH complex are also regulated, which is significantly altered in the presence of a pathogenic stimuli. At the same time, the stability of NDH complex is also compromised after a pathogenic insult. All these results indicate that the NDH complex is subject to a subtle regulation in accordance with the changing environment.

The ABA is a key player to activate plant defenses against *P. cucumerina* infection. Moreover, hemibiotrof pathogens such as *Pseudomonas syringae* DC3000 also cause increased synthesis of ABA. However, this increase leads to the suppression of defensive responses, which can be attributed a dual role. The regulation of dual role of this hormone, activator or repressor for different defenses, indicates the existence of a post-regulating hormone synthesis. In this Doctoral Thesis, PYR1 has been identified as a family member receptor ABA PYR/PYL/RCAR that perceives this hormone to trigger a defensive response to infection by a pathogen. PYR1 reduces the salicylic acid (SA)-dependent response against the biotrophic pathogens attack. Thus exerts a positive control of JA-dependent responses required to activate the defenses against necrotrophic pathogens. The loss of the perception of ABA through PYR1 activates the expression of SA-responsive genes, modifies the chromatin remodeling and increases the activation of MAP kinases after a biotrophic pathogen attack. Thus, ABA through PYR1 receptor and an epigenetic control, have a regulatory role in plant immunity depending on the lifestyle of pathogens infecting the plant.

ABREVIATURAS

ABA: ácido abscísico

ACC: 1-amynocilocpropane-1-carboxylic acid

AGO: Argonauta

ARF: Auxin Response Factor

ARNsi: ARN interferentes

ATP: adenosín trifosfato

ATT: Aberrant Induction of Type Three genes

BDG: Bodyguard

bHLH: basic hélix-loop-helix

BRE1/LACS: Long-Chain Acyl-CoA Synthetase

CalS: Calosa Sintasa

CEF: flujo cíclico de electrones

COI1: Coronatine Insensitive 1

CRR: Chlororespiratory Reduction

EAR: Associated Amphiphilic Repression

EGL: Enhancer of Glabra 3

elf18: péptido del factor de elongación bacteriano (EF)Tu

EMS: metano sulfonato de etilo

Ep5C: Peroxidasa Catiónica Extracelular de tomate

ET: Etileno

ETI: Effector Triggered Immunity

Flg22: Flagellina 22

FNR: ferredoxin–$NADP^+$ reductase

GL1: Glabra 1

GUS: **β**-glucuronidasa

H_2O_2: peróxido de hidrógeno

HDCA: Histona Deacetilasa

HR: Respuesta Hipersensible

ISR: Resistencia Sistémica Inducida

JA: Ácido Jasmónico

JAZ: Jasmonate Zim-Domain

LCR: Lacerata

LEF: Flujo Lineal de Electrones

LHCI: Comlejo de Captación de Luz I

LHCII: Complejo de Captación de Luz II

MAMP o **DAMP**: Microbe or Damage-Associated Molecular Patterns

MEcPP: Methylerythritol Cyclodiphosphate

MeJA: Jasmonato de Metilo

MORF: Multiple Organellar RNA Editing Factor

NADPH: Nicotinamida Adenina Dinucleótido Fosfato

NINJA: Novel Interactor of JAZ Protein

NPQ: Quenching No Fotoquímico

NPR: *Non expresser of Pathogenesis-Related genes*

NRPD2: NUCLEAR RNA POLYMERASE D2

$^{\cdot}OH$: hidroxilo

$^1O_2$: oxigeno singlete

$O_2^{\cdot-}$: superóxido

O_3: ozono

OCP: Overexpresion Cationic Peroxidase

ORRM: Organelle RRM protein

PAMP: Pathogen Associated Molecular Pattern

PAP: 3'-phosphoadenosine 5'-phosphate

PMR4: Powdery Mildew Resistant 4

PP2C: Protein Phosphatase 2C

PPR: motivo de repetición pentatricopeptido

PR: Pathogenesis Related

PRR: Pattern Recognition Receptors

PSI: fotosistema I

PSII: fotosistema II

PTI: PAMP-Trigger Immunity

PYL: Pyrabactin Resistance like

PYR: Pyrabactin Resistance

RC: Centro de Reacción

RCAR: Regulatory Components of ABA Receptor

RdDM: *RNA-directed DNA methylation*

RIP: RNA editing factor Interacting Protein

ROS: Especies Reactivas de Oxígeno

SA: Ácido Salicílico

SAR: Resistencia Sistémica Adquirida

SCF: Skp-Cullin-Fbox

SnRK2s: Snf1 (Sucrose-non-fermentation 1)-Related Kinases Subfamily 2

TPL: Topless

TT8: Transparent Testa 8

Introducción

1. Introducción general

En la naturaleza, las plantas responden de manera eficiente a los diferentes tipos de estrés que se dan en su ambiente con el fin de sobrevivir y reproducirse. Estos estreses se pueden clasificar en dos grandes grupos: abióticos o bióticos. Las condiciones causantes de estrés abiótico son factores inanimados que contribuyen a un ambiente desfavorable e incluyen cambios de temperatura, períodos de falta de disponibilidad de agua y nutrientes, así como un incremento en la salinidad. El estrés biótico es causado por otro organismo e incluye interacciones con patógenos de complejidad diferente (e.g., virus, bacterias, hongos, oomicetos), también con insectos, así como la interacción con otras plantas. Con el fin de sobrevivir, las plantas han desarrollado la capacidad de percibir y responder a estos estímulos de una manera rápida y adecuada. Por lo tanto, frente a una infección microbiana se desencadenan diferentes mecanismos dirigidos a impedir o reducir el daño producido por la infección, mientras que el patógeno desarrolla nuevas estrategias para tratar de evitar las defensas vegetales o favorecerse de las estructuras vegetales para concluir con éxito el proceso infectivo (Agrios 2005).

Los patógenos vegetales, en base a la estrategia que utilizan para la adquisición de nutrientes de sus huéspedes, se clasifican de manera general en biotrofos y necrotrofos. Los patógenos biotrofos obtienen nutrientes de la células vegetales vivas y han desarrollado sofisticadas estrategias con el fin de aprovechar la energía de su planta huésped mientras completan su ciclo vital (Glazebrook, 2005; Laluk and Mengiste, 2010). Por el contrario, los patógenos necrotrofos obtienen los nutrientes a partir de células muertas o en proceso de degenaración celular, lo que conlleva una maceración del tejido infectado con la manifestación final de un tejido necrosado en el órgano infectado (Laluk y Mengiste, 2010). Frente a este hecho diferencial, debido al estilo de vida de los patógenos vegetales, las plantas han desarrollado sofisticados mecanismo de defensa e inmunidad dependiendo del agente agresor.

Los hongos necrotrofos, tales como *Botrytis cinerea, Alternaria brassicicola* o *Plectosphaerella cucumerina*, son los responsables de causar enfermedades en un amplio rango de cultivos, así como en la planta modelo *Arabidopsis thaliana* (Cramer y Lawrence, 2004; Ramos *et al*., 2013; Williamson *et al*., 2007). Durante la colonización de los tejidos, algunos hongos necrotrofos desarrollan estructuras denominadas apresorios, que por acción mecánica y también ayudados por la secreción de enzimas hidrolíticas (e.g., cutinasas, celulasas) rompen y/o disuelven las barreras físicas preestablecidas en el huésped (e.g., cuticula, pared celular) (Howard y Valent, 1996, Ospina-Giraldo *et al*., 2003; Reignault *et al*., 2008). Por otra parte, también se ha descrito que este tipo de patógenos producen una serie de toxinas y proteínas, que interfieren con los mecanismos de defensa de la planta y permiten la colonización de ésta por parte del patógeno (Friesen *et al*., 2008; Lawrence *et al*., 2008).

2. Barreras defensivas de las plantas.

2.1 La cutícula

La cutícula recubre la pared celular de los tejidos aéreos y establece puentes de interacción entre las plantas y el medioambiente que les rodea. Así mismo, representa una primera barrera que permite minimizar la pérdida de agua y solutos así como proteger a las plantas frente a estreses bióticos y abióticos. Recientemente se ha observado que mutantes que presentan un incremento en la permeabilidad de la cutícula, como ocurre en los mutantes *lcr* (*lacerata*), *bdg* (*bodyguard*) y *lacs2 (long-chanin acyl-CoA 2)*, concurre una fuerte resistencia frente al hongo necrotofo *B. cinerea* (Bessire *et al*., 2007; Chassot *et al.,* 2008). Por ello se ha sugerido que un incremento en la permeabilidad de la cutícula permitiría una mejor percepción de los elicitores fúngicos. L´Haridon *et al*., (2011) han demostrado que una mayor permeabilidad de la cutícula va acompañada de un incremento en la aparición de especies reactivas de oxígeno (ROS) que actúan como moléculas señalizadoras para la inducción de defensas (Orozco-Cardenas *et al*., 2001). Por lo tanto, a la cutícula

se le podrían atribuir diferentes implicaciones en la defensa vegetal en función del momento concreto de la infección.

2.2 La pared celular

La pared celular muestra una organización dinámica y es esencial para los procesos de división celular, de elongación celular e incluso de diferenciación celular. Además, la pared celular también está implicada de una forma muy directa y activa en la señalización y en la respuesta a la infección por patógenos (Roberts, 1990; Nishimura *et al*., 2003; Vorwerk *et al*., 2004). La composición y la arquitectura de la pared, varía en función del estadío de desarrollo de la planta (Varner y Lin 1988), y por ello supeditada tanto a la proporción como al tipo de polímeros que la conforman, y que incluye, por orden de abundancia, a la celulosa, la lignina y la hemicelulosa, así como un complejo numero de proteínas y lípidos (Carpita and McCann, 2000).

En cuanto a la implicación de la pared celular en la defensa vegetal, se puede destacar que la pared es un gran reservorio de proteínas antifúngicas y de metabolitos secundarios que inhiben el crecimiento de gran cantidad de patógenos (Darvill y Albersheim, 1984). Sin embargo, algunos patógenos han desarrollado la capacidad de penetrar a través de la pared celular y acceder a los nutrientes celulares. En el concurrir evolutivo, las plantas han desarrollado, también, modificaciones específicas de la pared celular con objeto de impedir el avance del patógeno (van Kan 2006). Así, aquellos patógenos que son capaces de sobrepasar las primeras barreras estructurales (e.g., cutícula, pared celular), se van a enfrentar a otro tipo de barreras, que vienen a denominarse barreras post-invasivas, y que incluyen la inducción de la síntesis y deposición del polimero calosa, con la correspondiente formación de la denominada papila en la cara interna de la pared celular. También concurre con este mecanismo de modificación de la pared una rápida acumulación de especies de oxígeno reactivas (ROS) (Asselbergh *et al*., 2008). La deposición de calosa se cree que tiene como función actuar como segundo freno estructural al avance del patógeno desde su primer punto de penetración en la

epidermis. La calosa es mayoritariamente un polimero de β-1,3-glucano acompañado de una gran variedad de polisacáridos, compuestos fenólicos, intermediarios de oxigeno reactivos y proteínas, que de manera conjunta se amalgaman para formar la denominada papila (Bestwik *et al*., 1997, Thordal-Christensen *et al*., 1997; Hématy *et al*., 2009).

La calosa se encuentra presente en gran cantidad de tejidos y también está implicada en diferentes procesos del desarrollo y crecimiento vegetal. Es un componente mayoritario del polen y de la formación del tubo polínico (Ariizumi y Toriyama, 2011). Recientemente se ha demostrado que el factor de transcripción de respuesta a auxinas ARF17 (auxin response factor 17) regula la expresión de la calosa sintasa 5 (CalS5) responsable de la síntesis de la calosa implicada en la formación del polen (Yang *et al*., 2013). Por otro lado, la calosa asociada a los plasmodesmos está implicada en los canales de transporte (Simpson *et al*., 2009), donde forma parte de la placa cribosa en el floema en dormición y se piensa que participa en el sellado de los tubos criboso en respuesta a diferentes estímulos (Zavaliev *et al*., 2011). En el contexto de la interacción planta-patógeno es la calosa sintasa 12 (cals12) o PMR4 (powdery mildew resistant 4) la que juega un papel crucial. Tras establecerse el contacto entre la espora del hongo y la cara externa de la hoja, la calosa sintasa PMR4 es reclutada en vesículas desde la membrana plasmática hacia los sitios de penetración del patógeno para iniciar la biosíntesis y deposición de este polímero. Así, la sobreexpresión de PMR4 en *A. thaliana* incrementa la deposición de calosa en la zona de pentración del patógeno, incrementando la resistencia (Eggert *et al*., 2014). En sentido contrario, el mutante *pmr4*, que tiene bloqueada la actividad de la calosa sintasa 12 (Jacobs *et al*., 2003; Nishimura *et al*., 2003) presenta un incremento en la susceptibilidad a *A. Brassicicola*, *Pythium irregulare*, *Leptosphaeria maculans* y *P. cucumerina* (Adie *et al*., 2007; Flors *et al*., 2008; Kaliff *et al*., 2007; García-Andrade *et al*., 2011), indicando un importante papel de la calosa en la resistencia a enfermedades. En este sentido, recientemente, se ha identificado a PR2 (pathogenesis related 2) como una β-1,3-glucanasa implicada en

la degradación de la calosa inducida por el patógeno y su regulación transcripcional está regulada por el ácido abscísico (ABA) (Oide *et al*., 2013).

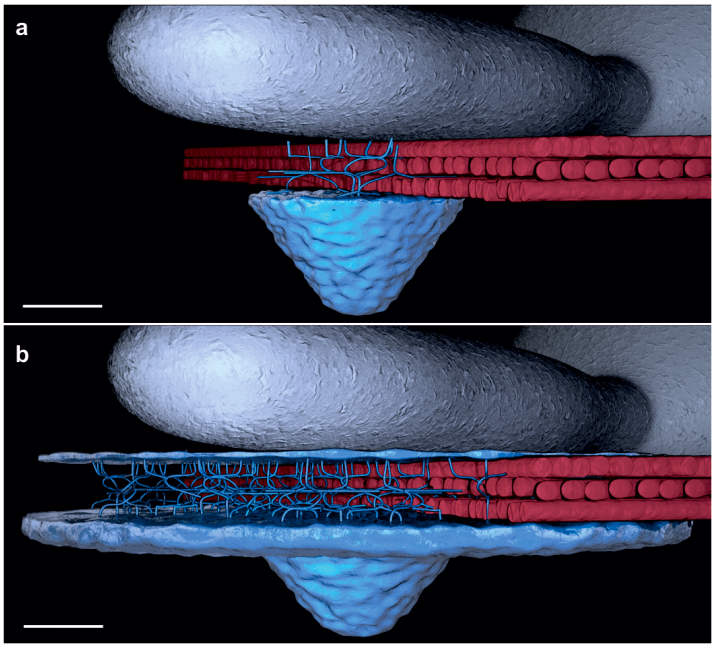


Figura 1: Modelo 3D de la deposición de calosa en los sitios de penetración del hongo. a) planta silvestre, b) sobreexpresión de *PMR4*. Imágenes de células epidérmicas a las 6 h post inoculación con powdery mildew. En color azul se observa la calosa, en color rojo la celulosa y en color gris las estructuras del hongo. Obtenido de Eggert *et al*., 2014.

3. Reconocimiento planta-patógeno

Las plantas notan la presencia de un patógeno a través de un conjunto de receptores de reconocimiento de patrones (PRRs, Pattern Recognition Receptors) situados en la membrana plasmática. Estos receptores pueden detectar la presencia de ligandos que actuan como patrones moleculares asociados a patógenos (PAMPs, Pathogen Associated Molecular Pattern). Entre los mejor caracterizados podemos encontrar, el péptido Flg22 que corresponde a una región de 22 aminoácidos de la proteína encargada del movimiento bacteriano conocida como flagellina; el elf18, un péptido

del factor de elongación bacteriano (EF)Tu; y la chitina, componente de la pared celular de hongos (Kunze *et al*., 2004; Wang *et al*., 2008). Tras la percepción del patógeno se activa una cascada de respuesta que incluye, entre otras, la producción de especies reactivas de oxigeno (ROS), cambios en la expresión génica, producción de componentes de defensa y cierre estomático lo que se conoce como inmunidad activada por PAMPs o PTI (PAMP-Trigger immunity). En la mayoría de los casos la activación de PTI es capaz de frenar la infección sin producir síntomas de la enfermedad. Sin embargo, algunos patógenos han desarrollado estrategias para sortear el efecto de la activación de la respuesta inmune, a través de la inyección de efectores, mediante el que consiguen suprimir la inmunidad e infectan la planta. A su vez, las plantas han desarrollado un sistema de proteínas de resistencia (R) que reconocen específicamente cada efector y activan la inmunidad activada por efectores o ETI (Effector-Triggered Immunity) (Jones y Dangl, 2006).

4. Activación de la señalización hormonal

La capacidad de las plantas para activar un mecanismo de defensa frente al ataque de un patógeno depende en gran medida de la señalización dirigida por ciertas hormonas vegetales. Tradicionalmente, el ácido salicílico (SA) es el responsable de activar la señalización frente a patógenos biotrofos y hemibiotrofos, mientras que el ácido jasmónico (JA) y el etileno (ET) son los responsables de la activación de la defensa frente a patógenos necrotrofos (Glazebrook J. 2005). Más recientemente, el ácido abscísico (ABA) ha sido identificado como un factor importante en la defensa frente a hongos necrotrofos (Mauch-Mani y Mauch 2005; Asselbergh *et al*., 2008; Fan *et al*., 2009; Adie *et al*., 2007). Además de estas hormonas, que juegan un papel central en la interacción planta-patógeno, otras hormonas vegetales como los brasinosteroides, giberelinas y auxinas están emergiendo como importantes correguladores de la resistencia a patógenos (Bari and Jones, 2009). Por lo tanto, un punto importante en la regulación de la respuesta defensiva de las plantas viene determinada por cómo dichas hormonas son sintetizadas y percibidas en un contexto patogénico, y también cómo las mismas pueden interactuar para potenciar o reprimir

de manera alternativa rutas de señalización a través de mecanismos de cross-talk hormonal (Robert-Seilaniantz *et al*., 2011).

4.1 Ácido salicílico (SA)

El ácido salicílico (SA) juega un papel central en la defensa vegetal y es percibido por el regulador NPR1 (nonexpresser of PR genes1). NPR1 regula tanto la supervivencia de la célula a través de la activación de la respuesta inmune frente al ataque de agentes patógenos biotrofos, como los fenómenos de respuesta hipersensible conducentes a la aparición de muerte celular en interacciones compatibles e incompatibles, respectivamente (Canet *et al*., 2010; Cao *et al*., 1994, 1997). Recientemente se ha propuesto a las proteínas NPR3 y NPR4 también como receptores adicionales del SA (Fu *et al*., 2012). NPR1 se identificó en un rastreo de doble híbrido por interaccionar con los factores de transcripción TGA y se propuso como un co-activador transcripcional de la expresión de genes implicados en la respuesta sistémica adquirida (SAR) (Zhang *et al*., 1999; Zhou *et al*., 2000). En ausencia de SA, NPR1 forma oligómeros en el citosol de tal forma que se previene la activación de las defensas. Tras un ataque patogénico o tratamientos con SA, la respuesta redox conduce a la reducción de NPR1 que se desensambla en los monómeros correspondientes y que se traslocan al núcleo donde regulan la expresión génica y activación transcripcional de genes de defensa (e.g., PRs) (Mou 2003). Por lo tanto NPR1 tienen un claro papel como cofactor transcripcional, de tal manera, que en estudios realizados con el mutante de pérdida de función *npr1* se observó que la respuesta transcripcional mediada por SA se redujo aproximadamente a un 10% y se vió comprometida la resistencia (Wang *et al*., 2006). Sin embargo, NPR3 y NPR4, homólogos de NPR1, jugarían un papel contrario al descrito para NPR1 ya que el doble mutante *npr3 npr4* presenta un incremento en la resistencia al contrario de lo descrito para *npr1*. Recientemente se ha propuesto que tanto NPR3 como NPR4 interaccionan con NPR1 para favorecer su degradación vía proteosoma y que esta interacción está regulada por SA. Mientras el SA reduce la interacción entre NPR4 y NPR1, éste, al mismo tiempo, favorece la interacción entre NPR3 y NPR1, por lo que se ha sugerido que NPR3 y

NPR4 unirían SA para regular la interacción con NPR1 de manera dependiente de la concentración de esta hormona. Siguiendo este modelo, en tejidos infectados se produce un incremento en la acumulación de SA lo que favorece la degradación de NPR1 tras interaccionar con NPR3 y se desencadena la muerte celular y la ETI. En tejidos adyacentes, los niveles de SA son bajos pero suficientes para evitar la interacción entre NPR4 y NPR1 lo que evita la degradación de NPR1 y se activa así la resistencia mediada por SA (Yang y Dong, 2014).

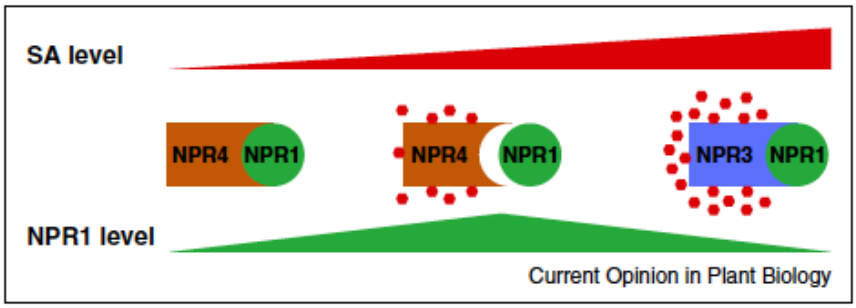


Figura 2: Modelo de la regulación de la percepción de SA en función de la concentración. Obtenido de Yang y Dong, 2014.

4.2 Ácido jasmónico (JA)

El ácido jasmónico (JA) y sus derivados (jasmonatos) son hormonas derivadas de ácidos grasos. Estos actúan como moléculas reguladoras en un gran número de procesos de desarrollo (e.g., fertilidad, determinación sexual, elongación radicular o incluso la maduración del fruto). El JA, y en particular su forma activa JA-Ile, es clave en la activación de las defensas frente a patógenos, frente a insectos masticadores, y también en fenómenos de respuesta a daños mecánicos y heridas y otros tipos de estrés abiótico (Wasternack, 2007; Acosta *et al*., 2009; Browse y Howe, 2008; Chico *et al*., 2008).

La percepción del JA se da a través de la proteína COI1 (Coronatin Insensitive 1). COI1 es una proteína F-box que forma parte del complejo SCF (SKIP-CULLIN-Fbox). Estos complejos multiprotéicos contienen una ligasa de ubiquitina E3 que

cataliza la ubiquitación de las proteínas y las marca para ser degradadas por el proteosoma. La proteína F-box es el componente que confiere especificidad por el substrato. En el caso de COI1 en presencia de la hormona (JA-Ile) reconoce a las proteínas JAZ (Jasmonate Zim Domain) y las marca para ser degradadas por el proteosoma 26S (Thines *et al*., 2007; Devoto *et al*., 2002; Xu *et al*., 2002; Chini *et al*., 2007). Las proteínas JAZ forman una familia de 12 miembros que actúan como reguladores negativos de la expresión de genes a bajas concentraciones de JA, tras un incremento en la concentración de JA el complejo SCF^{COI1} interacciona con los JAZ favoreciendo su degradación y provocando la activación trascripcional de genes de respuesta mediada por JA.

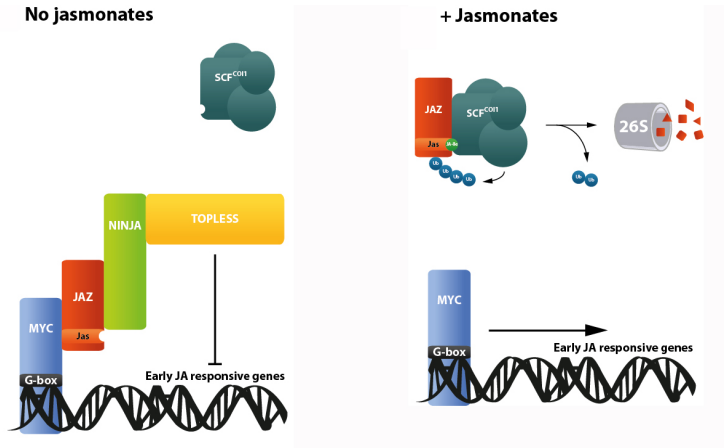


Figura 3: Modelo de la activación de la respuesta a JA tras la degradación del complejo de represión JAZ-NINJA-TOPLESS. Obtenido de la web de A. Goossen.

El factor de transcripción MYC2, perteneciente a la familia bHLH (basic hélix-loop-helix), fue el primer factor que se identificó reprimible por proteínas JAZ (Chini, A. *et al*., 2007). Posteriormente se identificaron los factores MYC3 y MYC4 que presentan funciones redundantes con MYC2 en la señalización por JA y que, al igual que en éste último, la interacción se da entre el dominio Jas del represor JAZ y el extremo N terminal de los factores MYC (Cheng *et al*., 2011; Fernández-Calvo *et*

al., 2011). Además de los factores MYC, las proteínas JAZ interaccionan con otros factores de transcripción: TT8 (Transparent Testa 8), GL3 (Glabra 3), EGL3 (Enhancer of Glabra 3), MYB75, GL1 (Glabra 1) que vienen a estar implicados en la síntesis de antocianos y en la diferenciación de tricomas. Sin embargo, en estos casos las proteínas JAZs interaccionan en el extremo C terminal (Qi *et al*., 2011; Kazan y Manners 2011). También se ha demostrado interacción de los JAZ con MYB21 y MYB24 implicados en el desarrollo (Mandaokar *et al*., 2006). Recientemente se ha publicado un estudio en el que se detalla el mapa de interacciones de Arabidopsis a través del que se han confirmado las interacciones descritas para las proteínas JAZ y se han identificado nuevas interacciones (Arabidopsis Interactome Mapping Consortium *et al*., 2011).

Los resultados comentados anteriormente refuerzan la idea de que la modulación de la respuesta a JA por las proteínas JAZ probablemente implican interacciones con un gran número de factores de transcripción, que regulan cada uno de manera separada o conjunta un tipo de la señalización de la ruta. Esta capacidad de las proteínas JAZ de interaccionar con diferentes activadores, formando homo- y heterodímeros a través del dominio ZIM puede dar lugar a un incremento en el número de factores de transcripción potencialmente susceptibles de ser reprimidos por las proteínas JAZ (Chini *et al*., 2009).

Por otra parte, ya que las proteínas JAZ no presentan un motivo de represión, se ha sugerido que dichas proteínas para reprimir la transcripción deben interaccionar con un co-represor. Así pues, recientes estudios han demostrado que las proteínas JAZ interaccionan con proteínas TPL, TPR2 y TPR3 (Topless, Topless related 2 y 3) (Fernández-Calvo *et al*., 2011; Pauwels, *et al*., 2010). Estas proteínas represoras forman complejos con enzimas modificadoras de las histonas, como las deacetilasas de histonas (HDCAs). La deacetilación de la histona provoca que la cromatina no esté accesible para la maquinaria celular encargada de la transcripción. La interacción entre el represor TPL y el complejo SCF^{COI1}-JAZ se da a través de un dominio EAR que contienen algunas proteínas JAZ o por medio de un intermediario conocido como NINJA (Novel Interactor of JAZ) (Payankaulam *et al*., 2010).

Por lo tanto, es biológicamente relevante conocer que interacción JAZ-factor de transcripción se rompe una vez percibido el JA a través de COI1. De esta forma, llegaríamos a entender cómo una misma hormona es capaz de regular tantos y diferentes procesos en un mismo organismo. Al mismo tiempo, permitiría identificar a los efectores de los patógenos que tratan de impedir la interacción del complejo SCF^{COI1} con las proteínas JAZ y alterar la señalización dependiente de JA y favorecer la infección por parte del patógeno (Grant *et al*., 2009; Robert-Seilaniantz *et al*., 2011).

4.3 Ácido abscísico (ABA)

El ácido abscísico (ABA) es una hormona implicada en la regulación de diferentes aspectos del desarrollo de las plantas, incluida la dormición de las semillas, embriogénesis y la senescencia foliar. Además, juega un papel importante en la respuesta defensiva de las plantas frente a estreses de tipo abiótico, en concreto salinidad y sequía (Fujita *et al*., 2006; Wasilewska *et al*., 2008) y es clave en la regulación del cierre estomático (Schroeder *et al*., 2001). Por otra parte, en esta última década han surgido diferentes trabajos que implican al ABA en la respuesta inmune de las plantas frente estrés biótico. Sin embargo, no está del todo claro el papel que juega dicha hormona, y cómo lo juega, ya que se han visto efectos tanto positivos como negativos en función del patógeno estudiado, el estado de desarrollo de las plantas utilizadas o las condiciones de inoculación y crecimiento de las plantas (Mauch-Mani y Mauch 2005; Asselbergh, *et al*., 2008; Fan *et al*., 2009; Adie *et al*., 2007; Flors *et al*., 2008; Jensen *et al*., 2008; Ton *et al*., 2009; García-Andrade *et al*., 2011; Luna *et al*., 2011; Sánchez-Vallet *et al*., 2012). De aquí la importancia de seguir trabajando para dilucidar cómo el ABA regula la defensa de las plantas.

Recientemente se han descrito los componentes esenciales de la percepción del ABA. El núcleo central de la percepción está compuesto por una familia de proteínas PYR/PYL/RCAR consideradas como el receptor del ABA. En Arabidopsis la familia de las proteínas PYL está formada por 14 miembros, entre los que se incluye PYR1 y los PYLs 1-13 (Park *et al.,* 2009; Ma *et al.,* 2009; Fujii *et al.,*

2009; Santiago *et al.,* 2009). Las proteínas PYL en presencia de ABA interaccionan e inhiben a las proteínas con actividad fosfatasa de tipo A (PP2C: ABI1, ABI2, HAB1, HAB2, PP2CA, HAI1, HAI2 y HAI3) (Santiago *et al.,* 2009; Yin *et al.,* 2009; Melcher *et al.,* 2009; Miyazono *et al.,* 2009; Melcher *et al.,* 2010). En ausencia de ABA las PP2C interaccionan, reduciendo su capacidad catalítica, con proteínas SnRK2s con actividad quinasa [Snf1 (Sucrose-non-fermentation 1)-related kinases subfamily 2] y estas reducen su capacidad de fosforilar las diferentes dianas de regulación y activar la señalización (Fujii *et al*., 2009; Umezawa *et al*., 2009). Tras un incremento en la concentración de ABA se activan los receptores PYLs que tras unirse al ABA modifican su estructura terciaria favoreciendo la interacción con las PP2Cs para inhibir su actividad. De esta forma, tras generarse el complejo ABA-PYL-PP2C, se interrumpe el efecto negativo que las PPC2s ejercen sobre las SnRKs permitiendo que estas quinasas se autofosforilen y activen a los factores de transcripción de tipo ABF, lo que conlleva a una activación de la señalización dependiente de ABA (Umezawa *et al.,* 2009; Mustilli *et al.,* 2002; Fujii *et al.,* 2009; Johnson *et al.,* 2002; Furihata *et al.,* 2006).

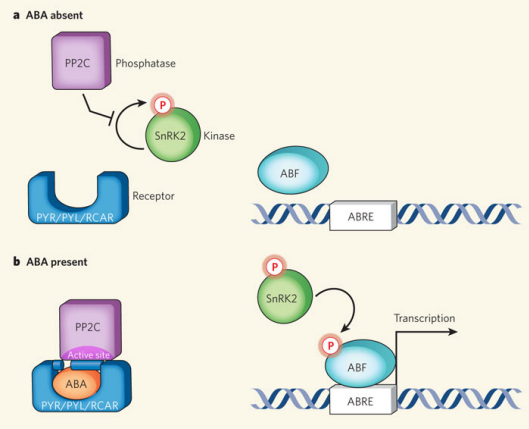


Figura 4: Ruta mínima de señalización dependiente del ABA. Obtenido de Sheard y Zheng 2009.

Los receptores PYR/PYL, en ensayos realizados en protoplastos, son capaces de activar la expresión de genes en respuesta a ABA (excepto PYL13) (Fujii *et al*.,

2009). Sin embargo, el patrón de expresión de estos genes presenta diferencias sustanciales entre ellos. En ensayos realizados utilizando el gen delator GUS, se observó que los genes *PYR1* y *PYL5* se expresaban en el córtex de la parte superior de la raíz, mientras que *PYL1*, *PYL4* y *PYL8* se expresaban en las células de la columnela (Gonzalez-Guzman *et al*., 2012). Por otra parte, análisis genéticos de los diferentes mutantes *pyr*/*pyl* sugieren que su función no es completamente redundante (Szostkiewicz *et al*., 2009; Antoni *et al*., 2012), aún cuando ha sido necesario generar cuádruples y séxtuples mutantes para observar una respuesta severa de insensibilidad al ABA (Park *et al*., 2009; Gonzalez-Guzman *et al*., 2012). En este sentido, PYL8 juega un papel no redundante en la regulación de la sensibilidad de la raíz al ABA; siendo el mutante de pérdida de función *pyl8* insensible a la reducción del crecimiento de la raíz en respuesta a ABA (Antoni *et al*., 2013). Esto hace pensar que la especificidad en la regulación de los diferentes receptores facilita el control de los diversos procesos en los que está implicada dicha hormona. En lo que hace referencia a la activación de la respuesta inmune se conoce poco sobre cómo el ABA regula dicha respuesta y a través de qué intermediarios.

5. Especies reactivas de oxígeno (ROS)

Las especies reactivas de oxigeno (ROS) juegan un papel importante en el crecimiento, desarrollo y metabolismo vegetal, así como en la respuesta a diferentes estreses de tipo biótico y ambiental y regulan, entre otros, procesos de muerte celular programada (Mittler *et al*., 2011; Inzé *et al*., 2012Baxter *et al*., 2012). Entre las ROS encontramos moléculas como el superóxido ($O_2^{\cdot-}$), hidroxilo ($^{\cdot}OH$), peróxido de hidrógeno (H_2O_2) y el oxigeno singlete ($^1O_2$) (Halliwell, 2006; Halliwell y Gutteridge, 2007). La enzima NADPH oxidasa de la membrana plasmática juega un papel relevante en la producción de ROS (Suzuki *et al*., 2011; Batxer *et al*., 2014), aunque orgánulos como el cloroplasto, la mitocondria y los peroxisomas también contribuyen tanto a la producción como a la regulación de la cascada de señalización provocada por este tipo de moléculas en lo que se conoce como señalización redox (Bolwell y Daudi, 2009; O'Brien *et al*., 2012; Foyer y Noctor, 2005). En concreto, los cloroplastos son importantes productores de $^1O_2$ a través de

diferentes reacciones fotodinámicas (Triantaphylidés y Havaux, 2009), como por ejemplo una reducción de la captación de energía por parte del fotosistema II (Mullineaux y Baker, 2010). En situaciones de estrés en las que se reduce el metabolismo fotosintético, se incrementan los niveles de $^1O_2$ generando una pérdida de integridad de los cloroplasto lo que precede a una ruptura de la vacuola y al colapso celular (Triantaphylidès *et al*., 2008). Además, este fenómeno está regulado de una manera similar a la inmunidad innata, respuesta hipersensible (HR) (Mateo *et al*. 2004; Mühlenbock *et al*. 2007, 2008; Chang *et al*. 2009; Frenkel *et al*. 2009) y a la resistencia sistémica adquirida (SAR) (Coll, Epple y Dangl 2011; Luna *et al*. 2012; Luna y Ton 2012).

Por lo tanto, existe una relación ente la señalización generada por un tipo de estrés biótico y la que se genera en condiciones de estrés abiótico como, por ejemplo, la derivada de un incremento brusco en la intensidad lumínica (Karpiński *et al*., 2012). En este sentido experimentos que conllevan el análisis transcriptómico en plantas sometidas a un exceso de iluminación mostraron una activación de genes implicados en la inmunidad vegetal. Así pues, se ha demostrado que cambios en el estado redox de los transportadores de electrones fotosintéticos son capaces de inducir tanto genes de respuesta a la aclimatación del estrés lumínico como genes de respuesta a la defensa frente a ptógenos, incluyendo genes implicados en el metabolismo y acción del, SA, ABA, ET y ROS (Fryer *et al*., 2003; Rossel *et al*., 2007; Mühlenbock *et al*., 2008).

Un papel importante de las ROS, entre ellas el H_2O_2 y el $^1O_2$, es su implicación en la comunicación entre el núcleo y el cloroplasto, lo que se conoce como señalización retrógrada. Aunque recientemente se han identificado nuevas moléculas con capacidad de regular la expresión de genes de respuesta a estrés, como por ejemplo el methylerythritol cyclodiphosphate (MEcPP) y el 3'-phosphoadenosine 5'-phosphate (PAP) (Gil *et al*., 2005; Estavillo *et al*., 2011). Otro compuesto cloroplástico como el *β*-cyclocitral también ha sido propuesto como un inductor de mecanismos de defensa a través de la señalización mediada por $^1O_2$ (Ramel *et al*., 2012) y que requiere de las proteínas EXECUTER 1 y 2 (Kim *et al*., 2012). Por ello,

identificar nuevos reguladores de la maquinaria fotosintética con capacidad de generar moléculas ROS con capacidad de señalización, permitiría entender que mecanismo(s) se activa(n) tras la percepción del patógeno y cuáles van dirigidos a frenar el avance de éste.

6. Implicación de la fotosíntesis en la defensa vegetal

Las plantas son uno de los poco organismos capaces de utilizar la energía proveniente del sol para convertir moléculas simples como el dióxido de carbono y el agua en moléculas más complejas como los carbohidratos. Además, en la fotosíntesis se produce oxígeno y otros compuestos relacionados con esta molécula los cuales muestran capacidad señalizadora, entre los que encontramos el H_2O_2 y el $^1O_2$.

6.1 El cloroplasto

El cloroplastos es un orgánulo fotosintético, que posee un genoma que codifica entre 60 y 120 proteínas dependiendo de la especie vegetal (Sugiura 1992). La estructura interna del cloroplasto consta de una membrana continua plegada que se denomina tilacoide, rodeada por el estroma que principalmente está compuesto por una solución acuosa. La membrana tilacoidal sufre plegamientos en forma de vesículas que se denominan grana y están interconectados entre ellos (Kieselbach *et al*. 1998; Peltier *et al*. 2000; Schubert *et al*. 2002). En las membranas tilacoidales se encuentran cuatro complejos multiprotéicos mayoritarios que participan activamente de los procesos fotosintéticos: el fotosistema I y II (PSI y PSII), el complejo citocromo b_6f y la ATP-sintasa.

Los complejos fotosintéticos se distribuyen a lo largo de la membrana de una manera heterogenea. La mayoría del PSII se encuentra agrupado en la parte interior de los grana, mientras que el PSI y la ATP-sintasa se localizan por el estroma y la membrana lamelar, además de la parte externa de los grana (Anderson and Andersson 1982). El citocromo b_6f se encuentra tanto de forma agrupada como no agrupada (Albertsson 2001).

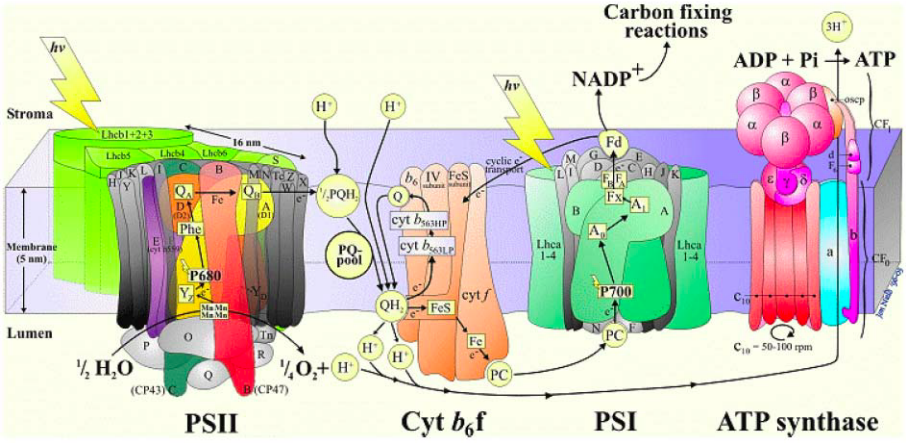


Figura 6: Esquema de la cadena de transporte electrónico de los tilacoides. Obtenido de Jon Nield, Imperial College London, 2000.

6.2 Fotosistema II

El PSII está formado por más de 25 subunidades diferentes. El centro de reacción (RC) del PSII consta de las proteínas D1 y D2, cada una contiene cinco hélices transmembrana con un N- terminal en la cara del estroma de la membrana tilacoidal (Svensson *et al*. 1996; Barber 1998). Además, el RC contiene el citocromo b559 compuesto por dos subunidades alfa y beta (codificadas por los genes *PsbF y PsbE*), la proteína PsbI y otras proteínas de bajo peso molecular (Sharma *et al*. 1997). El núcleo del PSII está rodeado por el complejo antena formado por 15 proteínas, las proteínas de unión a las clorofilas A y B forman el complejo de captación de luz II (LHCII) que está formado por complejos monoméricos y triméricos (Hobe *et al*. 1995; Kuttkat *et al*. 1996). Los genes *Lhcb1, 2, 3, 4, 5*, y *6* son los que codifican las proteínas principales de este supercomplejo del PSII (Boekema *et al*. 2000) y que están sometidos a un control retrogrado entre el núcleo y el cloroplasto (Fernández y Strand 2008).

6.3 Fotosistema I

La parte central del PSI está formada por 13 subunidades diferentes, que van desde la PSI-A hasta la PSI-N (la proteína PSI-M se ha perdido en la mayoría de las

plantas, aunque sí que se encuentra en las cianobacterias) y proteínas de captación de la luz I (LHCI) (Scheller *et al*. 2001). El LHCI de Arabidopsis, está formado por dímeros de la proteína LHCA codificada por 6 genes nucleares *Lhca1-6*. La parte más importante del PSI está formada por tres subunidades PSI-A, PSI-B y PSI-C que son las encargadas de unir los aceptores de electrones: A0 (chl a), A1 (phylloquinone) y FX, FA, FB. Por otra parte encontramos cuatro subunidades diferentes de Lhca que se encuentran en un semicírculo entre las subunidades del PSI PsaG y PsaK alrededor de la cara en la que se localizan la PsaF y PsaJ. Entre el complejo LHCI y el PSI encontramos una gran hendidura que es a través de la que se transfiere la energía vía PsaG, PsaK y PSaF donde los pigmentos se encuentran estrechamente unidos entre si (Ben-Shem *et al*. 2003).

6.4 Transferencia de electrones fotosintética

El primer paso de la fotosíntesis es la absorción de la luz por la clorofila o carotenoides asociados a los complejos PSII-LHCII y PSI-LHCI en la membrana. La energía absorbida de la luz se transfiere al RC del PSII o PSI. Esto da lugar a la separación de las cargas por la excitación de la clorofila del RC (P680) para dar lugar al estado singlete $^{1}P680^{*}$, de esta forma se dona un electrón al primer aceptor Pheophytin (Phe). En este proceso el RCII llega a oxidarse ($P680^{+}$). El electrón perdido por el P680 es reemplazado por un electrón transferido desde una molécula de agua al $P680^{+}$ vía un residuo activo de tirosina Y_Z, por lo que el agua se convierte en oxígeno y protones. Esta rotura de la molécula de agua ocurre en el PSII en la parte que se encuentra en el lumen en el complejo de oxígeno en evolución que alberga cuatro moléculas de manganeso como sitio catalítico. El electrón se transfiere desde Phe hasta la plastoquinona PQ_A y de la PQ_A^{-} a la plastoquinona PQ_B. Después de obtener la PQ_B^{-}, esta se reduce por un segundo electrón y también se protona para dar lugar a la PQ_BH_2 que abandona el PSII hacia el complejo b_6f.

La PQ_BH_2 perdida se reemplaza por una PQ_B oxidada proveniente de la reserva que se encuentra en la membrana tilacoidal. La plastoquinona reducida PQ_BH_2 libera sus electrones al citocromo b_6f y este simultáneamente los libera en el interior del lumen

del tilaciode. En este punto los electrones son transferidos desde el citocromo b_6f hacia el PSI ($P700^+$) en su estado de carga separado donde se reemplaza el electrón, este se transfiere a la cadena de oxido reducción del PSI. Esta cadena contiene A_0 una molécula de clorofila *a*, A_1 una plastoquinona y F_X, F_A y F_B tres agrupaciones de sulfuro-hierro.

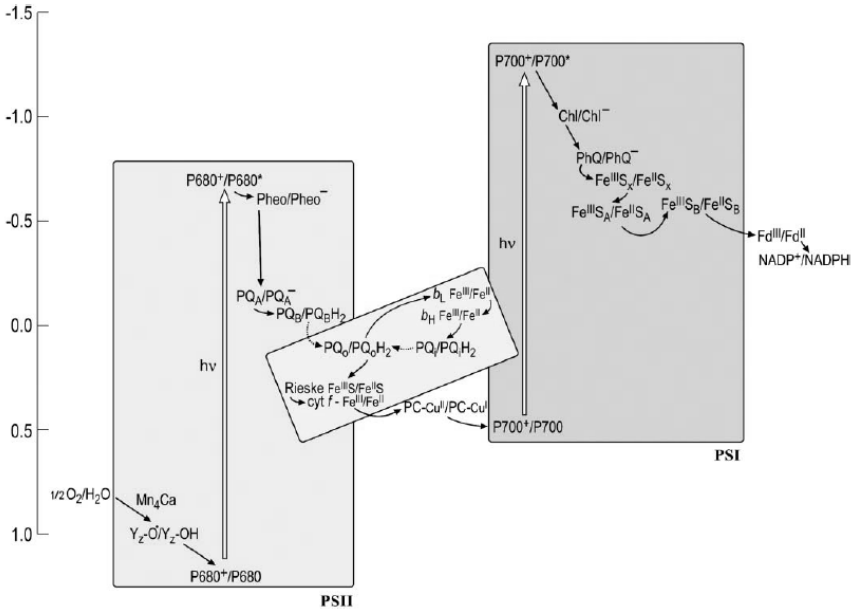


Figura 7: Descripción del esquema en Z del transorte de electrones fotosintético. Cada transportador se muestra en su estado de oxidación/reducción. Obtenido de Merchant y Sawaya 2005.

El aceptor final de los electrones es la ferredoxina (Fd) que está en la parte del estroma de la membrana tilacoidal. En el siguiente paso, las ferredoxina-$NADP^+$ oxidorreductasa (FNR) reduce el $NADP^+$ a NADPH. El movimiento de los electrones está acoplado a un pase de protones a través de la membrana tilacoidal, del estroma al lumen, formando un gradiente de pH a través de la membrana. Los protones acumulados en el lumen se mueven a favor de gradiente de concentración

desde el lumen hacia el estroma a través del complejo ATP-sintasa. De esta forma, el movimiento de protones favorece la síntesis de ATP (Boyer 1989).

Una ruta alternativa, y aún no del todo bien entendida, para el flujo de electrones es el flujo cíclico de electrones (CEF) en torno al PSI (Bendall y Manasse 1995). En este caso los electrones nunca alcanzan la $NADP^+$, sino que se mueven a través del complejo citocromo b_6f y los protones son bombeados del estroma hacia el lumen. El transporte cíclico de electrones no genera una producción de NADPH y oxigeno, únicamente produce ATP. Las moléculas de ATP y NADPH generadas en el proceso fotosintético son utilizadas como fuente de energía utilizada para llevar a cabo el resto de reacciones metabólicas.

6.5 Complejo NDH (NADPH deshidrogenasa)

En el CEF alrededor del PSI se produce un gradiente de pH a través de la membrana tilacoidal que es el responsable de la producción ATP sin acumulación de NADPH. En esta vía, los electrones del PSI vuelven al complejo citocromo b_6f y de ahí nuevamente al PSI vía la plastocianina. Una posible ruta por la cual los electrones pueden volver desde el aceptor del PSI al conjunto de PQ se conoce como la ruta dependiente de ferredoxina sensible a antimicina A y en la que participan PGR5 (Proton Gradient Regulation 5) y PGRL1 (PGR5-like1) (Munekage, *et al*. 2002; Hertle, *et al* 2013).

Sin embargo, hay evidencias de que existe una vía insensible a la antimicina A que implica al complejo NDH (NADPH deshidrogenasa) ubicado en la membrana tilacoidal. Este complejo es homólogo al complejo NADH deshidrogenasa (complejo I) de la mitocondria. La primera evidencia sobre la implicación del complejo NDH en el CEF proviene de la cianobacteria *Synechocystis* (PCC6803). Se observó que un mutante de pérdida de función en la proteína NdhB tenía bloqueado el CEF alrededor del PSI (Mi *et al*., 1995). Mediante la utilización de técnicas de transformación genética directa de cloroplastos, se ha observado que mutantes deficientes en el complejo NDH adolecen de un funcionamiento correcto del CEF, lo cual sugeriría una implicación funcional del complejo NDH en el transporte de

electrones del estroma al conjunto de PQ (Burrows *et al*., 1998; Shikanai *et al*., 1998; Suorsa *et al*., 2009).

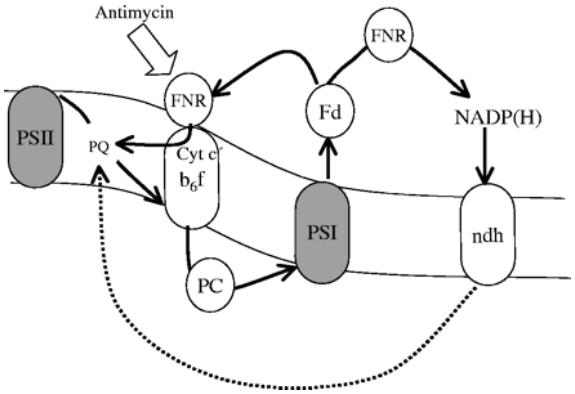


Figura 8: Posibles vías que siguen los electrones desde el aceptor del PSI al donador del PSI. Obtenido de Johnson 2005.

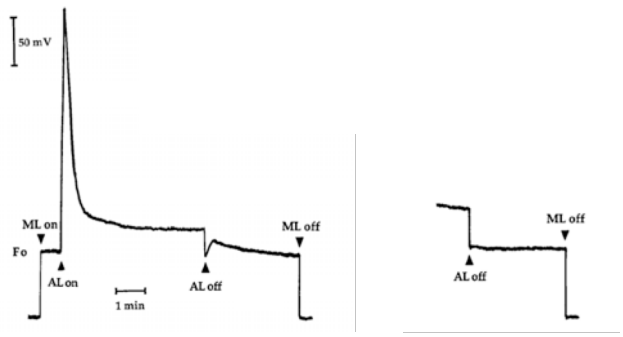
Los cloroplastos pertenecientes a plantas superiores conservan 11 genes que codifican para diferentes subunidades del complejo NDH (*ndhA*, *ndhB, ndhC, ndhD, ndhE, ndhF, ndhG, ndhH, ndhI, ndhJ, ndhK)*) (revisado en Shikanai, 2007, Ifuku *et al*., 2011) y que son homólogos a los genes *ndh* de bacterias. Todavía hoy en día se desconoce la naturaleza de la subunidad encargada encargada de la incorporación de los electrones en el complejo NDH del cloroplasto. Se ha propuesto que la FNR (ferredoxina-$NADP^+$ oxidorreductasa), implicada en la reducción del $NADP^+$ a NADPH, podría interaccionar con el complejo NDH proporcionando electrones desde la reducción del NADPH. Recientemente, se ha sugerido que la ferredoxina también podría ser una fuente de electrones, en este caso la enzima FNR jugaría un papel como transportador de electrones (Shikanai, 2007).

6.6 Medida del flujo cíclico de electrones CEF

Una de las mayores dificultades para cuantificar el CEF se deriva del hecho de que los transportadores de electrones implicados en el flujo cíclico son los mismos que

los implicados en el flujo lineal (citocromo b_6f, PSI, Fd y $NADP^+$). Por este motivo se han desarrollado diferentes técnicas para cuantificar, tanto *in vitro* como *in vivo*, la contribución del CEF en el flujo total de electrones.

Mediante la utilización de un flourómetro PAM (pulso de amplitud modulada), capaz de excitar la flourescencia de la clorofila sin afectar al estado de la fotosíntesis, se consigue monitorizar el flujo de electrones *in vivo* a través de un cambio en la fluorescencia de la clorofila. En particular, el flujo de electrones desde el complejo NDH hacia la cadena de transporte puede ser correctamente estimada a través de un incremento transitorio de la fluorescencia de la clorofila tras un período de iluminación con luz actínica (Schreiber *et al*., 1986). El hecho de que la luz roja lejana, que activa predominantemente la fotoquímica del PSI, sea capaz de apagar este incremento en la florescencia demuestra que el complejo NDH transfiere electrones hacia un grupo de plastoquinonas (Shikanai *et al*., 1998).



WT *ndh*

Figura 9: Fenotipo típico de fluorescencia de un mutante sin un complejo NDH de cloroplasto funcional: el incremento transitorio de la fluorescencia después de apagar la luz actínica (AL off) se reduce. Obtenido de Kofer *et al*., 1998.

El CEF cumple dos funciones principales:

1.- Durante la transición de oscuridad a luz, y en los primeros minutos de dicha transición, la síntesis de ATP generada a través de CEF permite la activación de las

proteínas implicadas en el ciclo de Calvin. Un ejemplo sería la propia activación de la Rubisco que requieres de ATP (Campbell and Ogren, 1992; Laisk *et al*., 2005; Joliot and Joliot, 2002).

2.- Por otra parte, el CEF, aún no aportando moléculas de NADPH, juega un papel importante en el balance/ratio de ATP/NADPH. El flujo lineal de electrones produce un ratio de 1.26 mientras que para el correcto funcionamiento del ciclo de Calvin se requieren un ratio de 1.5 o 1.66, y es precisamente este aporte extra de ATP el que vendría a ser aportado por la actividad CEF (Osmond, 1981)**.**

Otra de las implicaciones que se le atribuyen al CEF es la capacidad de prevenir la fotoinibición producida por un exceso de luz, regulando la disipación de energía y modificando el gradiente de pH (Heber y Walker, 1992).

Por otra parte, se ha visto que el CEF podría estar regulado por el estado redox del cloroplasto. En cloroplastos degradados, los electrones proporcionados por la ferredoxina o el NADPH son capaces de activar el flujo cíclico (Bukhov *et al*., 2002; Nandha *et al*., 2007). Ante otros tipos o situaciones de estrés, tal y como puede ser un exceso de iluminación, la actividad del CEF en relación con el flujo lineal de electrones (LEF) se ve incrementado, favoreciéndose la inducción de quenching no fotoquímico (NPQ) por la generación de un gradiente de pH a través de la membrana (Miyake *et al*., 2005). Así, mutantes de plantas de tabaco en el complejo NDH son más sensibles al estrés por exceso lumínico (Endo *et al*., 1999). Este mecanismo alternativo también lo utilizan las plantas ante situaciones de sequía o de altas temperaturas en las que el CEF se ve activado y se genera un gradiente de protones que inducen el NPQ (quenching no fotoquímico) que está implicado en disipar el exceso de energía (Golding and Johnson, 2004). Además, el CEF podría, en parte, regular el H_2O_2 que se genera tanto en procesos celulares del desarrollo como el producido en situaciones de estrés en las que se demandan mecanismos de defensa (Traverso, *et al*. 2013; O**'**Brien, *et al*. 2012; Karpi**n**ski, *et al*. 2013). En este sentido se ha demostrado que el H_2O_2 es capaz de activar la expresión del complejo NDH (Casano, *et al*. 2001).

En resumen, el CEF puede ser considerado como una válvula de seguridad en la que los electrones son reciclados a través del PSI generando un gradiente de protones y consecuentemente el oxígeno es reducido por lo que se minimiza la producción de especies reactivas de oxígeno (ROS). En este sentido, los dobles mutantes *pgr5 crr*, que tienen bloqueada tanto la vía dependiente de ferredoxina como la dependiente del complejo NDH, presentan claramente comprometido el correcto crecimiento y desarrollo de la planta. Esto sugiere que el transporte cíclico de electrones alrededor del PSI es necesario para una fotosíntesis eficiente y para un correcto crecimiento autotrófico (Munekage *et al*., 2004).

Figura 10: Fenotipos de crecimiento de plantas silvestres, mutantes simples *crr2-2*, *crr3*, *crr4-2* and *pgr5*, así como dobles mutantes *crr4-2 pgr5*, *crr3 pgr5* and *crr2-2 pgr5*. Obtenido de Munekage *et al*. 2004.

7. Edición del RNA

En las plantas vasculares se ha observado que determinadas citosinas (C) presentes en moléculas de RNA, pueden ser desaminadas a uracilos (U) mediante un proceso postranscripcional conocido como edición de RNA (Covello y Gray 1989; Gualberto *et al*., 1989; Hiesel, *et al*., 1989). De manera habitual, en una planta encontramos entre 30 y 40 residuos específicos de C presentes en el RNA cloroplástico que son susceptibles de ser editadas, y de manera más concreta dichos residuos de citosina modificables suman un total de 43 en el caso de *A. thaliana* (Ruwe *et al*., 2013).

Dicho número es inferior al encontrado en los RNA mitocondriales, para los cuales el número de residuos de citosinas editables asciende a 619 (Bentolila *et al*., 2013; Chateigner-Boutin y Small 2007). La edición del RNA da lugar a un cambio en el aminoácido codificado y que resulta, por ende, en la proteína funcional. En los casos más extremos de dicha conversión, se ha identificado que la edición del RNA puede generar codones de inicio o fin de la traducción en determinados mRNAs lo que es esencial para la correcta función génica (Okuda *et al*., 2007; Kotera *et al*., 2005). Este proceso se da como mecanismo corrector para restablecer el RNA mensajero funcional tanto en cloroplastos como en mitocondrias, cuyos genomas han sufrido previamente cambios de timina (T) a C (Gray y Covello 1993).

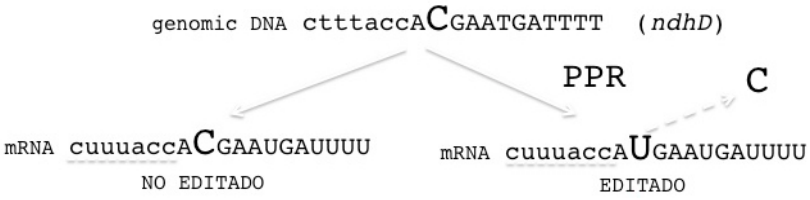


Figura 11: Ejemplo de la edición de RNA del transcrito *ndhD*, mediante el que se regula la aparición del codón de inicio de la traducción.

7.1 Edición del RNA del complejo NDH

La composición de la maquinaria molecular que lleva a cabo la edición del RNA en los cloroplastos, el editosoma, aún hoy en día se desconoce en su totalidad. La especificidad de la edición se da gracias al reconocimiento de un elemento 5'-*cis* próximo a la citosina editable presente en el mRNA diana y que es reconocido por una proteína que contiene un motivo de repetición pentatricopeptido (PPR). El primer factor *trans* que se identificó en Arabidopsis fue la proteína CRR4 (chlororespiratory reduction 4). De manera coincidente se observó que las plantas mutantes para este factor presentan una reducción en la actividad del complejo NDH (Kotera *et al*., 2005). Las plantas mutantes de pérdida de función de *crr4* presentan un defecto en la edición del RNA del tránscrito del gen *NdhD* lo que impide que se genere el codón de iniciación para la traducción y por lo tanto no se traduce la

proteína NdhD y no se acumula el complejo NDH. Estos factores *trans* también se encuentran y desempeñan el mismo papel en las mitocondrias (Zhu *et al*., 2012), así pues, se han descrito numerosas proteínas PPR implicadas en la edición del RNA tanto en cloroplastos como en mitocondrias, habiéndose identificado 450 miembros de la familia de las PPRs (Lurin *et al*., 2004).

CRR2 (chlororespiratory reduction 2) es un miembro de la familia de las PPRs, de los primeros en ser caracterizados (Hashimoto *et al*., 2003). Las plantas mutantes *crr2*, al igual que las plantas *crr4*, tienen un defecto en la actividad del complejo NDH. Las plantas mutantes *crr2* no acumulan el mRNA monocistrónico del gen *NdhB*, aunque sí su precursor junto con el gen *Rps7* situado en la zona delantera del mRNA. Por lo tanto CRR2 estaría implicado en el procesamiento del RNA y con la consiguiente eliminación de la región intergénica correspondiente al gen *Rps7*.

Puesto que hay más sitios editables que proteínas con capacidad de edición se ha sugerido que un mismo factor *trans* es capaz de reconocer más de dos sitios. Así pues, CRR22 y SOL2 están implicados en la edición de RNA de al menos tres sitios en el cloroplasto y de seis sitios en mitocondrias respectivamente (Okuda *et al*., 2009; Zhu *et al*., 2012). Esto podría ser debido a: 1) Las secuencias de reconocimiento de los distintos sitios diana, en cierta medida están conservadas (Pfalz *et al*., 2009); 2) Las proteínas PPRs contienen varios motivos PPR para reconocer diferentes dianas de RNA (Cai *et al*., 2011); 3) Algunas PPRs distinguen entre pirimidinas y purinas y no reconocen nucleótidos de manera exacta (Hammani *et al*., 2009).

En los cloroplastos, algunos de los sitios de edición de RNA además de las proteínas PPRs requieren también de la actividad de proteínas con las que forman los complejos proteína-RNA. En dichos complejos, denominados editosomas, se han identificado diferentes tipos o familias de proteínas acompañantes, entre las que destacarían las proteínas de la familia MORF (multiple organellar RNA editing factor), proteínas RIP (RNA editing factor interacting protein) que forman heterocomplejos y proteínas de la familia ORRM (organelle RRM protein)

(Takenaka *et al*., 2012; Bentolila *et al*., 2012; Sun *et al*., 2013). Estas proteínas son necesarias para la edición de algunas dianas pero no para otras. Por ello, la diversidad de editosomas existentes y encargados de la edición específica de todas y cada una de los distintos residuos de C en los mRNA de referencia vendría determinado, no únicamente por el sitio específico de reconocimiento por parte de la proteína PPR, sino también por los miembros adicionales que forman el complejo de edición. En este punto todavía quedan componentes de los editosomas por ser identificados que ayuden a entender la regulación de la edición del RNA frente a diferentes estímulos a los que están sometidas las plantas.

8. *OVEREXPRESSOR of CATIONIC PEROXIDASE* (*OCP3*)

Previamente, en el laboratorio en el que se ha realizado la presente Tesis Doctoral, se describió la implicación del gen *Ep5C* que codifica una peroxidasa catiónica extracelular en la respuesta defensiva de la planta. Se demostró que la expresión de dicho gen se induce en respuesta a la infección causada por la bacteria *Pseudomonas syringae* DC3000 como consecuencia directa de la producción de peróxido de hidrógeno (H_2O_2) (Coego *et al*., 2005a).

Las características más interesantes del gen *Ep5C* fueron su rápida inducción, encontrando un aumento de la transcripción incluso a las dos horas tras la aplicación del estímulo, y su especificidad en cuanto a la percepción de éste. La inducción de *Ep5C* es patente tras la infección con *P. syringae*, y curiosamente tras la aplicación de su hipotético co-sustrato, el H_2O_2. Sin embargo, *Ep5C* no muestra un aumento en su transcripción en respuesta a herida, ACC (precursor del etileno) o MeJA, consideradas hormonas clave en la defensa frente a patógenos necrotrofos; ni al SA, considerada la vía hormonal principal en respuesta a patógenos biotrofos.

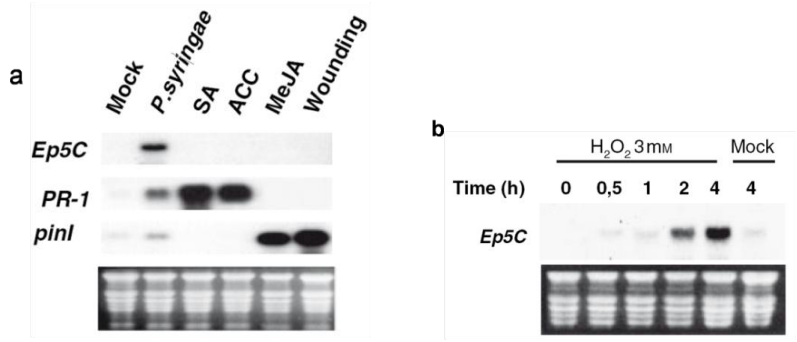


Figura 5: Inducción de *Ep5C* por *P. syringae* y H_2O_2 .Extraído de Coego *et al*., 2005a.

Debido al interés suscitado por dicho gen, y su expresión característica, el promotor de *Ep5C* fue aislado y fusionado al gen marcador ***β-glucuronidasa*** (*GUS*). Con esta construcción (*PEp5C::GUS*) se generaron plantas transgénicas de *Arabidopsis thaliana* con el objetivo de utilizar dichas líneas para profundizar en los mecanismos moleculares que regulan la expresión del gen *Ep5C* y por tanto la respuesta defensiva mediada por ROS. A partir de una población de semillas transgénicas *PEp5C:GUS*, se procedió a mutagenizarlas con metano-sulfonato de etilo (EMS) y en la M2 se seleccionaron plantas que mostraran expresión constitutiva del gen marcador *GUS*; a dichos mutantes se les denominó *ocp* (*overexpressor of cationic peroxidase*). En el laboratorio en el que se ha realizado la presente Tesis Doctoral, se han identificado y caracterizado los genes alterados en los mutantes *ocp11*, *ocp1* y *ocp3* (Coego *et al*., 2005b; Agorio y Vera 2007; Ramírez *et al*., 2009; Ramírez *et al*., 2010; López *et al*., 2011), estando todos ellos relacionados con diferentes aspectos de la respuesta defensiva.

El mutante *ocp11* contiene una mutación en el gen *ARGONAUTA4* (*AGO4*), componente de la ruta de metilación de ADN mediada por pequeños ARN interferentes (ARNsi). El análisis fenotípico del mutante *ocp11* (renombrado con posterioridad *ago4-2*) indica que AGO4 es esencial para la respuesta de las plantas al ataque de patógenos biotrofos, relacionando así la vía de RdDM con la respuesta defensiva de las plantas (Agorio y Vera, 2007). El mutante *ocp1* contiene una

alteración en la proteína NRPD2 que corresponde a la segunda subunidad mayor de las ARN polimerasas IV y V recientemente identificadas como específicas de plantas e implicadas en la RdDM y la inmunidad vegetal. Específicamente, mutantes en la ARN polimerasa V presentan un fenotipo de "priming", que consiste en una mayor activación de los genes de defensa gracias al incremento de la deposición de marcas positivas en las histonas lo que favorece la activación de la expresión génica (e.g., H3K4me3 y H3K9ac) (López *et al*., 2011).

El mutante *ocp3* codifica un factor transcripcional miembro de la familia de los Homeobox con el dominio HD o homeodominio característico (Coego *et al*., 2005b). En este caso, la caracterización fenotípica del mutante posiciona a la proteína OCP3 como un regulador negativo implicado en la resistencia de la planta frente a hongos necrotrofos, tales como *B. cinerea* y *P. cucumerina*, y de una manera dependiente de la percepción de JA a través de COI1 e independiente de SA o ET (Coego *et al*., 2005b). Además, OCP3 tiene un determinado papel en la Resistencia Sistémica Inducida (ISR) (Ramírez *et al*., 2010) y en la que la vía del JA es determinante. Por otra parte, se ha visto que OCP3 está implicada en la resistencia de las plantas a periodos prolongados de sequía, de tal forma que las plantas mutantes *ocp3* son más efectivas en el cierre estomático y soportan mejor períodos prolongados de sequía (Ramírez *et al*., 2009). Tales datos nos permiten hablar de una integración de la vía de señalización del ácido jasmónico (implicado en la respuesta a necrotrofos) y el control de la apertura estomática mediada por ABA.

OBJETIVOS

Con el fin de caracterizar de la implicación de OCP3 en los mecanismos defensivos de las plantas frente a patógenos necrotrofos, así como la función molecular de esta proteína, se establecieron los siguientes objetivos:

1. Análisis de la implicación de OCP3 en la regulación de la deposición de calosa frente al ataque de hongos necrotrofos. Evaluación de la implicación del ABA y el JA en este proceso.

2. Identificación del mecanismo molecular en el que está implicado la proteína OCP3, clasificada como un factor de transcripción nuclear miembro de la familia de los Homeobox.

3. Dada la importancia del ABA en los fenotipos descritos para las plantas *ocp3*, se propone el estudio de los diferentes receptores de ABA, con el fin de identificar una posible especificidad en la percepción del ABA patogénico.

Capítulo I

Arabidopsis *ocp3* mutant reveals a mechanism linking ABA and JA to pathogen-induced callose deposition

Javier García-Andrade, Vicente Ramírez, Victor Flors[(1)] and Pablo Vera[(*)]

Instituto de Biología Molecular y Celular de Plantas (IBMCP). Consejo Superior de Investigaciones Científicas (CSIC)-Universidad Politécnica de Valencia. Camino de Vera, s/n. 46022 Valencia, Spain.

[(1)] Present address: Plant Physiology Section, Dpt. Experimental Sciences, Universidad Jaime I, Castellón 12071, Spain.

The Plant Journal (2011) 67 (5):783-794

ABSTRACT

In the present study, we evaluated the role of the defense related gene *OCP3* in callose deposition as a response to two necrotrophic fungal pathogens, *Botrytis cinerea* and *Plectosphaerella cucumerina*. *ocp3* plants exhibited accelerated and intensified callose deposition in response to fungal infection associated with enhanced disease resistance to the two pathogens. A series of double mutant analyses showed potentiation of callose deposition and the heightened disease resistance phenotype in *ocp3* plants required the plant hormone abscisic acid (ABA) and the *PMR4* gene encoding a callose synthase. This was congruent with an observation that *ocp3* plants exhibited increased ABA accumulation, and ABA was rapidly synthesized following fungal infection in wild type plants. Furthermore, we determined that potentiation of callose deposition in *ocp3* plants, including enhanced disease resistance, also required jasmonic acid (JA) recognition though a COI1 receptor, however JA was not required for basal callose deposition following fungal infection. In addition, potentiation of callose deposition in *ocp3* plants appeared to follow a different mechanism than that proposed for callose β-amino-butyric acid (BABA)–induced resistance and priming, because *ocp3* plants responded to BABA-induced priming for callose deposition and induced resistance of a magnitude similar to that observed in wild type plants. Our results point to a model in which OCP3 represents a specific control point for callose deposition regulated by JA yet ultimately requiring ABA. These results provide new insights into the mechanism of callose deposition regulation in response to pathogen attack, however the complexities of the processes remain poorly understood.

INTRODUCTION

The plant cell wall exhibits a dynamic organization essential in cell division, enlargement, and differentiation, and is actively engaged in plant cell response to imposed biotic stresses (Ellis *et al*., 2002; Nishimura *et al.,* 2003; Roberts, 1990; Vorwerk *et al*., 2004). Many pathogens have evolved the capacity to directly penetrate the cell wall and gain access to cellular nutrients. In turn, plant cells have developed pre-invasive structural defenses, including a cuticular layer, and local modifications of cell walls that serve to obstruct the advance of an intruder (Mendgen *et al*., 1996; van Kan *et al*., 2006). The chemical nature of the modified cell wall, the deposition speed of new constituents, and degree of extracellular matrix compaction are crucial for a successful defense (Hückelhoven, 2007). Successful pathogens face a second barrier of early post-invasive defenses, including papillae formation and the rapid accumulation of reactive oxygen species (ROS) (revised by Asselbergh *et al*., 2008). These post-invasive defenses are finally followed by transcriptional reprogramming and corresponding changes in metabolic and hormonal profiles (Anderson *et al*., 2004; Lorenzo *et al*., 2004; Yadav *et al*., 2005). Therefore, the plant immune system is ultimately comprised of a complex web of signaling pathways and the interplay between different hormones (Pieterse *et al*., 2009).

The build up of papillae is a characteristic cellular response that occurs on the inner surface of the epidermal cell wall and directly below the attempted entry point of a pathogen. The major constituent of papillae is callose, a β-1,3-glucan, but other constituents including polysaccharides, phenolic compounds, reactive oxygen intermediates, and proteins have also been identified (Bestwick *et al.*, 1997; Thordal-Christensen *et al*., 1997; Hématy *et al*. 2009). Callose is distributed throughout a plant and is synthesized at different stages of plant development. It is also manufactured in response to different biotic and abiotic stress. Twelve genes encoding callose synthases (GSL/CalS; EC 2.4.1.34; UDP-glucose:1,3-β-D-glucosyl transferase) have been identified in *Arabidopsis* on the basis of sequence homology (Richmond and Somervillle, 2000; Verma and Hong, 2001). In particular, GSL5

(=PMR4) is responsible for pathogen-induced callose deposition (Jacobs *et al*., 2003; Nishimura *et al*., 2003); and *pmr4* plants that lack callose deposition following pathogen attack show enhanced susceptibility to the fungal pathogens *Alternaria brassicicola* and *Pythium irregulare* (Adie *et al*., 2007; Flors *et al*., 2008).

The plant hormone abcisic acid (ABA) has long been recognized as integral in the control of different aspects of plant development and abiotic stress. More recently, ABA, in addition to salicylic acid (SA) and jasmonic acid (JA), has emerged as an important regulator mediating crucial aspects of a plant's response to imposed biotic stress, which acts through a still poorly understood mechanism (Adie *et al*., 2007; Fan *et al*., 2009). ABA has been shown to positively regulate callose deposition, allowing an early and efficient build up of papillae at sites of infection, and in turn positively regulate disease resistance against several plant pathogens (Nishimura *et al*., 2003; Ton and Mauch-Mani, 2004). The respective ABA deficient and insensitive mutants, *aba1-3* and *abi1-1*, display enhanced susceptibility to *Leptospheria maculans,* along with a significantly decreased level of callose deposition (Kaliff *et al*., 2007). In addition*,* the *cds2-1D* mutant, where *9-CIS-EPOXYCAROTENOID DIOXIGENASE5 (NCED5)* (one of the six genes encoding the ABA biosynthetic enzyme NCED) is overexpressed, shows both enhanced endogenous levels of ABA and increased resistance to *A. brassicicola* (Fan *et al*., 2009). Furthermore, exogenous ABA application protects Arabidopsis plants against the fungal pathogens *A. brassicicola* and *Plectosphaerella cucumerina* (Ton and Mauch-Mani, 2004). The non-protein amino acid *β*-amino-butyric acid (BABA) protects plants against a wide range of pathogens through a BABA-induced resistance (BABA-IR) mechanism, where the protective effect is based primarily on a primed accumulation of callose. Mutants impaired in ABA signaling, as well as the callose-deficient mutant *pmr4*, failed to express BABA-IR against the necrotrophic fungus *P. cucumerina*, demonstrating that BABA-IR requires intact ABA signaling and callose biosynthesis (Ton and Mauch-Mani, 2004; Flors *et al.* 2008).

Despite the importance of JA as a regulator of disease resistance to fungal

pathogens, the regulation of callose deposition by JA remains largely unexplored, and its interaction with ABA signaling is not well understood. Genetic investigation of disease resistance in Arabidopsis determined that COI1 is a key player in the JA pathway. Furthermore, COI1 was identified as a JA receptor involved in a proteaosome-meditated protein degradation pathway that integrates cellular responses triggered by JA (Xie *et al*., 1998; Xu *et al*., 2002; Chini *et al*., 2007; Thines *et al*., 2007; Sheard *et al*., 2010). In addition, COI1 loss-of-function was previously identified to result in a marked susceptibility to fungal pathogens (Thomma *et al*., 1998).

Previous evidence indicated that OCP3, a protein with structural similarities to transcription factors of the homeodomain (HD) family, functions as a negative regulator to necrotrophic fungal pathogen disease resistance through a JA-mediated mechanism requiring COI1 (Coego *et al.,* 2005b; Ramirez *et al*., 2010). Also Ramirez *et al.* (2009) demonstrated OCP3 serves a regulatory role in an adaptive response to drought in an ABA-dependent manner. In the present work, we unveiled a connection between callose deposition, ABA, and JA signaling, where OCP3 played an important role in fine-tuning callose deposition following recognition of necrotrophic fungal pathogens such as *Botrytis cinerea* and *Plectosphaerella cucumerina*.

RESULTS

ocp3 plants exhibit heightened deposition of callose at fungal infection sites

Previous reports indicated a recessive mutation in the Arabidopsis *OCP3* locus promoted an enhanced disease resistance response to necrotrophic fungal pathogens (Coego *et al*., 2005b; Ramirez *et al*., 2009). The present study served to demonstrate if callose deposition was involved in an observed increased disease resistance in *ocp3* plants. The participation of callose was assessed by challenging parental wild-type Arabidopsis (Col-0) and *ocp3* plants with either *Botrytis cinerea* or

Plectosphaerella cucumerina. Subsequently, comparative cytological observations were performed at the sites of attempted fungal infection at various times following inoculation. The degree of callose deposition induction in inoculated leaves was monitored after staining with aniline blue, and examination by UV fluorescence microscopy. Callose deposition was quantified by determining the relative number of yellow pixels on digital photographs that corresponded to pathogen-induced callose. Results indicated neither Arabidopsis Col-0 nor *ocp3* plants exhibited aniline blue staining in control leaves at fungal inoculation sites (Fig. 1A, B). However, Col-0 plants deposited callose locally at the site of fungal inoculation and deposition progressively increased at 24 and 48 hours post inoculation (h.p.i.) (Fig. 1A-C). Callose deposition was observed near fungal infection sites and occurred following inoculation with either *B. cinerea* and *P. cucumerina*. In susceptible plants symptoms of fungal disease appear in the form of necrotic lesions in inoculated leaves. Lesions are routinely measured following inoculation, and are used to quantify the degree of plant susceptibility (Fig. 1D).

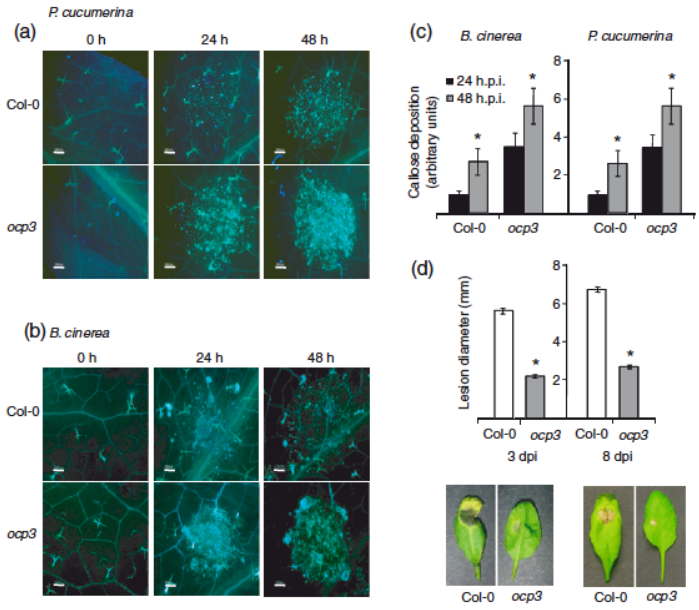


Figure 1. *ocp3* mutant plants demonstrate a rapid and strong callose deposition against the necrotrophic fungi *B. cinerea* and *P. cucumerina*. Aniline blue staining and epifluorescence microscopy was applied to visualize callose accumulation.

Micrographs showing callose deposition following *P. cucumerina* (a) and *B. cinerea* (b) infection in Arabidopsis Col-0 and *ocp3* plants at 0, 24 and 48 h post inoculation (h.p.i.). Scale bars represent 500 µm. (c) Pathogen-induced callose deposition was calculated as arbitrary units by quantifying the number of yellows pixels per million on digital micrographs of infected leaves at 24 and 48 hpi. Bars represent mean ± SD, n = 15 independent replicates. (d) Lesion size resulting from *B. cinerea* and *P. cucumerina* at 3 and 8 days post inoculation (dpi), respectively. Values are means and ±SE (n = 50). Asterisks indicate significant differences between the wild-type and *ocp3* plants least significant difference (LSD) test; $P < 0.05$. Below are shown representative leaves from Col-0 and *ocp3* plants after inoculation with *B. cinerea* (left) or with *P. cucumerina* (right).

It is notable that concurrent analysis in *ocp3* plants revealed the mutant reacted to the presence of either *B. cinerea* or *P. cucumerina* with increased callose deposition relative to parental Col-0 (Fig. 1A-C). Furthermore, greater callose deposition at earlier fungal infection stages (24 h.p.i.) indicated that callose deposition timing was also altered in *ocp3* plants. In the case of *B. cinerea* infection, where the appearance and abundance of hyphae was easily observed by staining with calcofluor (Fig. S1), callose deposition in inoculated *ocp3* leaves demarcated zones where fungal growth and colonization was impeded. This suggested a role of early callose induction as a structural barrier to block fungal invasion on the leaf surface. Wild-type plants exhibited decreased callose deposition around fungal infection sites, and hyphae grew faster and colonized the host tissues more readily (Fig. S1).

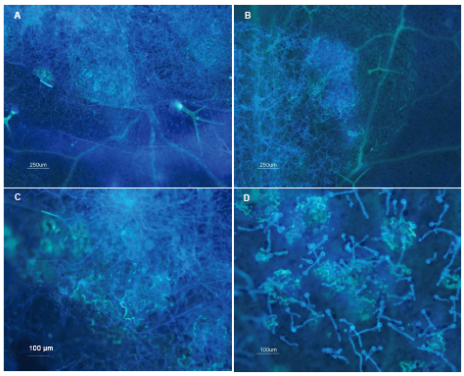


Figure S1. Different tissue colonization by *Botrytis cinerea* in Col-0 and *ocp3* plants. Micrographs show callose accumulation visualized by calcofluor and aniline blue staining at 48 hpi. Fungal structures are stained bright blue and callose is stained yellow. (a, c) are Col-0 plants, and (b, d) are *ocp3* plants infected by the fungus (at different scales).

The earlier and heightened production of callose in *ocp3* mutant plants was correlated with an enhanced disease resistance to both fungal pathogens. These results suggested that OCP3 could be negatively regulating, in a linked manner, deposition of callose and disease resistance.

Enhanced callose deposition and disease resistance in *ocp3* plants require PMR4

Twelve *callose synthase* (*GSL/CalS*) genes have been identified in the Arabidopsis genome, and only *GSL5/PMR4* is responsible for pathogen-induced callose deposition (Jacobs *et al*., 2003; Nishimura *et al.,* 2003). Therefore, we examined the role of PMR4 in *ocp3*-associated disease resistance. We generated a homozygous double mutant *ocp3 pmr4* Arabidopsis strain that allowed us to evaluate its response to fungal infection relative to single mutant parental lines. Compared to Col-0 plants, *pmr4* plants revealed a notable increase in disease susceptibility to *P. cucumerina* (Fig. 2A, B). This result was consistent with previous reports for other fungal pathogens (Adie *et al.,* 2007; Flors *et al.,* 2008). The behavior of *pmr4* plants is thus opposed to that observed for *ocp3* plants. However, the *pmr4* mutation suppressed the enhanced disease resistance attributable to the *ocp3* mutation since *ocp3 pmr4* plants behaved as single *pmr4* mutant plants showing the remarkable increase in disease susceptibility towards *P.cucumerina* (Figure 2A-B). Likewise, the increased callose deposition observed in *ocp3* plants following fungal infection becomes abrogated when assayed in a *pmr4* background (Figure 2C). For *pmr4* and for *ocp3 pmr4* plants we observed a complete inhibition of callose deposition when compared to Col-0 plants. These results suggested an intact PMR4 is required for the *ocp3* mutant to mount the heightened disease resistant response.

GSL5/PMR4 expression measured by real-time RT-qPCR in both Col-0 and *ocp3* plants revealed that transcript levels for the *GSL5/PMR4* gene experienced no significant changes following fungal inoculation (Fig. 2D). This is in marked contrast with the induction of *PDF1.2a* expression, a disease resistance marker to necrotrophs (Penninckx *et al*., 1998). The induced expression of *PDF1.2a* is remarkable in *ocp3* plants (Fig. 2D). The lack of statistical significant induction in

GSL5/PMR4 gene expression following fungal inoculation suggested that heightened callose deposition, which occurs in plants following fungal infection is most likely regulated at the post transcriptional level.

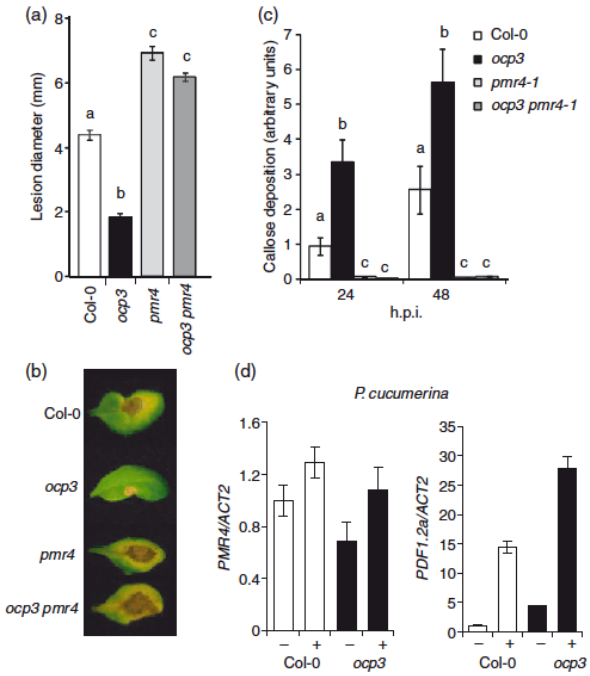


Figure 2. *ocp3*-associated disease resistance requires PMR4. (a) Lesion size resulting from *P. cucumerina* infection in Col-0, *ocp3*, *pmr4*, and *ocp3 pmr4* plants at 8 days post inoculation (dpi). Values are means and ± SE (n = 50). Different letters indicate significant differences (LSD test; $P < 0.05$). (b) Representative leaves from each genotype at 12 days following inoculation with *P. cucumerina*. (c) Quantification of *P. cucumerina*-induced callose deposition was performed as described in Figure 1. (d) RT-qPCR analysis for *PMR4* and *PDF1.2a* gene expression at 48 h following *P. cucumerina* inoculation in Col-0 (open bars) and *ocp3* plants (filled bars). *PMR4* and *PDF1.2a* expression was normalized to *ACTIN2* expression. (-) and (+) indicate mock- and *P. cucumerina*-inoculated plants, respectively. Bars represent mean ± SD, n=3 independent replicates.

Enhanced callose deposition and disease resistance in *ocp3* plants require ABA biosynthesis

Exogenous application of ABA has been reported to prime callose deposition during the initial stages of fungal colonization (Asselbergh *et al*., 2008; Anderson *et al*.,

2004; Flors *et al*., 2008; Mohr and Cahill, 2007, Fan *et al*., 2009). Furthermore, it has been shown ABA is required for JA production and subsequent activation of plant defenses against *Pitium irregular*e (Adie *et al*. 2007). ABA2 encodes a short-chain dehydrogenase/reductase 1 (SDR1), which catalyzes a key step in ABA biosynthesis. Accordingly, *aba2* plants exhibit highly reduced ABA levels (González-Guzmán *et al.,* 2002). These observations, in addition to previous results indicating that *ocp3* plants show an increased sensitivity to ABA (Ramírez *et al*., 2009), led us to determine if ABA is pivotal for the enhancement of callose deposition observed in *ocp3* plants. We therefore crossed *ocp3* with *aba2* plants and generated the double mutant *ocp3 aba2*. We subsequently compared the response of Col-0, *ocp3*, *aba2*, and *ocp3 aba2* plants following inoculation with *P. cucumerina*. Results indicated *aba2* plants possessed increased *P. cucumerina* disease susceptibility (Fig. 3A).

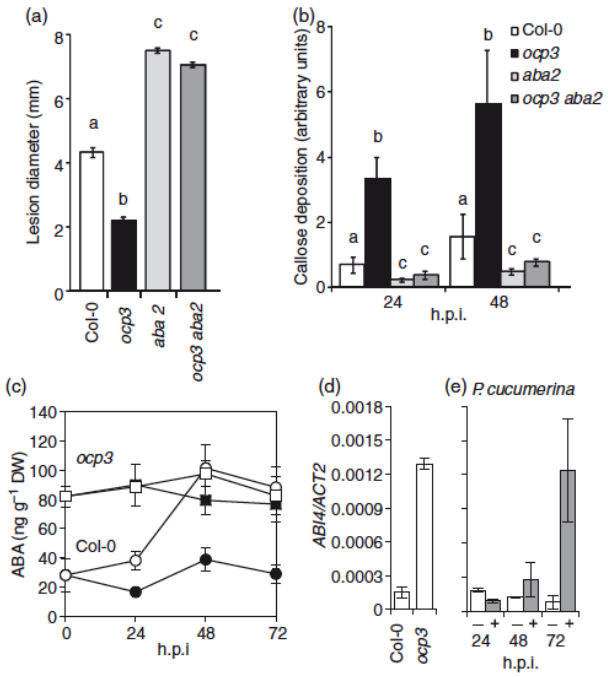


Figure 3. Effect of impaired ABA biosynthesis on *ocp3*-mediated callose deposition and disease resistance response. (a) Lesion size resulting from *P. cucumerina* infection in Col-0, *ocp3*, *aba2* and *ocp3 aba2* plants. Lesion measurements were performed as described in previous figures at 8 days post

inoculation (dpi). (b) Quantification of *P. cucumerina*-induced callose deposition performed as described in Figure 1. (c) Relative ABA accumulation in Col-0 (circles) and *ocp3* (squares) plants at different times following infection with *P. cucumerina* (open symbols) and mock-inoculated (filled symbols). Plants were inoculated as described in Figure 1. Plant tissue was collected and ABA levels were determined in freeze-dried material by HPLC-MS. Results are means ± SD (n = 5). (d) RT-qPCR analysis for constitutive *ABI4* gene expression in Col-0 and *ocp3* non-inoculated plants. (e) RT-qPCR analysis for induced expression of *ABI4* gene expression at 24, 48, and 72 hpi with *P. cucumerina*. (-) indicates mock-inoculation and (+) indicates inoculation with *P. cucumerina*. *ABI4* expression in (d) and (e) was normalized to *ACTIN2* expression. Bars represent mean ± SD, n=3 independent replicates.

In addition, the *aba2* mutation completely impaired the heightened resistance of *ocp3* plants, as demonstrated by a markedly susceptible *ocp3 aba2-1* double mutant phenotype. Callose deposition was notably reduced in *aba2* mutant plants to levels below those attained in Col-0 (Fig. 3B). Analysis of *ocp3 aba2* plants revealed that callose deposition following pathogen infection was similarly compromised (Fig. 3B), and paralleled the observed loss of *ocp3*-associated increased disease resistance when assayed in the highly reduced ABA level *aba2* plants (Fig. 3A). These results highlight the importance of ABA for disease resistance and callose deposition in response to *P. cucumerina*, and also in particular indicate the *ocp3*-associated phenotype requires ABA.

ocp3 plants accumulate increased ABA levels

The *ocp3*-asociated phenotype required ABA, and ABA participated in priming for callose deposition, therefore it was possible ABA levels would be up-regulated in *ocp3* plants. Alternatively, ABA biosynthesis would be induced following fungal infection, and if so, induction would be heightened in *ocp3* plants. To test between ABA up-regulation or increased induction in *ocp3* plants, we measured endogenous ABA content by HPLC-MS in leaf extracts derived from Col-0 and *ocp3* plants at 0, 24, 48, and 72 h following inoculation with *P. cucumerina* and extracts from mock-inoculated plants. Results showed fungal inoculation in Col-0 plants promoted an increase in ABA accumulation; which was negligible between o and 24 h after inoculation and peaked at 48 h with a 3-fold ABA accumulation (Fig. 3C). Interestingly, a concurrent analysis performed in *ocp3* plants revealed a 3-fold

increase in the constitutive levels of endogenous ABA, with no further enhancement at any period following fungal inoculation (Fig. 3C). Quantitative RT-PCR expression analysis for *ABI4*, which encodes a transcription factor required for chemical-induced priming of callose deposition (Ton and Mauch-mani, 2004), revealed its constitutive up-regulation in healthy *ocp3* plants compared to Col-0 (Fig. 3D). Furthermore, in wild type plants expression of *ABI4* was progressively up-regulated following inoculation with *P. cucumerina*, and reached a maximum level at 72 h post-inoculation (hpi) (Fig. 3E). The results therefore indicated that ABA biosynthesis and signaling was in fact triggered by *P. cucumerina* infection in Col-0 plants, which reinforces the hypothesis that the sharp increase in ABA may prime callose biosynthesis and deposition. In *ocp3* plants, where ABA levels are already high, the priming effect might already be established and thus allow a more efficient deployment or potentiation of callose deposition once the pathogenic signal is recognized.

COI1 is not essential for basal callose deposition but is required for potentiated callose deposition in *ocp3* plants

JA is the essential plant hormone in the response to necrotrophic fungal pathogens (Browse 2009), and COI1 is key in controlling the JA response pathway (Sheard *et al*., 2010). Results demonstrated *ocp3*-associated disease resistance was dependent on appropriate recognition of JA, and correspondingly, the double mutant *ocp3 coi1* plants were hyper-susceptible to fungal necrotrophs (Coego *et al.,* 2005b). Therefore, if *ocp3* plants require JA, it is possible JA levels in *ocp3* plants are higher relative to Col-0 plants. We measured endogenous levels of JA-Ile, the active form of the hormone *in vivo*, following inoculation with *B. cinerea*. Figure 4A shows that *ocp3* plants did not exhibit enhanced accumulation of JA-Ile under resting conditions. Furthermore, fungal inoculation in Col-0 plants promoted a transient increase in JA-Ile levels that peaked at 48 h with a subsequent decline. *ocp3* plants responded similarly to *B. cinerea* with a similar spike in JA-Ile accumulation . Concurrently, we determined camalexin accumulation, which is also an important molecule for plant defense (Glazebrook *et al*., 1996; Thomma *et al*., 1999), and is

required for *B. cinerea* basal resistance in Arabidopsis (Ferrari *et al*., 2003, 2007; Kliebenstein *et al*., 2005). Significant differences between Col-0 and *ocp3* plants were not observed at early stages of infection (Fig. 4B).

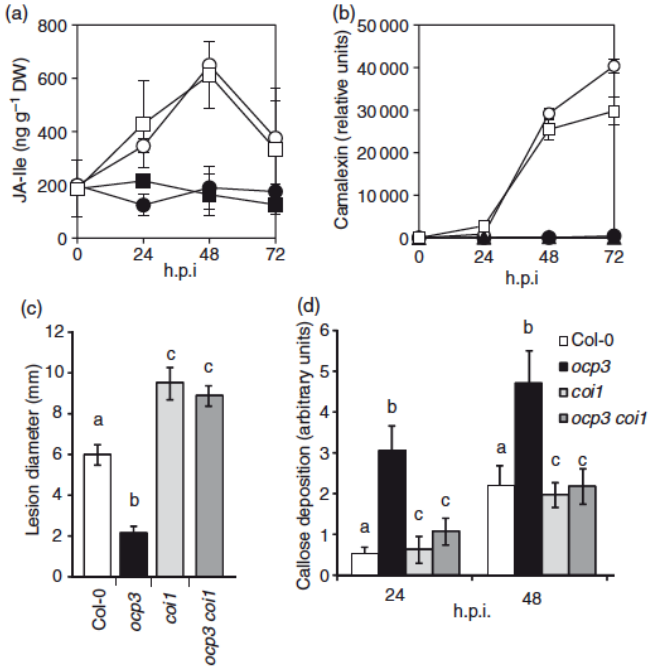


Figure 4. Deductions regarding JA signaling via COI1 in the *ocp3*-callose deposition phenotype. Relative JA-Ile (a) and camalexin (b) accumulation in Col-0 (circles) and *ocp3* (squares) plants at different times following infection with *B. cinerea* (open symbols) compared to mock-inoculated plants (filled symbols). Plants were inoculated and hormone levels quantified as described in Figure 3. (c) Lesion size resulting from *B. cinerea* infection in Col-0, *ocp3*, *coi1* and *ocp3 coi1* plants at 3 days post inoculation (dpi). Lesion measurements were performed as previously described. (d) Quantification of *B. cinerea*-induced callose deposition performed as described previously.

We further investigated the role of JA in the potentiation of callose deposition in *ocp3* plants by measuring pathogen-elicited callose deposition at 0, 24, and 48 h in Col-0, *ocp3*, *coi1-1*, and *ocp3 coi1-1* plants following inoculation with *B. cinerea*. Figure 4D shows that at 24 and 48 h.p.i., *ocp3* plants retained the characteristic increased callose deposition. Interestingly, *coi1* callose deposition was similar to that observed in Col-0 plants (Fig. 4D), indicating that COI1 is not required for basal

callose deposition following fungal attack. Nevertheless, basal callose deposition was not sufficient to arrest pathogen growth as *coi1* plants show hypersusceptibility to the pathogen (Fig. 4C). It is worth noting the observed potentiation of callose deposition that occurred in *ocp3* plants returned to wild-type levels when assayed in a *coi1-1* mutant background (i.e., *ocp3 coi1-1* mutant) (Fig. 4D), but instead the enhanced resistance associated with the *ocp3* mutation was completely lost (Fig. 4C). These results suggest that in wild-type plants, basal callose deposition is not dependent on COI1, and callose is only effective against pathogens if COI1-dependent defenses remain intact. Furthermore, *coi1* brings the increased accumulation of callose in *ocp3* plants to a wild-type level, suggesting the COI1-dependent pathway is only required for the potentiation of callose deposition characteristic of *ocp3* plants, but not for basal deposition. Therefore, enhanced resistance in *ocp3* plants, in addition to potentiated callose deposition, acts in concert to effectively defend the plant from the pathogen.

These results enabled us to discern between basal and potentiated callose deposition as two separate yet additive processes. The latter negatively regulated by OCP3 in a COI1-dependent manner, the former COI1-independent; both requiring of ABA and PMR4.

Flg22, but not DCB, potentiates callose deposition in *ocp3* plants

Different elicitors rather than fungal pathogens can also trigger the deposition of callose in plants. Therefore, we tested the response to the bacterial flagellar peptide elicitor Flg22 (Gomez-Gomez *et al*., 1999), and the chemical compound dichlorobenzinil (DCB) (Nishimura *et al*., 2003), which are unrelated inducers of callose deposition. Flg22 was also chosen because of its effectiveness in protecting Arabidopsis against *B. cinerea* (Ferrari *et al*., 2007). In addition, Flg22 and DCB-induced callose deposition require the callose synthase PMR4. We utilized a seedling assay to study elicitor-mediated induction of callose deposition in cotyledons. Results indicated callose deposition induced by either 1µM Flg22 or 100µM DCB were readily detectable above background levels in Col-0 seedlings following staining with aniline blue (Fig. 5A, B). Interestingly, *ocp3* seedlings

responded to Flg22 treatment, but not DCB, with a notable potentiation in the induced deposition of callose observed in Col-0. Flg22-induced potentiation effect in *ocp3* plants was strongly reduced when assayed in the *ocp3 aba2* plants, and completely lost in the double *ocp3 pmr4* mutant plants, indicating the requirement of intact ABA and PMR4 for potentiation to occur. Conversely, the DCB-induced callose deposition observed in *ocp3* plants was not compromised when assayed in the double *ocp3 aba2* mutant, while it was abated in *ocp3 pmr4* plants. These results suggest that Flg22 and DCB function at different levels in the pathway leading to callose deposition, but communicate via PMR4.

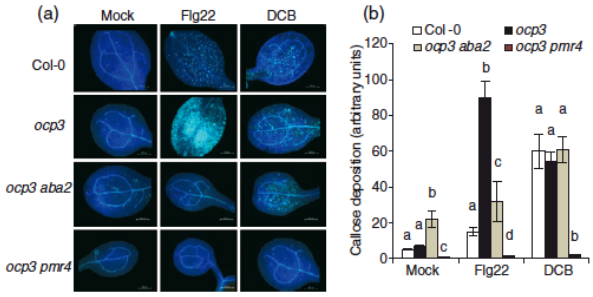


Figure 5. Genetic requirements for Flg22-induced callose formation in *opc3* plants. One week old plants grown in MS medium were treated with water (mock), 1 µM Flg22 or 100 µM DCB. Callose accumulation was visualized 24 h post treatment by aniline blue staining and epifluorescence microscopy. (a) Representative micrographs showing plant responses. (b) Quantification of callose accumulation. Bars represent mean ± SD (n = 15). Different letters indicate statistically significant differences (LSD test; $P < 0.05$).

In addition, the Flg22-mediated potentiation of callose deposition in *ocp3* plants was similar to the potentiation effect when infected by fungi. Moreover, and accordingly with this, we observed that *OCP3* expression in wild-type plants was down-regulated after treatment with Flg22 (Fig. S2) in a manner similar to what occurs following fungal inoculation (Coego *et al*., 2005). This may provide insights into understanding why Flg22, fulfilling its role as a pathogen-associated molecular pattern (PAMPs) molecule, shows efficacy in protecting Arabidopsis against *B. cinerea* (Ferrari *et al*., 2007).

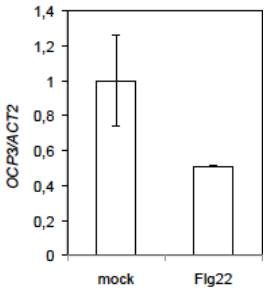


Figure S2. RT-qPCR analysis for *OCP3* gene expression following Fgl22 treatment in Col-0 plants (filled bars). *OCP3* expression was normalized to *ACTIN2* expression Bars represent mean ± SE, $n = 3$ independent replicates.

The potentiated deposition of callose in *ocp3* plants is unrelated to the priming effect mediated by BABA

The non-protein amino acid *β*-amino-butyric acid (BABA) protects Arabidopsis against a wide range of pathogens, and this effect is primarily based on ABA-dependent priming for callose (Flors *et al.,* 2008). Ton and Mauch-Mani (2004) blocked BABA-induced resistance (BABA-IR) and primed callose deposition in *pmr4* and *aba1* plants after infection with *P. cucumerina*. The similarities with *ocp3*-associated responses prompted us to determine if *OCP3* is involved in regulating BABA priming for callose deposition and fungal disease resistance. Therefore, we tested BABA effectiveness in *ocp3* plants. Mutant treatment with 150 µM BABA resulted in a 40% reduction in lesion size following infection with *P. cucumerina*, which was additive to that already shown in water-treated *ocp3* plants (Fig. 6B). This added primed resistance was of a magnitud similar to that attained in BABA-treated Col-0 plants. Cytological observations performed at the infection sites in water and BABA-treated Col-0 and *ocp3* plants revealed BABA-IR expression against *P. cucumerina* was associated with primed deposition of callose as indicated by aniline blue staining (Fig. 6A). In *ocp3* plants, BABA-priming for callose deposition following *P. cucumerina* infection was additive to that already potentiated in water-treated *ocp3* plants (Fig. 6C). These results demonstrated that OCP3 was not required for the expression of BABA-IR and priming for callose.

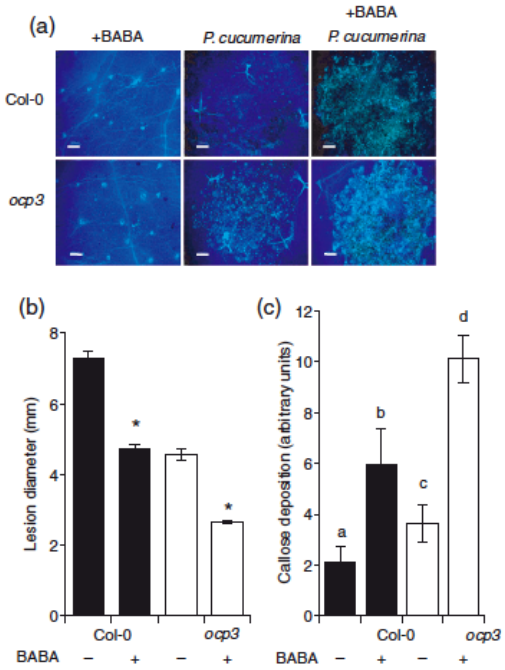


Figure 6. Quantification of BABA-induced resistance against *P. cucumerina*.in Col-0 and *ocp3* plants. Four-week-old plants were soil-drenched with water or 150 µM BABA. Two days after chemical treatment, plants were challenged with *P. cucumerina* as described in Figure 1. (a) Micrographs show callose accumulation visualized by calcofluor and aniline blue staining. Scale bars represent 500 µm. (b) Lesion size resulting from the inoculation in the two genotypes at 8 days post inoculation (dpi). Bars represent mean ± SE (n = 50). Asterisks indicate significant differences (LSD test; $P < 0.05$). (c) Quantification of callose accumulation at 48 hpi measured as described previously. Asterisks and different letters indicate significant differences between wild-type (filled bars) and *ocp3* (open bars) plants (LSD test; $P < 0.05$, n = 15). (-) indicates plants drenched with water and (+) indicates plants drenched with BABA.

The disease resistance mechanism and linked callose deposition controlled by OCP3 appeared to operate through a distinct signaling pathway, separate from BABA-mediated priming, and where only the former required intact JA. In any case, both pathways were ultimately under the control of ABA and both mediated callose deposition through PMR4.

DISCUSSION

In the present study we demonstrated that resistant *ocp3* plants responded to inoculation with *B. cinerea* and *P. cucumerina* spores with heightened deposition of callose at fungal infection sites. The success of a plant to combat microbial pathogens depends on the speed and intensity of response in activating primary defenses around the pathogen invasion sites. A simple explanation of why pathogen colonization in *ocp3* plants was severely compromised is that the mutant plants were pre-alerted in a more efficient manner to fungal presence. An efficient deployment of first line defenses, in which rapid deposition of callose is primary and serves as a structural barrier to block fungal invasion in the leaf surface, may be the basis for the enhanced fungal resistance of *ocp3* plants. Since this resistance was lost when assayed in a *pmr4* genetic background which lacked pathogen-induced callose deposition (Nishimura *et al.,* 2003) our finding reinforced the importance of callose deposition for the overall disease resistance response in *ocp3* plants. To our surprise, and despite the observed accelerated PMR4-dependent callose deposition, no significant variation was observed in *GSL5/PMR4* gene transcript levels of *ocp3* or Col-0 plants following fungal inoculation. This observation suggests a posttranscriptional regulation of gene expression controlling callose deposition following fungal attack. The nature of this regulation, and whether it operates at the translational or post-translational levels, merits future research. Regardless of this regulatory mechanism, a plant must rapidly regulate gene expression to adapt its physiology to changing environmental conditions. Our results suggested that in wild type plants, part of the signal pathway responsible for regulating rapid deployment of defense resources (i.e. callose deposition) is under negative control by *OCP3*. Subsequently, *ocp3* mutant plants are relieved of this negative control and respond more efficiently to the presence of pathogens. This hypothesis is consistent with our inference that the enhanced deposition of callose observed in *ocp3* relies on signals already present in the mutant, or which must be rapidly produced in wild type plants, and precedes efficient deposition of the ß-1,3-glucan polymer through GSL5/PMR4. Distress signals that alert cells to synthesize callose can be numerous. However, the

correlation that exists between H_2O_2 production and activation of callose deposition, not only during plant-pathogen interactions but also during plant development, is high (Benitez-Alfonso *et al*., 2009; Luna *et al*., 2011). Since healthy *ocp3* plants exhibited increased accumulation of H_2O_2, and constitutively expressed the H_2O_2-inducible *Ep5C* and *Glutathione S-transferase1* marker genes (Coego *et al*., 2005a; 2005b) our results support for the fungal-induced potentiatiation of callose deposition observed in *ocp3* plants preexistence of increased levels of H_2O_2 might be mandatory.

In this same line, the finding that callose deposition is blocked when ABA biosynthesis was impaired suggests a functional link between ABA and the potentiation of callose deposition in the *ocp3* mutant. A positive influence of ABA on callose deposition in response to infection by different pathogens has been reported (Flors *et al*., 2005; Asselbergh *et al*., 2008; Ton *et al*., 2009). In addition, You *et al*. (2010) characterized a DNA/RNA nuclease that likely post-transcriptionally controls an ABA-dependent stimulation of callose deposition during *B. cinerea* infection. Interestingly, Luna et al. (2011) recently reported Arabidopsis growth conditions leading to ABA-induced potentiation of callose, which resulted in increased H_2O_2 accumulation. Likewise, the callose-promoting effect that follows exogenous application of ABA has been explained by ABA-induced ROS production (Ghassemian *et al*., 2008; Xing *et al*., 2008). Thus, one is tempted to propose that the enhanced disease resistance in *ocp3* might be caused by the demonstrated increased levels of ABA and the associated H_2O_2 accumulation that concur in this mutant under resting conditions. This is congruent with the observation that increased biosynthesis of ABA occurred in wild type plants soon after fungal attack. A further link between ABA and the promotion of disease resistance in *ocp3* plants comes from the observation that expression of ABI4, a transcription factor that integrates ROS and ABA signaling (Staneloni *et al*., 2008) and required for chemical-induced priming of callose deposition (Ton and Mauch-mani, 2004), is up-regulated in *ocp3* plants. Moreover, *ABI4* expression is up-regulated following fungal infection in wild type plants; this up-regulation is concomitant with the previously demonstrated down-regulation of OCP3 that occurs

in wild-type plants following fungal infection (Coego *et al*., 2005b). These results further substantiate our proposal that part of the network mediating the *ocp3*-associated potentiation of callose deposition is already established in the mutant plants before recognition of fungal structures, and reinforces our deduction that OCP3 is a negative regulator of callose deposition, and in turn of disease resistance. The fact that overexpression of *OCP3* in transgenic plants does not render increase in the susceptibility towards fungal attack and neither affect the normal deposition of callose (see supplemental Figure S3), indicates that in wild-type plants the endogenous level of OCP3 is perfectly adjusted to maximize its role as a regulator during plant immunity.

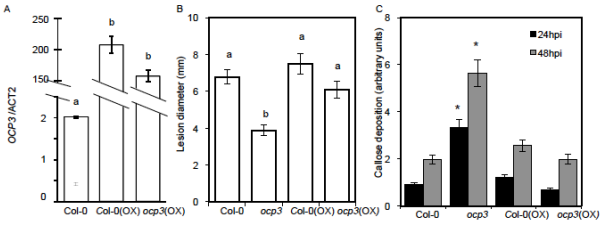


Figure S3. Overexpression of *OCP3* cDNA under the control of the 35S promoter (OX lines). Col-0 and *ocp3* plants were transformed with *a 35S::OCP3* gene construct and homozygous lines were generated. The construct was functional as it fully complemented the *ocp3* mutant. Only one representative transgenic line for each genetic background is shown. (a) RT-qPCR analysis for *OCP3* gene expression in Col-0 and in representative transgenic lines generated in a Col-0 background (Col-0(OX)) and in a *ocp3* background (*ocp3*(OX)). *OCP3* expression was normalized to *ACTIN2* expression. (b) Lesion size resulting from *Plectosphaerella cucumerina* infection in Col-0, *ocp3*, Col-0(OX) and *ocp3*(OX) plants. Lesion measurements were performed at 8 dpi. (c) Quantification of *P. cucumerina*-induced callose deposition in in Col-0, *ocp3*, Col-0(OX) and *ocp3*(OX) plants.

Our results indicated resistance to *B. cinerea* in *coi1-1* plants was severely compromised without affecting the normal deposition of basal callose. Therefore, the JA pathway appears pivotal for resistance but not through the control of basal callose deposition. Interestingly, we observed *ocp3*-associated resistance and the potentiated deposition of callose were lost when assayed in a *coi1-1* background. *ocp3 coi1-1* double mutant plants became hypersusceptible to *B. cinerea* and expressed only wild-type basal levels of callose deposition, similar to the parental *coi1-1* single mutant plants. This suggests deposition of only basal levels of callose

may not be sufficient to impede the pathogen, which may explain the susceptibility of wild type plants to this type of pathogen. However, *aba2* and *pmr4* mutant characterization, and the corresponding double mutants in an *ocp3* background revealed that if basal callose deposition is blocked, the plants became hypersusceptible. Therefore, both basal callose and potentiated callose deposition characterized in the *ocp3* mutant were required, but only the latter conferred enhanced resistance. Our results suggest a potentiated callose deposition, such as in *ocp3* plants, exhibits the most efficacy to arrest the advance of a pathogen. Moreover, our study further indicated JA controlled the potentiation mechanism of callose deposition and ABA controlled basal callose deposition. The former is effective in conferring resistance if the latter remains intact. Consequently, our results point towards a new layer of complexity in the regulation of pathogen-induced deposition of callose. This pathway specialization requires the interplay between ABA and JA and is negatively controlled by OCP3. When OCP3 is blunt, the pathway is relieved, signals commence, and the plant is more prone to respond to pathogenic signals that set in motion early resistance responses. How a cross-talk between JA and ABA is established remains unknown. However, it is pertinent to note here that a positive effect of ABA in regulating expression of a gene asset under control of the JA signal pathway branch that depends on the MYC2 transcriptional activator has been described (Adie *et al*., 2007). Moreover, MYC2 was originally described as an activator of ABA signaling (Abe *et al*., 2003), and was proposed to be a key regulator in the crosstalk mechanism between ABA and MeJA signalling pathways (Lorenzo *et al*., 2004). More recently, Ramirez *et al*. (2009) reported that *MYC2* gene expression is up-regulated in Col-0 plants following treatments with ABA, being the induction even more prominent in the case of *ocp3* plants (Ramirez *et al*., 2009). All these observations reconciles with the results showing that ABA levels are constitutively high in *ocp3* plants, and likely indicate the mutant is more prone to activate signaling through MYC2. Whether OCP3 is key in the interconnection of ABA and JA pathways upon signal perception, and whether or not it exherts control on other activators (i.e., MYC3 and MYC4) of the JA-regulated programs that act additively with MYC2 (Fernández-

Calvo *et al*., 2011) merits future research.

Finally, the parallelism between the potentiation of callose deposition observed in *ocp3* plants with the so-called priming for callose deposition embedded in the induced resistance (IR) mediated by the BABA mechanism is worth noting (Ton and Mauch-Mani, 2004). However, we observed that *ocp3* plants remain intact in overcoming the priming mechanism in response to BABA treatment, which is of a magnitude similar to that achieved in wild type plants. This indicates that BABA-induced priming for callose deposition is distinct from the mechanism we referred to as potentiation of callose in *ocp3* plants. In any case, both mechanisms for enhancing callose deposition over the normal basal levels in wild type plants convey through PMR4, and both require intact ABA signaling. However, BABA induced priming for callose does not require JA signaling (Ton and Mauch-Mani, 2004) and the potentiated mechanism of callose deposition in *ocp3* plants entirely rely on JA signaling. Therefore, our results strongly support both mechanisms as different branches of a complex network controlling pathogen-induced callose deposition in plants. BABA is presumably a chemical priming mechanism, and our results suggest that OCP3 may represent a regulator of a biological priming mechanism. Initially we propose it as a potentiation mechanism being controlled by the interplay of ABA, JA, and OCP3.

MATERIALS AND METHODS

Plant materials and growth.

Seeds of mutants and wild-type *Arabidopsis thaliana* were kept at 4°C for 3 days and sown in jiffy7 peat pellets (Clause-Tezier Ibérica, Paterna, Spain). Plants were grown in a growth phytochamber with a light intensity of approximately 150-200 µE $m^{-2}s^{-1}$ at 23°C under 10 h light/14 h dark cycles and 60% humidity.

PCR-based detection of *ocp3, aba2-1* and *coi1-1* were performed as described (Coego *et al.* 2005b; Ramirez *et al.* 2009). Genomic DNA was extracted from young leaves of Arabidopsis as described by Edwards *et al*. (1991). For the *ocp3 pmr4-1*

double mutant, the primers used were 5'-TTACCAGCCCAACCAATTTC-3' and 5'-AGATCAGGGACATGGGACAG-3'. The 799 bp PCR product was digested with NheI (New England Biolabs) resulting in 483, 316 bp in *pmr4-1* (Nhisimura *et al.* 2003). In double mutant analysis the same phenotype was observed for, at least, two independent double mutant lines generated.

Botrytis cinerea and Plectosphaerella cucumerina bioassays

In both *B. cinerea* and *P. cucumerina* infections, five-week-old plants were inoculated as described by Coego *et al*. (2005b), with a suspension of fungal spores of $2.5x10^4$ and $5x10^6$ spores/mL respectively. The challenged plants were maintained at 100% relative humidity. Disease symptoms were evaluated by determining the lesion diameter of at least 50 lesions 3 or 8 days after inoculation. For pathogen-induced callose deposition analyses, infected leaves were stained with aniline blue at 24 and 48 h.p.i and callose deposition were quantified by epifluorescence microscopy as described by Ton and Mauch-Mani (2004). These experiments were repeated at least three times with similar results.

RNA isolation and qRT-PCR analysis

RNA was isolated with Trizol (Invitrogen. www.invitrogen.com) following manufacturer´s instructions. RNA was quantified with a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies. www.nanodrop.com). RNA quality was assessed with a 2100 Bioanalyzer from Agilent Technologies (www.agilent.com). For reverse transcription, RevertAid M-MuLV Reverse Transcriptase (Fermentas. www.fermentas.com) was used according to the manufacturer's instructions. RT-qPCRs were carried out with gene specific primers designed using the Primer Express 2.0 software (Applied Biosystems. www.appliedbiosystems.com): PDF1.2a (AT5G4442): 5´-CTTGTTCTCTTTGCTGCTTTC-3´ and 5´-CATGTTTGGCTCCTTCAAG-3´, PMR4 (AT4G03550): 5´-GGACGGCATTCATAGATTGAAGC-3´ and 5´-ACCGTCACATCCCAGGAATTCT-3´, ABI4 (AT2G40220) 5'-ACCAAACCCTAACCCATAATAATCC-3' and 5'-

CGTTGAGCGGAGGAAGTTGAT-3', OCP3 (AT5g11270) 5´-AAGCTGGGCGTCGTAAAACTAGTA-3´ and 5´-TGGCGGTTTTTCATCTGGTAGTGT-3´, ACT2 (AT3g18780) 5´-TCTTCCGCTCTTTCTTTCCAAGC-3 and 5´-ACCATTGTCACACACGATTGGTT-3´. RT-qPCRs were performed using the SybrGreen PCR Master Mix (Applied Biosystems. www.appliedbiosystems.com) in a ABI PRISM 7000 sequence detector. Cts were obtained using the 7000 System SDS Software Core Application Version 1.2.3 (Applied Biosystems. www.appliedbiosystems.com) and the data was transformed with the formula $2^{\wedge(40-Ct)}$. RT-qPCR analyses were performed at least three times using sets of cDNA samples from independent experiments.

Determination of ABA and JA-Ile levels

Fresh material was frozen in liquid nitrogen and lyophilized. Before extraction, a mixture of internal standards containing 100 ng [$^2H_6$] ABA and 100 ng of dhJA (Flors *et al.* 2008) was added. Dry tissue (0.05 g) was immediately homogenized in 2.5 ml of ultrapure water. After centrifugation (5000 g, 40 min), the supernatant was recovered and adjusted to pH 2.8 with 6% acetic acid, and subsequently partitioned twice against an equal volume of diethyl ether. The aqueous phase was discarded, and the organic fraction was evaporated in a Speed Vaccuum Concentrator (Eppendorf; www.eppendorf.com) at room temperature and the solid residue re-suspended in 1 ml of a methanol/water (10:90) solution and filtered through a 0.22 µm cellulose acetate filter. A 20 µL aliquot of this solution was then directly injected into the HPLC system. Analyses were carried out using a Waters Alliance 2690 HPLC system (Milford, MA, USA) with Kromasil reversed-phase column (100 x 2 mm i.d.; 5 µm; Scharlab, Barcelona, Spain; http:// www.scharlab.es). The chromatographic system was interfaced to a Quatro LC (quadrupole-hexapole-quadrupole) mass spectrometer (Micromass; http://www.micromass.co.uk). The MASSLYNX NT software version 4.1 (Micromass) was used to process the quantitative data from calibration standards and the plant samples. The calibration curves were obtained by using solutions containing increasing amounts of (±)-2-

cis,4-*trans*-abscisic acid (obtained from Sigma-Aldrich (Madrid, Spain)) and JA-Ile (kindly provided by Edward Farmer (University of Lausanne)) and a fixed amount of the corresponding internal standard.

BABA treatments

Induction treatment with *β*-amino-butyric acid (BABA) was performed in five-week-old plants. Two days before inoculation with fungal spores, plants were treated by soil-drenching with a solution containing water (mock) or BABA at a final concentration of 250 μM.

In vitro assays, growth conditions and Flg22 and DCB treatments

Approximately 15 sterilized Col-0, *ocp3* and double mutants *ocp3 aba2-1* and *ocp3 pmr4-1* seeds were sown per well in sterile 12-wells plates, containing filter-sterilized MS mediums without Gamborg's vitamins and with 0,5% of sucrose. Seedlings were cultivated under standard growth conditions (15 h day cycle; 20ºC /17ºC) with a light intensity of 150 μM/m2/s. After 7 days, the growth medium was replaced by fresh MS medium. One day later, plants were challenged with Flg22 and DCB at the final concentration of 1 μM and 100 μM in the growth medium. At day 9, the samples were stained with aniline blue and callose deposition quantifications were performed as described by Luna *et al.* (2011).

ACKNOWLEDGEMENTS

We thank E. Farmer and B. Mauch-Mani for providing a sample of JA-Ile. This was work supported by Spanish MICINN (Grants BFU2009-09771 and Consolider-TRANSPLANTA to P.V.) and by Generalitat Valenciana (Prometeo2010/020 to P.V.).

REFERENCES

Abe, H., Urao, T., Ito, T., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2003) Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. *Plant Cell*, **15**, 63-78.

Adie, B.A., Perez-Perez, J., Perez-Perez, M.M., Godoy, M., Sanchez-Serrano, J.J., Schmelz, E.A. and Solano, R. (2007) ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in Arabidopsis. *Plant Cell*, **19**, 1665-1681.

Anderson JP, Badruzsaufari E, Schenk PM, Manners JM, Desmond OJ, Ehlert C, Maclean DJ, Ebert PR, Kazan K. (2004) Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in Arabidopsis. *Plant Cell,* 16:3460-3479.

Asselbergh, B., De Vleesschauwer, D. and Hofte, M. (2008) Global switches and fine-tuning-ABA modulates plant pathogen defense. *Mol Plant Microbe Interact*, **21**, 709-719.

Benitez-Alfonso Y, Cilia M, San Roman A, Thomas C, Maule A, Hearn S, Jackson D. (2009) Redox homeostasis regulates plasmodesmal communication in Arabidopsis meristems. *Proc Natl Acad Sci U S A*, **106**, 3615-3620.

Bestwick, C.S., Brown, I.R., Bennett, M.H. and Mansfield, J.W. (1997) Localization of hydrogen peroxide accumulation during the hypersensitive reaction of lettuce cells to Pseudomonas syringae pv phaseolicola. *Plant Cell*, **9**, 209-221.

Browse, J. (2009) Jasmonate passes muster: A receptor and targets for the defense hormone. Ann Rev Plant Biol, 60: 183-205.

Coego, A., Ramirez, V., Ellul, P., Mayda, E. and Vera, P. (2005a) The H2O2-regulated Ep5C gene encodes a peroxidase required for bacterial speck susceptibility in tomato. *Plant J*, **42**, 283-293.

Coego, A., Ramirez, V., Gil, M.J., Flors, V., Mauch-Mani, B. and Vera, P. (2005b) An Arabidopsis homeodomain transcription factor, OVEREXPRESSOR OF CATIONIC PEROXIDASE 3, mediates resistance to infection by necrotrophic pathogens. *Plant Cell*, **17**, 2123-2137.

Chini, A., Fonseca, S., Fernandez, G., Adie, B., Chico, J.M., Lorenzo, O., Garcia-Casado, G., Lopez-Vidriero, I., Lozano, F.M., Ponce, M.R., Micol, J.L. and Solano, R. (2007) The JAZ family of repressors is the missing link in jasmonate signalling. *Nature*, **448**, 666-671.

Edwards, K., Johnstone, C. and Thompson, C. (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Res.* **19**, 1349.

Ellis, C., Karafyllidis, I., Wasternack, C. and Turner, J.G. (2002) The Arabidopsis mutant cev1 links cell wall signaling to jasmonate and ethylene responses. *Plant Cell*, **14**, 1557-1566.

Fan, J., Hill, L., Crooks, C., Doerner, P. and Lamb, C. (2009) Abscisic Acid Has a Key Role in Modulating Diverse Plant-Pathogen Interactions. *Plant Physiol*, **150**, 1750-1761.

Fernández-Calvo, P., Chini, A., Fernández-Barbero, G., Chico, J-M., Gimenez-Ibanez, S., Geerinck, J., Eeckhout, D., Schweizer, F., Godoy, M., Franco-Zorrilla, JM., Pauwels, L., Witters, E., Puga, MI., Paz-Ares, J., Goossens, A., Reymond, P., De Jaeger, G. Solano, R. (2011) The Arabidopsis bHLH Transcription Factors MYC3 and MYC4 Are Targets of JAZ Repressors and Act Additively with MYC2 in the Activation of Jasmonate Responses. *Plant Cell* **23**, 701-715.

Ferrari, S., Plotnikova, JM., De Lorenzo, G. and Ausubel, F.M. (2003) Arabidopsis local resistance to Botrytis cinerea involves salicylic acid and camalexin and requires EDS4 and PAD2, but not SID2, EDS5 or PAD4. *Plant J,* **35**, 193–205.

Ferrari, S., Galletti, R., Denoux, C., De Lorenzo, G., Ausubel, F.M. and Dewdney, J. (2007) Resistance to Botrytis cinerea induced in Arabidopsis by elicitors is independent of salicylic acid, ethylene, or jasmonate signaling but requires PHYTOALEXIN DEFICIENT3. *Plant Physiol*, **144**, 367-379.

Flors, V., Ton, J., van Doorn, R., Jakab, G., Garcia-Agustin, P. and Mauch-Mani, B. (2008) Interplay between JA, SA and ABA signalling during basal and induced resistance against Pseudomonas syringae and Alternaria brassicicola. *Plant J*, **54**, 81-92.

Ghassemian, M., Lutes, J., Chang, H.S., Lange, I., Chen, W., Zhu, T., Wang, X. and Lange, B.M. (2008) Abscisic acid-induced modulation of metabolic and redox control pathways in Arabidopsis thaliana. *Phytochemistry*, **69**, 2899-2911.

Glazebrook, J., Rogers, E.E. and Ausubel, F.M. (1996) Isolation of Arabidopsis Mutants With Enhanced Disease Susceptibility by Direct Screening. *Genetics*, **143**, 973-982.

Gomez-Gomez, L., Felix, G. and Boller, T. (1999) A single locus determines sensitivity to bacterial flagellin in Arabidopsis thaliana. *Plant J*, **18**, 277-284.

Gonzalez-Guzman, M., Apostolova, N., Belles, J.M., Barrero, J.M., Piqueras, P., Ponce, M.R., Micol, J.L., Serrano, R. and Rodriguez, P.L. (2002) The short-chain alcohol dehydrogenase ABA2 catalyzes the conversion of xanthoxin to abscisic aldehyde. *Plant Cell*, **14**, 1833-1846.

Hématy, K., Cherk, C. and Somerville, S. (2009) Host-pathogen warfare at the plant cell wall. *Current Opinion in Plant Biology*, **12**, 406-413.

Húckelhoven, R. (2007) Cell wall-associated mechanisms of disease resistance and susceptibility. *Annu Rev Phytopathol*, **45**, 101-127.

Jacobs, A.K., Lipka, V., Burton, R.A., Panstruga, R., Strizhov, N., Schulze-Lefert, P. and Fincher, G.B. (2003) An Arabidopsis Callose Synthase, GSL5, Is Required for Wound and Papillary Callose Formation. *Plant Cell*, **15**, 2503-2513.

Kaliff M, Staal J, Myrenås M, Dixelius C. (2007) ABA is required for *Leptosphaeria maculans* resistance via ABI1- and ABI4-dependent signaling. *Mol Plant Microbe Interact*, **20,** 335-345.

Kliebenstein, D.J., Rowe, H.C. and Denby, K.J. (2005) Secondary metabolites influence Arabidopsis/Botrytis interactions: variation in host production and pathogen sensitivity. *Plant J*, **44**, 25-36.

Lorenzo, O., Chico, J.M., Sanchez-Serrano, J.J. and Solano, R. (2004) JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. *Plant Cell*, **16**, 1938-1950.

Luna, E., Pastor, V., Robert, J., Flors, V., Mauch-Mani, B. and Ton, J. (2011) Callose deposition: a multifaceted plant defense response. *Mol Plant Microbe Interact*, **24**, 183-193.

Mendgen, K., Hahn, M. and Deising, H. (1996) Morphogenesis and mechanisms of penetration by plant pathogenic fungi. *Annu Rev Phytopathol*, **34**, 367-386.

Mohr, P.G. and Cahill, D.M. (2007) Suppression by ABA of salicylic acid and lignin accumulation and the expression of multiple genes, in Arabidopsis infected with Pseudomonas syringae pv. tomato. *Funct Integr Genomics*, **7**, 181-191.

Nishimura, M.T., Stein, M., Hou, B.H., Vogel, J.P., Edwards, H. and Somerville, S.C. (2003) Loss of a callose synthase results in salicylic acid-dependent disease resistance. *Science*, **301**, 969-972.

Penninckx, I.A., Thomma, B.P., Buchala, A., Metraux, J.P. and Broekaert, W.F. (1998) Concomitant activation of jasmonate and ethylene response pathways

is required for induction of a plant defensin gene in Arabidopsis. *Plant Cell*, **10**, 2103-2113.

Pieterse, C.M.J., Leon-Reyes, A., Van der Ent, S. and Van Wees, S.C.M. (2009) Networking by small-molecule hormones in plant immunity. *Nat Chem Biol*, **5**, 308-316.

Ramirez, V., Van der Ent, S., Garcia-Andrade, J., Coego, A., Pieterse, C.M. and Vera, P. (2010) OCP3 is an important modulator of NPR1-mediated jasmonic acid-dependent induced defenses in Arabidopsis. *BMC Plant Biol*, **10**:199.

Ramirez, V., Coego, A., Lopez, A., Agorio, A., Flors, V. and Vera, P. (2009) Drought tolerance in Arabidopsis is controlled by the OCP3 disease resistance regulator. *Plant J*, **58**, 578-591.

Richmond, T.A. and Somerville, C.R. (2000) The cellulose synthase superfamily. *Plant Physiol*, **124**, 495-498.

Roberts, K. (1990) Structures at the plant cell surface. *Curr Opin Cell Biol*, **2**, 920-928.

Sheard, L.B., Tan, X., Mao, H., Withers, J., Ben-Nissan, G., Hinds, T.R., Kobayashi, Y., Hsu, F-F., Sharon, M., Browse, J., He, S.Y., Rizo, J., Howe, G.A. and Zheng, N. (2010) Jasmonate perception by inositol-phosphate-potentiated COI1-JAZ co-receptor. *Nature*, **468**, 400-405.

Staneloni, R.J., Rodriguez-Batiller, M.J. and Casal, J.J. (2008) Abscisic acid, high-light, and oxidative stress down-regulate a photosynthetic gene via a promoter motif not involved in phytochrome-mediated transcriptional regulation. *Mol Plant*, **1**, 75-83.

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., Nomura, K., He, S.Y., Howe, G.A. and Browse, J. (2007) JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. *Nature*, **448**, 661-665.

Thomma, B.P.H.J., Eggermont, K. Penninckx, I.A.M.A., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A. and Broekaert , W.F. (1998) Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. *Proc. Natl. Acad. Sci*. U S A. **95**, 15107-15111.

Thomma, B.P., Nelissen, I., Eggermont, K. and Broekaert, W.F. (1999) Deficiency in phytoalexin production causes enhanced susceptibility of Arabidopsis thaliana to the fungus Alternaria brassicicola. *Plant J*, **19**, 163-171.

Thordal-Christensen, H., Zhang, Z., Wei, Y., and Collinge, D.B. (1997) Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *Plant Journal*, **11**, 1187–1194

Ton, J. and Mauch-Mani, B. (2004) Beta-amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose. *Plant J*, **38**, 119-130.

Ton, J., Flors, V. and Mauch-Mani, B. (2009) The multifaceted role of ABA in disease resistance. *Trends Plant Sci*, **14**, 310-317.

van Kan, J.A. (2006) Licensed to kill: the lifestyle of a necrotrophic plant pathogen. *Trends Plant Sci*, **11**, 247-253.

Verma, D.P. and Hong, Z. (2001) Plant callose synthase complexes. *Plant Mol Biol*, **47**, 693-701.

Vorwerk, S., Somerville, S. and Somerville, C. (2004) The role of plant cell wall polysaccharide composition in disease resistance. *Trends Plant Sci*, **9**, 203-209.

Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M. and Turner, J.G. (1998) COI1: an Arabidopsis gene required for jasmonate-regulated defense and fertility. *Science*, **280**, 1091-1094.

Xing, Y., Jia, W. and Zhang, J. (2008) AtMKK1 mediates ABA-induced CAT1 expression and H2O2 production via AtMPK6-coupled signaling in Arabidopsis. *Plant J*, **54**, 440-451.

Xu, L.H., Liu, F.Q., Lechner, E., Genschik, P., Crosby, W.L., Ma, H., Peng, W., Huang, D.F. and Xie, D.X. (2002) The SCFCOl1 ubiquitin-ligase complexes are required for jasmonate response in Arabidopsis. *Plant Cell*, **14**, 1919-1935.

Yadav, V., Mallappa, C., Gangappa, S.N., Bhatia, S. and Chattopadhyay, S. (2005) A basic helix-loop-helix transcription factor in Arabidopsis, MYC2, acts as a repressor of blue light-mediated photomorphogenic growth. *Plant Cell*, **17**, 1953-1966.

You, M.K., Shin, H.Y., Kim, Y.J., Ok, S.H., Cho, S.K., Jeung, J.U., Yoo, S.D., Kim, J.K. and Shin, J.S. (2010) Novel bifunctional nucleases, OmBBD and AtBBD1, are involved in abscisic acid-mediated callose deposition in Arabidopsis. *Plant Physiol*, **152**, 1015-1029.

Capítulo II

Mediated Plastid RNA Editing in Plant Immunity

Javier García-Andrade, Vicente Ramírez, Ana López and Pablo Vera*

Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia-C.S.I.C, Ciudad Politécnica de la Innovación, Edificio 8E, Ingeniero Fausto Elio, s/n, 46022 Valencia**,** Spain.

*Corresponding author: Pablo Vera. E-mail: vera@ibmcp.upv.es

PLoS Pathogens (2013) 9(10): e1003713

ABSTRACT

Plant regulatory circuits coordinating nuclear and plastid gene expression have evolved in response to external stimuli. RNA editing is one of such control mechanism. We determined the Arabidopsis nuclear-encoded homeodomain-containing protein OCP3 is incorporated into the chloroplast, and contributes to control over the extent of *ndhB* transcript editing. *ndhB* encodes the B subunit of the chloroplast NADH dehydrogenase-like complex (NDH) involved in cyclic electron flow (CEF) around photosystem I. In *ocp3* mutant strains, *ndhB* editing efficiency decays, CEF is impaired and disease resistance to fungal pathogens substantially enhanced, a process recapitulated in plants defective in editing plastid RNAs encoding NDH complex subunits due to mutations in previously described nuclear-encoded pentatricopeptide-related proteins (i.e. CRR21, CRR2). Furthermore, we observed that following a pathogenic challenge wild type plants respond with editing inhibition of *ndhB* transcript. In parallel, rapid destabilization of the plastidial NDH complex is also observed in the plant following perception of a pathogenic cue. Therefore, NDH complex activity and plant immunity appear as interlinked processes. We propose editing regulation and its effect in NDH activity in the chloroplast is an intrinsic component of a plant´s defense program.

AUTHOR SUMMARY

Plastids originated from cyanobacteria that were incorporated into the eukaryotic cell through an endosymbiotic relationship. During the gradual evolution from endosymbiont to organelle most genes of the cyanobacterial genome were transferred to the nuclear genome. Therefore, plastid biogenesis and function relies on nuclear gene expression and the import of these gene products into plastids, with the molecular dialogue between these two plant cell compartments therefore needed of a precise coordination. Nuclei-to-chloroplast communication, and *vice versa*, are thus regulated through anterograde and retrograde signaling pathways, respectively. Post-transcriptional RNA editing of plastid RNAs by nuclear encoded regulatory proteins, such as pentatricopetide-containing proteins (PPRs), represents one of such mechanism of control. Through the characterization of the nuclear-encoded OCP3 protein, previously found to function as a disease resistance regulator in Arabidopsis, we have discovered a pathogen-sensitive editing-mediated control of the plastidial NDH complex involved in cyclic electron flow (CEF) around photosystem I. This led us to find that different PPRs controlling editing extent of transcripts for plastidial NDH complex are modulated by pathogenic cues. Our results thus represent the first series of evidences indicating that chloroplast RNA editing and NDH activity are crucial components for the activation of a nuclear-encoded plant immune program.

INTRODUCTION

Plastid function relies on nuclear gene expression, and the import of nuclear gene products into plastids [1]. In fact, the plastid genome of current land plants encodes 75-80 proteins [2], whereas nuclear-encoded chloroplast proteins are estimated between 3500 and 4000 [3]. Current data approximates several hundred nuclear-encoded proteins are involved in post-transcriptional regulation of plastid gene expression [4,5]. One such regulation is mediated through RNA editing, a post-transcriptional process that alters specific cytidine residues to uridine (C-to-U) in different plastid RNAs [6]. Thirty-four sites are edited in 18 transcripts of Arabidopsis plastids [7]. Among the nuclear-encoded proteins regulating RNA editing, the pentatricopeptide repeat (PPR) protein family has attracted notable interest [8]. This family comprises 450 members defined by a tandem array of PPR motifs. PPRs are also involved in almost all stages of plastid gene expression, including splicing, RNA cleavage, translation, and RNA stabilization [9]. The pioneer work of Kotera *et al*. [10,11] revealed the Arabidopsis PPR protein CHLORORESPIRATORY REDUCTION4 (CRR4) acts as a site-specific recognition factor for RNA editing of the site 1 (ndhD-1) in the plastid *ndhD* transcript. *ndhD* encodes the D subunit of the chloroplast NADH dehydrogenase-like complex (NDH), involved in cyclic electron flow (CEF) around photosystem I (PSI) [11, 12]. Consequently, *crr4* mutants are defective in *ndhD* transcript editing at the ndhD-1 site, and CEF is compromised [10,11]. Subsequently, the number of PPR-encoding genes participating in editing control in the chloroplast has enlarged [9]. Although empirical evidence has been demonstrated for only a few PPR proteins, it is currently accepted that PPR proteins act as sequence-specific RNA binding adaptors, and hypothetical inferences suggest PPRs recruit effector enzymes or proteins to the target RNAs [13,14]. While the mechanism by which specific PPR proteins recognize specific editing sites is becoming understood, questions still remain to be completely solved including the characterization of the molecular components that conform the RNA editing apparatus (editosome) or the still unsolved identification of editing enzyme itself. Therefore, identification of

additional components modulating editing activities in plastids, and ascertaining how control of the post-transcriptional mechanism of chloroplast function influences other biological processes, in particular immune responses, is of great importance.

Despite the critical role of chloroplasts as a site for production of integral mediators of plant immunity such as salicylic acid, jasmonic acid, and ABA [15] , the molecular link between chloroplasts and the nuclear-encoded immune system remains largely unexplored. MEcPP, a plastidial metabolite previously shown to be involved in activating plant immunity in Arabidopsis [16] has been shown to mediate a retrograde signaling regulating expression of nuclear stress-response genes (17). Nomura *et al*. [18] recently reported the chloroplast calcium-sensing receptor (CAS), involved in transducing changes in cytosol Ca^{2+} concentrations into chloroplast responses, regulates plant immunity in Arabidopsis, possibly through chloroplast-derived ROS signals (i.e. $^1O_2$ and H_2O_2; [19]). These ROS signals may function through a retrograde signaling pathway to activate the expression of nuclear genes. Terashima *et al*. [20] demonstrated CAS is a crucial component of the machinery driving CEF around photosystem I in *Chlamydomonas reinhardtii*, and suggested CAS mediates changes in CEF activity, however the mechanism remains unresolved. In contrast to linear photosynthetic electron flow, where light drives ATP and NADPH synthesis; during CEF, light only drives ATP production by cycling electrons around PSI and Cyt *b6f* complexes, providing the molecular basis for this major energetic switch. Concurrently, CEF leads to the reduction of the plastoquinone pool, thereby increasing the frequency of charge recombination events in PSII; and as a result, altering the chloroplast redox status [21]. Consequently, CAS and Ca^{2+} via CEF alter ROS homeostasis, and may activate ROS-mediated retrograde signaling, which in a plant-pathogen interaction may have an impact on the outcome of plant disease resistance. CEF is also interrelated with nonphotochemical quenching (NPQ) which protects plants against damage resulting from ROS formation [22]. Göhre *et al*. [23] recently reported defense activation during PAMP-triggered immunity (PTI) in Arabidopsis resulted in rapid NPQ decrease, and NPQ also influenced immune responses, suggesting that NPQ and CEF are integral components regulating plant defense response. Similarly,

chloroplast-generated ROS following the recognition of pathogen-derived effectors by plant R proteins, resulted in HR-type programmed cell death (PCD), demonstrating chloroplast contribution to effector-triggered immunity (ETI) [24]. These evidences emphasize the importance of chloroplasts in plant immunity, and indicate the potential for future discoveries in this area of research.

We show the disease resistance regulator OVEREXPRESSOR OF CATIONIC PEROXIDASE3 (OCP3) is targeted to the chloroplast, and controls editing efficiency of plastid *ndhB* transcripts. We also show that NDH activity, and therefore CEF around PSI, is an important control point in plant immunity. Furthermore, a previously undescribed signaling pathway linking editing control with plant immunity via CEF activity modulation in the chloroplast was elucidated in this study.

RESULTS

OCP3 is targeted to plastids

OCP3 was classified as a transcription factor as it contained a 60-amino acid domain resembling a homeodomain and carried also two canonical bipartite nuclear localization signals [25] (Figure 1A). These features were interpreted as indicative of targeting OCP3 to nuclei, where it would be functioning as a negative regulator of plant immunity and was congruent with *ocp3* plants exhibiting a remarkable enhanced resistant to fungal pathogens due to a primed immune state [25-27]. However, when *Arabidopsis* were transformed with a gene construct carrying the fluorescent YFP protein fused to the OCP3 N-terminus or, alternatively, to the C-terminus (i.e. *35S::YFP-OCP3* and *35S::OCP3-YPF* constructs, respectively), confocal microscopy revealed different subcellular localizations for each protein (Figure 1B). YFP-OCP3 expression led to YFP-specific fluorescence at dispersed positions within the cell, while that derived from OCP3-YFP was unequivocally localized to the chloroplast (Figure 1B).

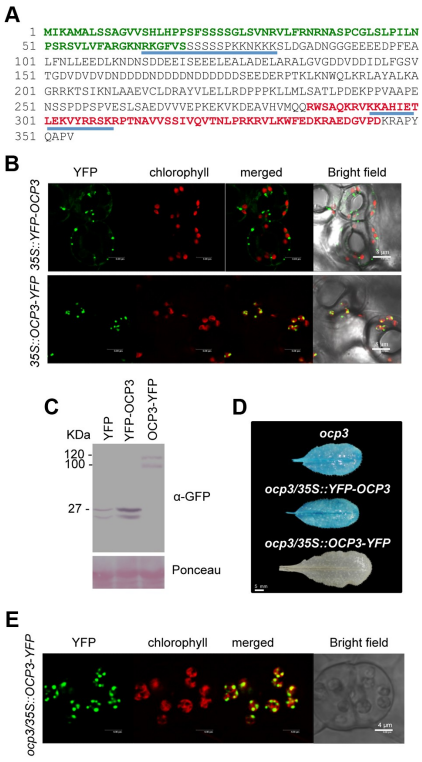


Figure 1. Functional OCP3 resides in the chloroplast. (**A**) OCP3 amino acid sequence. The 60 amino acid residues conforming the homeodomain are indicated in red letters. The N-terminal signal peptide sequence to chloroplast targeting, as predicted by TargetP, is indicated in green letters. Two canonical bipartite nuclear localization signals (RK-(X)10-KKNKKK and KK-(X)10-RRSKR) are underlined in blue. (**B**) Fluorescent confocal microscopy evaluation of protein localization in transgenic Arabidopsis plants transformed with a *35S::YFP-OCP3* construct (upper panel) and a *35S::OCP3-YPF* constructs (lower panel). YFP-specific fluorescence is shown in green and chlorophyll-derived fluorescence is shown in red. (**C**) Western blot analysis using anti-GFP antibodies of crude protein extracts derived from Arabidopsis plants transformed with *35S::YFP*, *35S::YFP-OCP3* and *35S::OCP3-YPF* genes constructs, respectively. Molecular mass markers are shown on the left. (**D**) Characteristic GUS expression pattern, as driven by the *Ep5C* gene promoter, in leaves of the *ocp3* mutant. Complementation of this molecular phenotype in *ocp3* plants upon transformation with *35S::OCP3-YFP* but not upon transformation with *35S::YFP-OCP3*. (**E**) Fluorescent confocal microscopy evaluation of protein localization in transgenic *ocp3* plants transformed with a *35S::OCP3-YPF* construct.

These different protein localizations were reproduced upon transfecting tobacco protoplasts using the same constructs (Supplemental Figure S1). Western blot using an anti-GFP antibody, revealed YFP-OCP3 accumulated as a YFP immunoreactive band similar to that observed for free YFP (Figure 1C). This was interpreted as partial trimming or processing of YFP-OCP3. Conversely, the OCP3-YFP protein was stable, and accumulated as two low migrating immunoreactive bands; the molecular weight of these polypeptides congruent with that expected for a fusion OCP3 with YFP (Figure 1C).

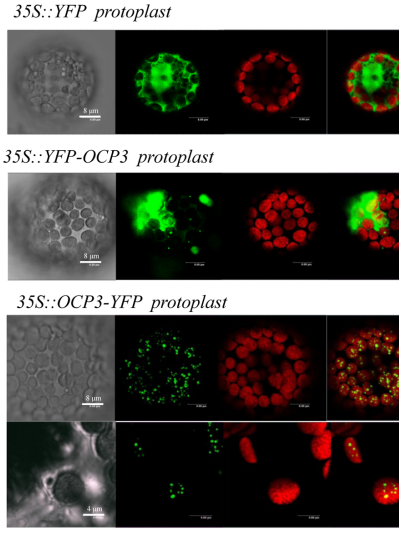


Figure S1. Protein localization in *N. benthamiana* protoplast. Fluorescent confocal microscopy evaluation of protein localization in transfected protoplast of *N. benthamiana* with a 35S::YFP construct (upper panel), a *35S::YFP-OCP3* construct (middle panel) and a *35S::OCP3-YPF* constructs (lower panel). YFP-specific fluorescence is shown in green and chlorophyll-derived fluorescence is shown in red.

Furthermore, the *35S::OCP3-YFP* gene construct, but not *35S::YFP-OCP3*, complemented the *ocp3* phenotype following stable transformation (Figure 1D). The *ocp3* mutant line carried a copy of the pathogen- and H_2O_2-responsive *Ep5C::GUS* transgene that became constitutively active in the mutant (revealed after staining with X-gluc) [25, 28], therefore, complementation was recorded as *Ep5C::GUS* expression repression (Figure 1D). Eight independent *ocp3/35S::OCP3-YFP* lines were assayed, and all showed repressed GUS expression (Figure 1D), indicative of effective complementation; in all cases the OCP3-YFP protein was targeted to the chloroplast (Figure 1E). However, all 12 independent *ocp3/35S::YFP-OCP3* transformed lines we generated retained GUS expression driven by the *Ep5C* gene promoter, demonstrating the inability of YFP-OCP3 to complement *ocp3*.

Inspection of OCP3 with TargetP (http://www.cbs.dtu.dk/services/ChloroP/) revealed a predicted 69 amino acid chloroplast signal peptide (SP) (Figure 1A). Correspondingly, N-terminal amino acid sequencing using Edman degradation of OCP3-YFP protein revealed processing at the predicted site (Polyp2; Supplemental Figure S2A). Furthermore, fusion of the first 81 amino acids of OCP3 to YFP (i.e. $OCP3_{1-81}$-YFP) was sufficient to target and internalize YFP to the chloroplast (Supplemental Figure S2B-C). Interestingly, chloroplast targeting, but not internalization, occurred when a short deletion (from aa 68-to-74) was introduced in

the constructs (OCP3$\Delta_{68\text{-}74}$-YFP; Supplemental Figure S2B-C), indicating amino acids at position 68-to-74 were critical for proteolytic maturation of OCP3 in the chloroplast. These results were congruent with the absence of *ocp3* complementation with deleted OCP3$\Delta_{68\text{-}74}$-YFP, and lack of mature OCP3-YFP protein accumulation in transformed plants (Supplemental Figure S2D-E). Immunoblot analysis of chloroplast suborganellar fractionations derived from plants expressing OCP3-YFP revealed incorporation of the protein into the chloroplast, and enrichment in the stroma and thylakoid fractions (Supplemental Figure S2F). Collectively, these results indicated a functional OCP3 protein resides in the chloroplast.

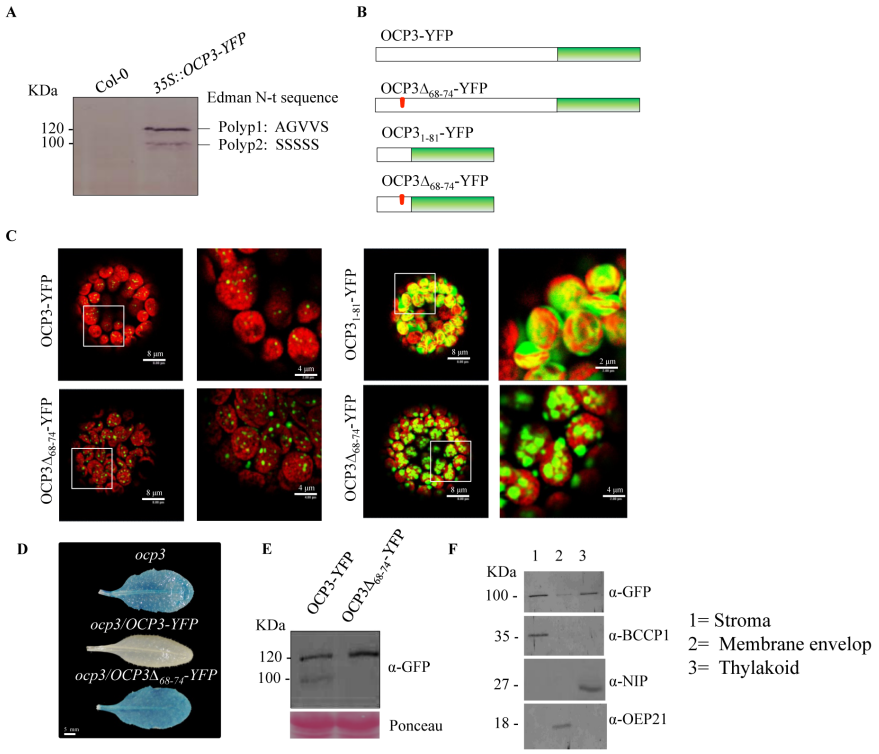


Figure S2. Functional characterization of the signal peptide sequence of OCP3. (A) SDS-PAGE and protein immunoblot with anti-GFP antibody indicating protein band position of OCP3-GFP precursors (Polyp1 and Polypep2) that were used for N-terminal amino acid sequence determination by Edman sequential degradation. Five rounds of degradation were conducted for each polypeptide which rendered the indicated 5 amino acid long N-terminal sequence. (B) Scheme depicting the different gene construct used for testing functionality of

the OCP3 signal peptide. Green: YFP protein; white: OCP3 protein; red symbol: relative position where the $\Delta_{68\text{-}74}$ internal deletion in the signal peptide sequence was created. (C) Confocal microscopy localization of the relevant constructs shown in (B). (D) Comparative complementation test of the *ocp3* mutant (which show constitutive GUS expression as driven by the Ep5C gene promoter) with construct *35S::OCP3-YFP* and construct *35S::OCP3*$_{68\text{-}74}$*-YFP*. The latter carries a deletion of 7 amino acids in the Signal peptide of OCP3 and was unable to be processed in the chloroplast. (E) Western blot with anti-GFP antibodies of proteins extracts derived from *ocp3* plants transformed with *35S::OCP3-YFP* and *35S::OCP3*$_{68\text{-}74}$*-YFP* gene constructs. (F) Western blots of the indicated chloroplast compartments obtained from chloroplasts preparations derived from *ocp3* plants transformed with the *35S::OCP3-YFP* gene construct. Westerns were developed using anti-GFP; anti-BCCP1 (as marker for the stroma (lane 1; loaded with 4 µg total protein)); anti-OEP21 (as marker for membrane envelop (lane 2; loaded 1 µg total protein)); and anti-NIP (as marker for thylakoids (lane 3; loaded with 1 µg total protein).

OCP3 localization overlaps with pTAC2, a pentatricopeptide repeat (PPR) protein

OCP3-YFP distribution within the chloroplast showed protein accumulated in the form of speckles or punctate patterns. We noted similarities among proteins targeting different plastid structures or molecules, including plastoglobules (i.e. PGL34, [29]), plastid nucleoids (PEND, [30]), targeting associated with introns containing RNAs (i.e. WHIRLY, [31]) and with RNAs undergoing editing (i.e. pTAC2) (Figure 2A). We co-expressed the OCP3-mCherry protein with PGL34-YFP, PEND-RFP, WHIRLY-GFP, or pTAC2-YFP in protoplasts to demonstrate possible co-localizations. OCP3-mCherry fluorescence overlapped consistently with the fluorescence derived from the pentatricopeptide repeat (PPR) protein pTAC2-YFP (Figure 2B).

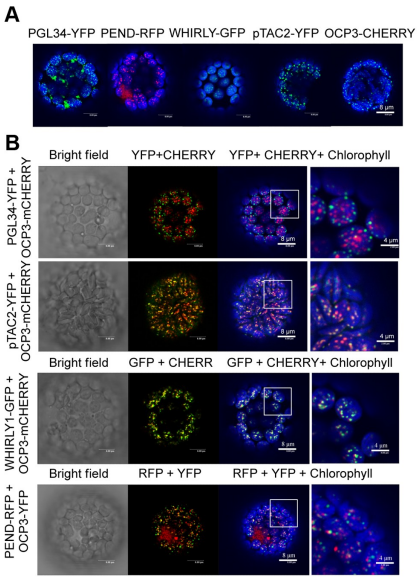


Figure 2. OCP3 co-localizes with pTAC2. (**A**) Chloroplast localization pattern of PGL34-YFP, PEND-RFP, WHIRLY-GFP, pTAC2-YFP and OCP3-mCHERRY in protoplasts from *N. benthamiana* evaluated by confocal microscopy. (**B**) Co-localization patterns of OCP3 with each of the proteins shown in (A). YFP- and GFP-specific fluorescence is shown in green, RFP- and mCHERRY-specific fluorescence is shown in red and chlorophyll-derived fluorescence is shown in blue.

OCP3 is co-expressed with a cluster of nuclear genes encoding plastid PPR proteins

Transcriptionally coordinated genes tend to be functionally related [32]. We hypothesized that identification of genes that are co-expressed with *OCP3* (at5g11270) in Arabidopsis would provide clues into the biological processes of OCP3 in the chloroplast. Therefore, we initially identified a co-expressed gene vicinity network for *OCP3* using the AraGenNet platform (http://aranet.mpimp-golm.mpg.de/aranet/Home) [33] where *OCP3* matched cluster 49 (Supplemental Figure S3). Based on functional annotation using MapMan ontology terms (http://aranet.mpimp-golm.mpg.de/), the co-expression network contained 207 genes significantly enriched for biochemical and regulatory aspects related to chloroplast development and function (see Table SI). A closer vicinity network including only genes two steps away from *OCP3*, identified 31 genes that were all related to plastid processes (Figure 3A and Table SII).

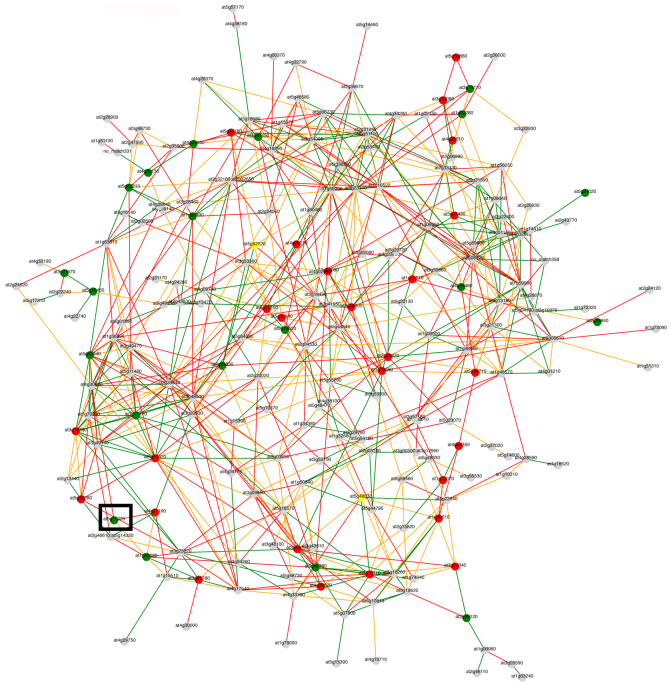


Figure S3. Co-expression gene vicinity network for OCP3. Nodes indicate individual genes, and edges indicate whether two genes are co-expressed above a certain mutual rank. Red, yellow, green, and grey nodes indicate whether mutations in the gene cause embryo lethality (red), gametophytic lethality (yellow), any other biological phenotype (green), or if no mutant phenotype currently is available (grey) according to TAIR. The color edges indicate strength of the coexpression based on mutual rank relationships between the individual gene pairs. Green, orange, and red edges indicate a mutual rank relationship ≤10 (green), between 11 and 20 (orange) and 21 and 30 (red), respectively, for each connected gene. The network was generated, and modified from AraGenNet (http://aranet.mpimp-golm.mpg.de/aranet; Mutwil et al., 2010).

Nine of these genes encoded PPR proteins, and the biological role in only one of these *PPR* genes, *CRR21* (at5g55740), has been elucidated. CRR21 acts as a site-specific factor recognizing RNA editing site 2 (ndhD-2 site) in plastid *ndhD* transcript, suggesting that the Ser128Leu change has important consequences for the function of the NDH complex [13]. The remaining eight *PPR* genes, were tentatively named as follows: *PPRa* (at4g21190), *PPRb* (at4g30825), *PPRc* (at3g29230), *PPRd* (at3g46610), *PPRe* (at5g14350), *PPRf* (at1g15510), *PPRg* (at3g14330), and *PPRh* (at3g49140) (Figure 3A).

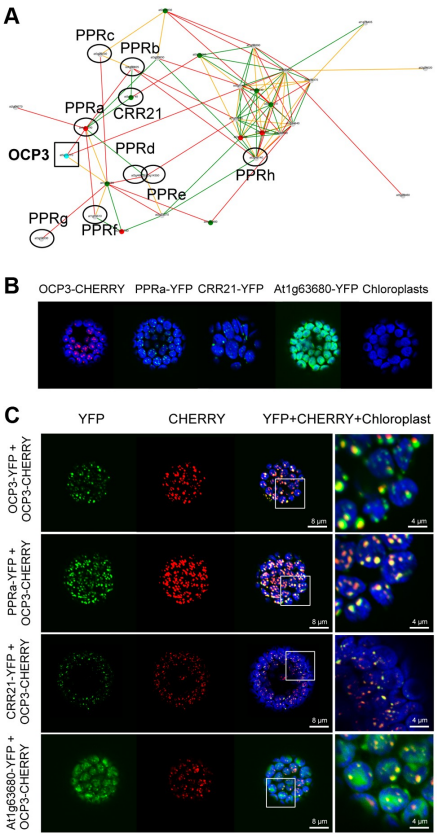


Figure 3. OCP3 is co-regulated with a subset of nuclear-encoded chloroplast PPR proteins. (**A**) Co-expression gene vicinity network around the *OCP3* node. This gene cluster was partitioned from the complex network illustrated in Supplementary Figure S3 and shows co-expressed genes that are only two steps away from *OCP3*. Nine out of the 31 genes of the cluster (see Table SII) encode PPR proteins and are indicated by circles, while *OCP3* is indicated by a square. In addition to *CRR21* (at5g55740), the other 8 *PPRs* surrounding *OCP3* node have been tentatively name as *PPRa* (at4g21190), *PPRb* (at4g30825), *PPRc* (at3g29230), *PPRd* (at3g46610), *PPRe* (at5g14350), *PPRf* (at1g15510), *PPRg* (at3g14330) and *PPRh* (at3g49140). Nodes indicate individual genes, and edges indicate whether two genes are co-expressed above a certain mutual rank. Color codes for nodes and edges as in Suplementay Figure S3. Green, orange and red edges indicate a mutual rank relationship 10 (green), 10 but 20 (orange) and 20 but 30 (red), respectively, for each connected gene pair. GO characterization of the 31 genes is shown in Table II. The network was generated, and modified from AraGenNet (http://aranet.mpimp-golm.mpg.de/aranet). (**B**) Chloroplast localization pattern of OCP3-CHERRY, PPRa (at4g21190)-YFP, CRR21-GFP, and At1g63680-YFP in protoplasts from *N. benthamiana* transfected with each respective construct as evaluated by confocal microscopy. (**C**) Chloroplast co-localization patterns of OCP3-CHERRY with OCP3-YFP and of OCP3-CHERRY with each of the other proteins shown in (B) in protoplast co-transfected with each of the indicated construct pairs. YFP-specific fluorescence is shown in green, CHERRY-specific fluorescence is shown in red and chlorophyll-derived fluorescence is shown in blue.

As for pTAC2, plastidial overlapping localization pattern was observed for OCP3-mCHERRY and PPRa-YFP (Figure 3B-C). Similarly, OCP3-mCHERRY overlaps with CRR21-YFP both following the same punctate distribution pattern (Figure 3B-C). The common co-localization pattern of OCP3 with different PPR proteins was not followed by all other proteins whose genes where co-expressed with OCP3, as deduced from the non-overlapping localization pattern in OCP3-mCHERRY and AT1G63680-YFP (Figure 3B). The observed common localization of OCP3 and different PPR proteins suggested involvement of OCP3 in some aspects of plastidial RNA editing processes

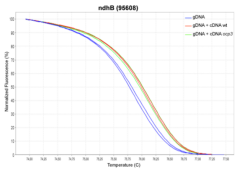
OCP3 is required for RNA editing of *ndhB* transcript in plastids

To test the involvement of OCP3 in RNA editing, we systematically examined the editing status of chloroplast transcripts derived from wild type and *ocp3* plants using high-resolution melting (HRM) screen of the 34 sites undergoing editing in Arabidopsis [7]. We identified major defects in the RNA editing of *ndhB-6*, *nhdB-4*, *ndhB-3*, and *ndhB-2* sites in *ocp3* plants (Figure S4). The comparison of the sequencing electrophoregrams of the RT-PCR products surrounding the editing sites confirmed that editing was compromised at the four indicated sites, if not totally at least partially, in *ocp3* plants (Figure 4A and Figure S5). All other known sites appeared similarly edited in *ocp3* plants as in Col-0 plants. Editing defects were further confirmed by poisoned primer extension (PPE) assays (Figure 4B-E). ndhB-6, ndhB-4, and ndhB-3 sites were edited in Col-0 at estimated efficiencies of approximately 72%, 95%, and 88%, respectively, while in *ocp3* plants efficiencies were reduced approximately to 55%, 89%, and 72%, respectively (Figure 4B and 4D).

A

Locus (transcript)	Genome position	Col-0	*ocp3*
AtCg00040 (matK)	2931	+	+
AtC00130 (atpF)	12707	+	+
AtCg00180 (rpoC1)	21806	+	+
AtCg00170 (rpoB)	23898	+	+
AtCg00170 (rpoB)	25779	+	+
AtCg00170 (rpoB)	25992	+	+
AtCg00300 (psbZ)	35800	+	+
AtCg00330 (rps14)	37092	+	+
AtCg00330 (rps14)	37161	+	+
AtCg00500 (accD)	57868	+	+
AtCg00500 (accD)	58642	+	+
AtCg00570 (psbF)	63985	+	+
AtCg00580 (psbE)	64109	+	+
AtCg00590 (petL)	65716	+	+
AtCg00065 (rps12)	69553	+	+
AtCg00670 (clpP)	69942	+	+
AtCg00740 (rpoA)	78691	+	+
AtCg00840 (rpl23)	86056	+	+
AtCg00890 (ndhB-9)	94999	+	+
AtCg00890 (ndhB-8)	95225	+	+
AtCg00890 (ndhB-7)	95608	+	+
AtCg00890 (ndhB-6)	95644	+	-
AtCg00890 (ndhB-5)	95650	+	+
AtCg00890 (ndhB-4)	96419	+	+/-
AtCg00890 (ndhB-3)	96579	+	-
AtCg00890 (ndhB-2)	96698	+	-
AtCg00890 (ndhB-1)	97016	+	+
AtCg01010 (ndhF)	112349	+	+
AtCg01050 (ndhD-5)	116281	+	+
AtCg01050 (ndhD-4)	116290	+	+
AtCg01050 (ndhD-3)	116494	+	+
AtCg01050 (ndhD-2)	116785	+	+
AtCg01050 (ndhD-1; (ATG)	117166	+	+
AtCg01080 (ndhG)	118858	+	+

B



C

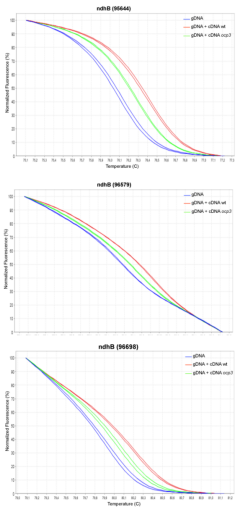


Figure S4. Analysis of the editotype of the *ocp3* mutant as revealed by high resolution melting (HRM). (**A**) Editing of the current 34 sites in *A. thaliana* chloroplast transcripts. Editing regulated genes are shown in the first column. The exact positions in the chloroplast genome sequence of each edited nucleotide is shown in the next column. Observed changes by HRM between Col-0 and *ocp3* plants are marked with (+) symbols, and absence of differences are marked with (-) symbol. The four editing sites found to be affected in *ocp3* plants are dashed in grey. (**B**) Example of HRM analysis, monitored by decrease in fluorescence as the temperature increase, for the amplicon encompassing *ndhB* transcript at position 95608 (ndhB-7 site) which suffers no variation between Col-0 and *ocp3*. (**C**) Examples of HRM analysis where the presence of less thermostable heteroduplexes in a sample alters the shape of the melting curves such as occurs with amplicons for transcript *ndhB* at positions 95644 (dnhB-6 site), 96579 (ndhB-3 site) or 96698 (ndhB-2 site) which suffer variation between Col-0 and *ocp3*.

The ndhB-2 editing site possessed a contiguous cytosine residue adjacent to the cytosine to be edited, which impeded a reliable PPE assay. Therefore, ndhB-2 was not further studied by this method. The ndhB-5 site exhibited no editing variation between Col-0 and *ocp3*, with efficiencies in the range of 82.5% and 81%, respectively; therefore, it served as an internal editing control site for the *ndhB* transcript not affected in *ocp3* plants.

Sequencing of individual cDNA clones, in sufficient quantities, is considered the most accurate method to measure editing extent, but is not cost effective for large-scale studies. We sequenced individual cDNA clones derived from RNAs obtained from equivalent leaves from Col-0 and *ocp3* plants. cDNA cloning strategy was designed to include the ndhB-4, ndhB-3, and ndhB-2 sites in one amplicon

(amplicon I) and the ndhB-6 site, along with non-altered ndhB-5, and ndhB-7 sites, in another amplicon (amplicon II).

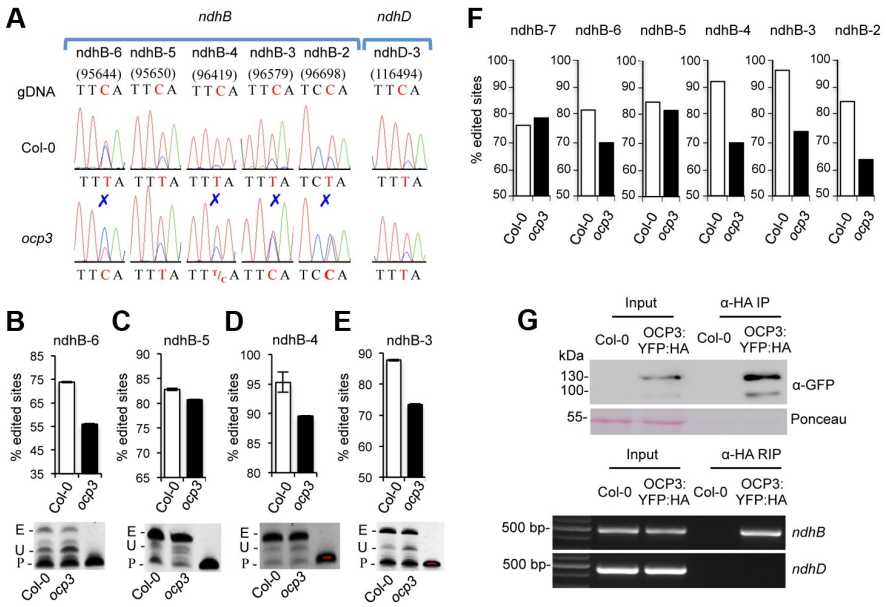


Figure 4. Editing defects in *ocp3* plants and *in vivo* association of OCP3 with *ndhB* RNA. (**A**) Nucleotide sequences surrounding the RNA editing sites of ndhB-6 (95644), ndhB-5 (95650), ndhB-4 (96419), ndhB-3 (96579), ndhB-2 (96698) and ndhD-2 (116494) are shown as sequence chromatograms. The editing sites are specified relative to the nucleotide sequence of the complete Arabidopsis chloroplast genome (Genebank accession number AP000423). Editing sites are indicated by a red C residue in the genomic (gDNA) sequence and its conversion or not to a U(T) residue in Col-0 and *ocp3* derived RNA samples. Editing defects in *ocp3* are indicated by a blue mark above the corresponding peaks. (**B-E**) Poisoned primer extension (PPE) assays were conducted on the editing sites ndhB-6 (B), ndhB-5 (C), ndhB-4 (D) and ndhB-3 (E). RT-PCR products were obtained with labeled 6-carboxyfluorescein primers that anneals next to the target editing site (forward PPE primers were used for all sites). Acrylamide gels (below panels) were visualized under UV light, and intensity of bands quantified calculated and plotted. Bars represent mean ± SD, n=3 independent replicates. Experiments were repeated at least three times with similar results. E, edited; U, unedited, P; primer. (**F**) Comparative RNA editing efficiency in Col-0 and *ocp3* plants as quantified from direct DNA sequencing of 100 independent cDNAs per genotype encompassing each of the indicated editing sites. (**G**) RNA immunoprecipitation (RIP) of anti-HA precipitated protein complexes from leaves derived from Col-0 and a 35S::OCP3:YFP:HA transgenic line. The upper panel shows a Western blot of protein present in crude leaf extracts and proteins immunoprecipitated (IP) with anti-HA antibody. The blot was developed with anti-GFP antibody and shows enrichment of the OCP3:YFP:HA protein in samples derived from the transgenic line. In the lower panel RT-PCR was used to detect association of *ndhB* transcripts with OCP3-enriched complexes in comparison to the corresponding input sample. Lack of association of *ndhD* transcripts with OCP3-enriched complexes is shown as a negative control.

One hundred cDNA clones for each amplicon, and for each genotype were analyzed by direct sequencing. Editing efficiency comparison is shown in Figure 4F. For the ndhB-4 site, Col-0 showed a 92% (92 of 100 sequenced clones) editing extent, which was reduced to 71% in *ocp3* plants. Col-0 showed a 94% editing extent for ndhB-3, reduced to 77% in *ocp3*. The ndhB-2 site exhibited an editing extent of 86% in Col-0, which was reduced to 64% in *ocp3*. These values were comparable to those observed for the PPE assays. Interestingly, *ocp3* plants exhibited concurrent editing inhibition at two sites within the same cDNA clone (of the three potential ones in amplicon I) in 21% of the sequenced clones while in Col-0 it was only 3%. Furthermore, lack of concurrent editing at the three sites remained notable in *ocp3*, and was observed in 8% of the sequence clones while in Col-0 it was 0%. These results suggested the concomitant editing inhibition at more than one site on the same *ndhB* transcript was a common feature in editing defects in *ocp3* plants. The ndhB-6 site showed an editing extent of 81% in Col-0 plants, which was reduced to a 61% in *ocp3* plants (Figure 4F). ndhB-5 and ndhB-7 served as controls for non-variation sites within the same transcript, and the editing extent was similar between Col-0 and *ocp3* plants (83% reduced to 81% for ndhB-5; and 76% increased to 78% for ndhB-7) (Figure 4F). Collectively these data indicated that OCP3 is required for efficient *ndhB* transcript edition.

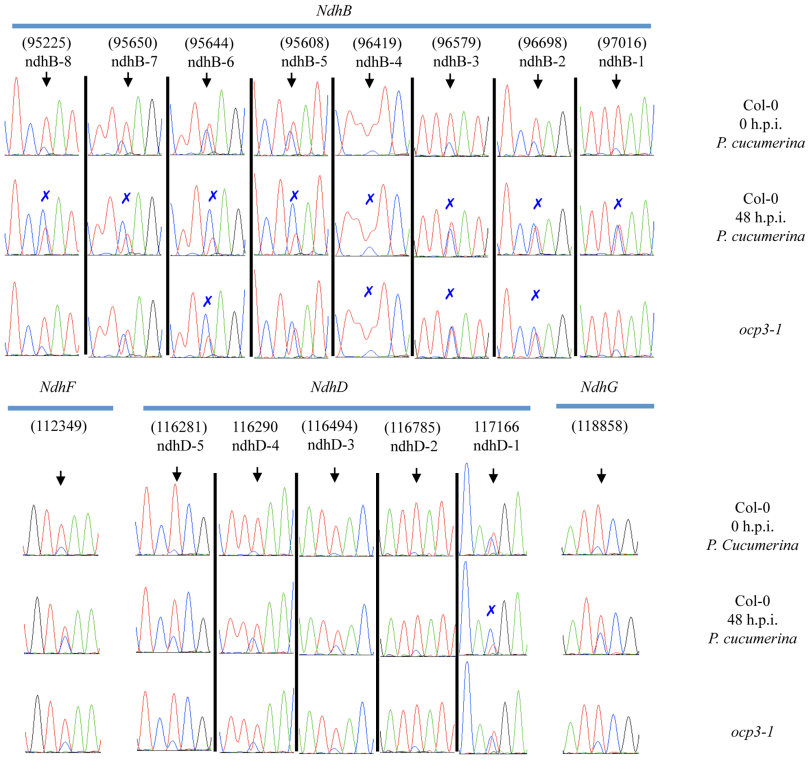


Figure S5. *P. cucumerina*-mediated editing inhibition. Nucleotide sequences of RT-PCR products obtained from Col-0 plants at 0 hours and at 48 hours post-inoculation with *P. cucumerina*, and its comparison with non-inoculated *ocp3-1* plants, are shown as sequencing electrophoregrams. Editing sites for the four transcript encoding the chloroplast-encoded NDH complex subunits (i.e., NdhB, NdhD, NdhF, and NdhG) are indicated by arrows pointing to the corresponding peaks. Observed editing inhibition following *P. cucumerina* infection are marked with a blue cross above the corresponding editing site.

In vivo association of OCP3 with *ndhB* RNA

To directly assess the association of OCP3 with *ndhB* transcripts, leaves from Col-0 and from a transgenic line expressing a *35S::OCP3:GFP:HA* gene construct were treated with folmaldehyde to generate protein-RNA cross-links and subsequently subjected to RNA immunoprecipitation (RIP), an analysis that serves to detect the presence of the corresponding RNA in the protein immunoprecipitate by reverse transcription PCR (RT-PCR). Immunoprecipitation of crude protein extracts with an anti-HA antibody selectively enriched the chimeric OCP3 protein in samples derived

from the 35S::OCP3:YFP:HA overexpressing line (Figure 4G, upper panel). Interestingly, the immunoprecipitated OCP3 complexes were shown to specifically co-precipitate *ndhB* transcripts as revealed by comparative RT-PCR analysis of the corresponding samples derived from the transgenic line and Col-0 plants (Figure 4G, lower panel). *ndhD* transcripts, which served as a negative control, did not show association with the OCP3 complex (Figure 4G, lower panel). The results thus indicate that OCP3 associates *in vivo* with *ndhB* transcript. Whether this association is the result of a direct interaction of OCP3 with the RNA molecule, or rather a consequence of the interaction of OCP3 with an RNA binding protein recognizing specificifically RNA sequences of the *ndhB* transcript remains unknown. Future characterization of such protein complex and the elucidation of its associated biochemical function will shed light on how editing of the *ndhB* RNA at their multiple editing sites is regulated.

ocp3 plants are impaired in NDH activity

Normal RNA editing at *ndhB-6*, *ndhB-4*, *ndhB-3* and *ndhB-2* sites converts a Ser codon to a Leu codon at aa279, a Ser to Phe at aa249, a His to Tyr at aa196, and a Pro to a Leu at aa156 in the NdhB protein. NdhB is one of the eleven chloroplast-encoded subunits of the chloroplast NDH complex. We hypothesized *ndhB* editing defects observed in *ocp3* plants would affect encoded protein function, which in turn would alter photosynthetic parameters in the mutant. NDH complex activity can be monitored as a transient increase in chlorophyll fluorescence reflecting plastoquinone pool reduction after turning off actinic light, as originally demonstrated by Shikanai et al. [11] . Figure 5A shows a typical chlorophyll fluorescence trace from Arabidopsis Col-0 and its comparison with *crr21*, a mutant lacking NDH activity. In *ocp3*, the post-illumination increase in chlorophyll fluorescence was modified in a manner similar to what occurred in *crr21* plants, indicating that NDH activity was compromised. This result strongly indicated OCP3 is a chloroplast factor pivotal in normal NDH complex function. This important phenotype was confirmed by employing additional mutant alleles. Due to the absence of T-DNA insertions mutants for the *OCP3* locus, and being the *ocp3*

mutant currently used a loss-of-function EMS mutant, we generated additional mutant alleles of this gene by artificial microRNA (amiRNA) interference. Two independent homozygous amiRNA lines (i.e. amiRNA-2 and amiRNA-3) phenocopying the original *ocp3* mutant (Figure S6A-D), were selected. These lines were designated *ocp3-2* and *ocp3-3*, respectively, and the original *ocp3* now designated *ocp3-1*. Defective NDH activity was recorded in these mutants (Figure 5A). Complementation of *ocp3-1* plants with an OCP3 wild-type sequence fully restored the post-illumination increase of chlorophyll fluorescence (Figure 5A). These results confirmed the importance of OCP3 for appropriate NDH complex function.

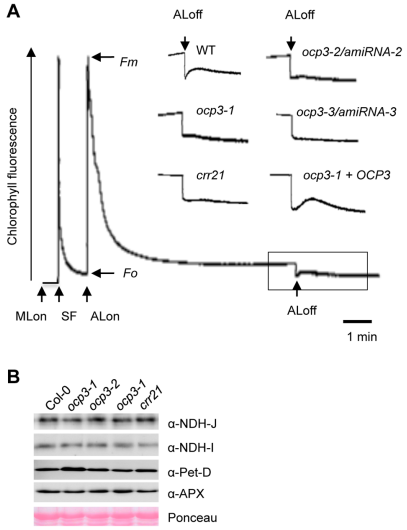


Figure 5. Monitoring of NDH activity by using chlorophyll fluorescence analysis. (**A**) Analysis of the transient increase in chlorophyll fluorescence (apparent Fo) after termination of actinic light (AL) illumination. The bottom curve indicates a typical trace of chlorophyll fluorescence in the wild type (WT). Leaves were exposed to AL (50 µmol photons m^{-2} s^{-1}) for 5 min. AL was turned off and the subsequent transient rise in fluorescence caused by plastoquinone reduction based on NDH activity monitored. Insets are magnified traces from the boxed area. The fluorescence levels were normalized by the maximum fluorescence at close PSII centers in the dark (*Fm*) levels; ML, measuring light; SF, a saturating flash of white light. The fluorescence was monitored using a pulse-amplitude-modulation chlrophyll fluorometer. *ocp3-1+OCP3*, *ocp3-1* allele transformed with a wild type *OCP3* cDNA. (**B**) Immunoblot analysis of NDH NdhI and NdhJ subunits, the subunit IV of cytochrome *b6f* (Pet-D) and ascorbate peroxidase (APX). Proteins were extracted from chloroplast preparations from each genotype and lanes loaded with 20 µg protein.

RNA editing results in amino acid changes that directly alter protein translation, function, or even may act to destabilize multiprotein complexes. The NDH complex is unstable when NdhD subunit is absent due to editing-mediated translation defects [34,35]. The NdhB subunit defects observed in *ocp3* plants were evaluated to determine the effects on NDH stability *in vivo*. Protein blots were analyzed using antibodies against the NdhI and NdhJ subunits, which served to monitor NDH complex stability (Figure 5B). NdhI and NdhJ accumulation levels did not experience noticeable changes in *ocp3* mutants compared to Col-0 plants. Similarly, NDH complex stability remained intact in *crr21* plants (Figure 5B). Although the exact function and organization of the whole set of subunits of the NDH complex in plants remains to be fully elucidated [36], our results indicate that the four amino acid residues in the NdhB subunit which were derived from editing-mediated codon conversion appear important for activity, but not for assembly of the NDH complex.

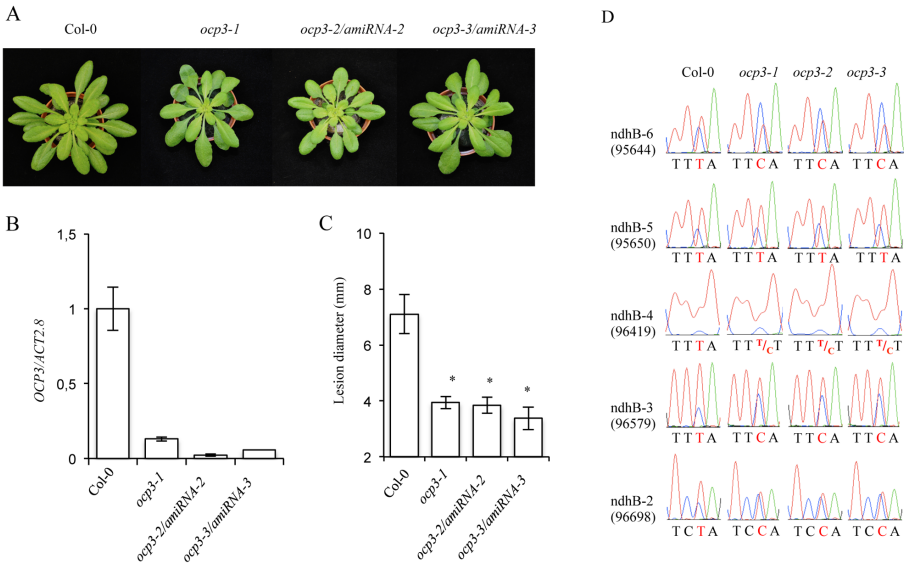
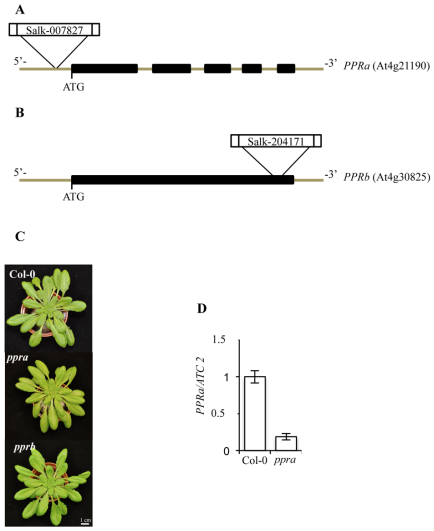


Figure S6. Characterization of Arabidopsis strains silenced in *OCP3* and generated artifitial microRNAs (amiRNAs). The new *ocp3* mutant strains (*ocp3-2/amiRNA-2* and *ocp3-2/amiRNA-3*) were generated by artifitial microRNAs (amiRNAs) designed to specifically target and down-regulate *OCP3*. The resulting mutant phenotypes were compared to the EMS-induced *ocp3-1* mutant and Col-0. (**A**) Comparison of vegetative growth and anatomical appearance between Col-0, *ocp3-1*, *ocp3-2* and *ocp3-3*. (**B**) RT-qPCR of *OCP3* transcript levels in the four indicated genetic backgrounds. *OCP3* expression was normalized to *ACTIN2*.8 expression. Bars represent mean ± SD, n=3 independent replicates. (**C**) Lesion size resulting from *P. cucumerina* infection in Col-0, *ocp3-1*, *ocp3-2*, and *ocp3-3*

plants at 12 days post-inoculation. Values are means and ± SE (n = 50). Asterisks indicate significant differences (LSD test; $P < 0.05$). (**D**) Sequence electrophoregrams corresponding to the RNA editing sites of ndhB-6 (95644), ndhB-5 (95650), ndhB-4 (96419), ndhB-3 (96579), ndhB-2 (96698) as derived from bulk RT-PCR sequencing of amplicons from Col-0, *ocp3-1*, *ocp3-2*, and *ocp3-3* plants mRNA preparations. Editing sites are indicated by a red T residue and unedited sites by a red C residue. Partial editing inhibition is indicated by red T/C.

Chloroplast NDH activity-defective mutants show enhanced disease resistance

We hypothesized that via NDH complex inhibition, plants could develop an alerted immune status. This might explain why *ocp3* plants exhibited enhanced disease resistance to fungal pathogens resulting from earlier and more intense callose synthesis and deposition following pathogen exposure [25,26]. If so, then mutants showing similar chloroplast NDH complex defects would activate the same immune status, and become resistant to fungal attack. Consequently, we challenged *crr21* and *crr2* mutants with *P. cucumerina*, and studied disease susceptibility in comparison to the resistant *ocp3* plants, and the susceptible Col-0 plants. CRR2 is a distinct PPR protein that functions in the intergenic RNA cleavage between *rps7* and *ndhB*, which is essential for subunit B translation, and *crr2* mutants are compromised in NDH activity [35]. *ppra*, a previously uncharacterized T-DNA mutant, defective in the expression of *PPRa* (Figure S8) encoding a PPR protein of unknown function that is highly co-expressed with *CRR21* and *OCP3* (Figure 3A), was also evaluated. Similarly, *pprb*, a T-DNA mutant defective in another co-expressed PPR of unknown function (Figure S8) was included in these experiments for comparison.



Supplemental Figure S8. *PPRa* (At4g2119) and *PPRb* (At4g3082) T-DNA insertion mutants. (**A**) The *ppra* mutant (strain Salk-007827) carries a T-DNA insertion at 340 nt upstream of the ATG initiation codon and therefore could affect expression of the gene. (**B**) The *pprb* mutant (strain Salk-204171) carries a T-DNA insertion internal to the unique exon, close to the ATG initiation codon, and therefore disrupts the ORF. Exons are indicated with solid rectangles. T-DNA insertions are indicated with white rectangles. (**C**) None of the mutations affect the normal growth of the plants and both mutants resemble Col-0 plants in morphological phenotype. (**D**) RT-qPCR of *PPRa* transcript levels in Col-0 and in *ppra* mutant reveal that expression of *PPRa* was down-regulated in the mutant. *PPRa* expression was normalized to *ACTIN2*.8 expression. Bars represent mean ± SD, n=3 independent replicates.

Following inoculation with *P. cucumerina,* disease was scored 12 d after inoculation by following necrosis and chlorosis extent present in inoculated leaves. As expected, Col-0 plants were highly susceptible to *P. cucumerina*, and all inoculated plants showed extended necrosis accompanied by extensive proliferation of fungal mycelia (Figure 6A-B). The same disease susceptibility was observed in the *pprb* mutant, indicating this *PPR* gene is not essential in plant´s defense activation (Figure 6A-B). In marked contrast, the inoculated *crr21*, *crr2,* and *ppra* plants responded with a substantial increase in disease resistance to *P. cucumerina* infection that was of a magnitude similar to that attained in *ocp3* plants (Figure 6A-B). Comparative cytological observations were performed at the sites of attempted fungal infection and the degree of induced callose deposition induction in inoculated leaves was monitored after staining with aniline blue, and examination by fluorescence microscopy. Results indicated none of the mutants exhibited aniline blue staining in control leaves (Figure 6C). Col-0 and *pprb* plants deposited callose locally at sites demarcating the zones of extended fungal growth. In marked contrast, *crr21*, *crr2*, *ppra,* and *ocp3* plants all exhibited intensified and highly localized callose deposition in response to fungal infection, which occurred at zones where fungal growth and colonization was impeded (Figure 6C-D). Consequently, heightened

disease resistance, and increased callose deposition were concurring traits in mutants defective in the correct editing of RNAs encoding subunits of the chloroplast NDH complex.

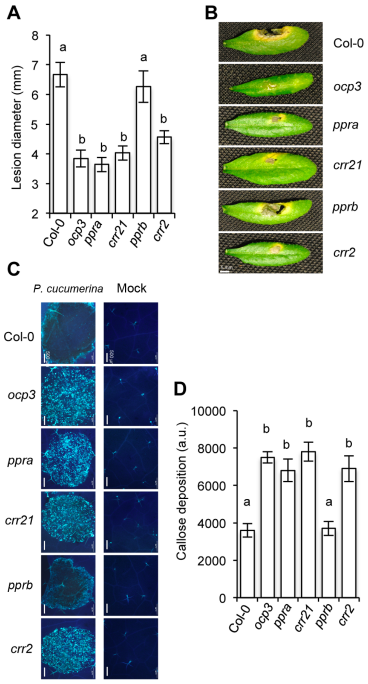


Figure 6. Comparative immune responses of plastid PPR-related mutants to inoculation with *P. cucumerina*. (**A**) *crr2*, *crr21*, *ppra*, and *pprb* disease resistance responses to *P. cucumerina* compared with *ocp3* and wild-type (Col-0) plants. Lesion diameter of 20 plants per genotype and four leaves per plant were determined 12 d following inoculation with *P. cucumerina*. Values are means and ± SE (n = 80). ANOVA detected significant differences at the P<0.05 level. Experiments were repeated three times with similar results. (**B**) Representative leaves from each genotype at 12 days following inoculation with *P. cucumerina*. Bar represents 5 mm. (**C**) Aniline blue staining and epifluorescence microscopy was applied to visualize callose accumulation. Micrographs indicate *P. cucumerina* inoculation and infection site in the different *Arabidopsis* genotypes at 0 h.p.i (right panel) and at 48 h.p.i. (left panel). Bar represents 500 µm. (**D**) The number of yellows pixels (corresponding to pathogen-induced callose) per million on digital photographs of infected leaves were used as a means to express arbitrary units (i.e. to quantify the image) at 48 h.p.i. Data are visible microscopy averages from Col-0 and mutant plants (±SE). Different letters above bars indicate statistically significant differences between genotypes, according to one-way ANOVA (P < 0.05, n = 15).

Fungal infection interferes with editing in plastids

The above results indicated that editing efficiency, chloroplast NDH activity, and disease resistance to fungal pathogens are linked traits mediated by OCP3. Fungal infection provokes local down regulation of *OCP3* in wild type plants [25], therefore we hypothesized that following Col-0 inoculation with a fungal pathogen, editing inhibition of *ndhB* would very likely arise and, the NDH complex would consequently be affected. Therefore, we inoculated Col-0 plants with the fungal pathogen *P. cucumerina* and examined the editing status of RNAs corresponding to chloroplast-encoded subunits of the NDH complex (i.e, NdhB, NdhF, NdhG and NdhD) by bulk sequencing of RT-PCR products. We identified major alterations in the RNA edition of *ndhB*. Eight sites normally edited in the *ndhB* transcripts (i.e. ndhB-1 to ndhB-8) showed inhibition at 48 h.p.i. with *P. cucumerina* (Figure S5). Results for the other NDH subunit RNAs indicated only the editing status of *ndhD* transcript was notably affected, and only at position 117166 (ndhD-1 site) which controls NdhD translation (Figure S5). These results surpass the four distinct defective editing sites identified in the *ocp3* mutant (Figure S5 and Figure 4). Therefore, in addition to OCP3, other factors appeared to be targeted for the realization of the fungal-promoted editing inhibition.

Temporal recording in a time course experiment following *P. cucumerina* inoculation revealed that editing inhibition is an early plant response to fungal attack. Most inhibition changes at the identified pathogen-sensitive *ndhB* editing sites were induced early following *P. cucumerina* inoculation (at 12 h.p.i.), and were sustained up to 48 h.p.i (Figure 7A), indicating the special vulnerability of *ndhB* editing to pathogenic cues. Results showed the specific editing defects at the ndhD-1 site lagged behind *ndhB* editing inhibition, reaching maximal inhibition at 48 h.p.i (Figure 7A). Some of these early effects were further corroborated by specific PPE assays, which provided estimates that editing at ndhB-6, ndhB-5, and ndhB-3 sites were inhibited following pathogen inoculation at different efficiencies and declining rates. ndhB-6 editing inhibition was the most prominent, with an efficiency that abruptly dropped at 12 h.p.i. and progressively decayed thereafter (Figure 7B).

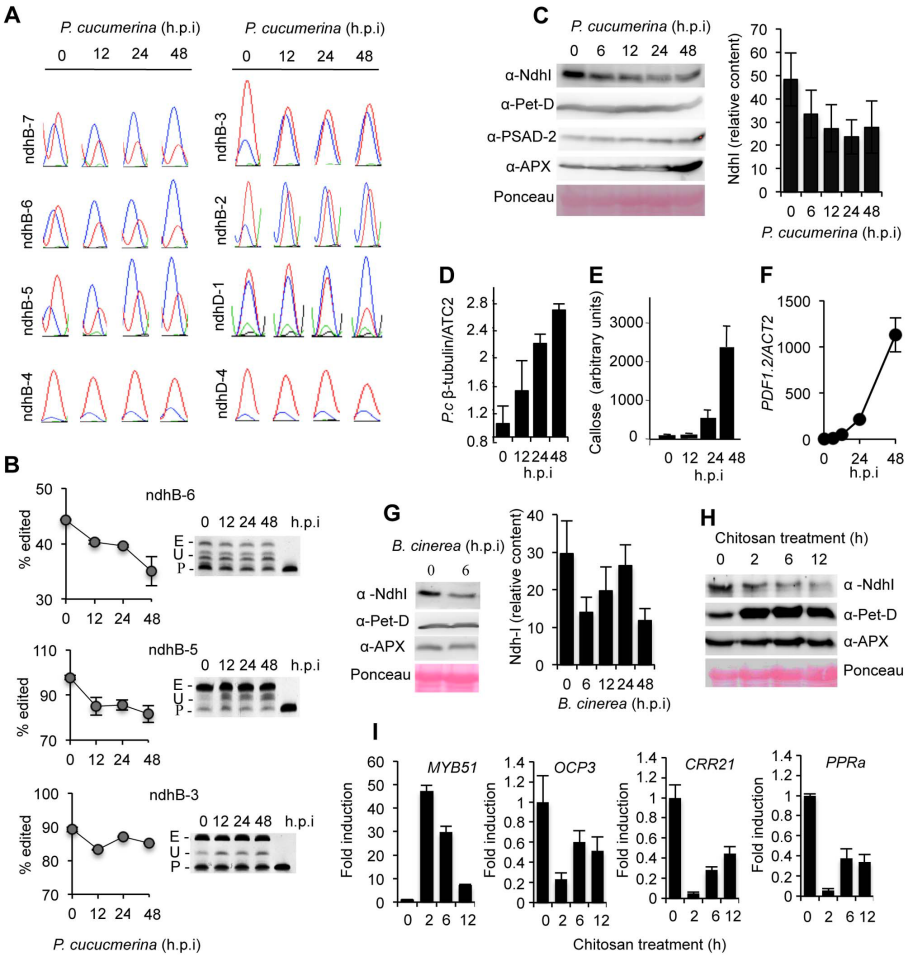


Figure 7. Pathogen-triggered editing inhibition in plastid *ndhB* and *ndhD* transcripts. (**A**) Portion of the electrophoretograms from RT-PCR bulk sequencing corresponding to the editable cytosine residue at sites ndhB-7, ndhB-6, ndhB-5, ndhB-4, ndhB-3, ndhB-2, ndhD-1, and ndhD-4 are shown for Col-0 plants at 0,12, 24 and 48 h post-inoculation with *P. cucumerina*. (**B**) PPE assays following fungal infection for ndhB-6, ndhB-5 and ndhB-3 sites confirms the reduction of editing extent as detected by bulk sequencing. The PPE products run on acrylamide gels are shown on the right. E, edited; U, unedited; P, primer. (**C**) Immunoblot analysis of NDH subunit I (NdhI), subunit IV of cytochrome *b6f* (Pet-D), PSI subunit D-2 (PSAD-2) and ascorbate peroxidase (APX) from Co-0 plants at 0, 6, 12, 24 and 48 h following inoculation with *P. cucumerina*. Intensity of NdhI immunoreactive bands was quantified and plotted on the right graph. Bars represent mean ± SD, n=3 independent replicates. The experiment was repeated three times with similar results (**D**) Extent of *P. cucumerina* growth on inoculated leaves. At the times indicated DNA was extracted from leaves and the amount of the *P. cucumerina β-tubulin* gene quantified by qPCR. Data are standardized for the presence of the *P. cucumerina β-tubulin* gene in Col-0 at time 0. Data represent the mean ± SD; n=3 biological replicates. (**E**) Determination of *P. cucumerina*-

induced callose deposition in inoculated leaves of Col-0 plants. (**F**) *P. cucumerina*-induced expression of the defense-related *PDF1.2* gene as determined by RT-qPCR. Data represent the mean ± SD; n=3 biological replicates. (**G**) Reduction of NdhI subunit content following inoculation of Col-0 with *B. cinerea*. NdhI content was quantified as in (C). On the right a Western blot detail revealing early (at 6 h.p.i) fungal-induced down regulation of NdhI subunit accumulation is shown. (**H**) Early induced down-regulation of NdhI protein accumulation in Arabidopsis seedlings by chitosan. Chitosan was applied for the times indicated to Arabidopsis seedlings and proteins analyzed by Western blot with anti-NhHI, anti-Pet-D and anti-APX antibodies. The experiment was repeated three times with similar results. (**I**) Chitosan-induced gene activation of *MYB51* and concomitant gene repression of *OCP3*, *CRR21* and *PPRa* as determined by RT-qPCR. Data represent the mean ± SD; n=3 biological replicates.

Early chloroplast NDH complex destabilization is part of the immune response

Following these previous observations we asked if stability of the NDH complex could become also altered following fungal infection. To assess this, NDH complex stability was monitored by Western blots using antibodies against one of the NDH subunits (i.e. NdhI). We observed NdhI accumulation level decayed very early following pathogen inoculation, with apparent reduction occurring at 6 h.p.i. (Figure 7C). The decay was progressive and showed an approximate 50% reduction in NdhI protein at 24 h.p.i. (Figure 7C). The results suggested decay specificity for NDH complex protein, since chloroplast integrity, measured using other marker proteins (i.e., Pet-D, PSAD-2 and APX), did not change or even increase in response to the fungus (Figure 7C). The decay process was set in motion at early stages of infection, and was inversely correlated with fungal growth (Figure 7D). Furthermore, the observed pathogen-triggered dismantling of the NDH complex subunit preceded the activation of other plant responses, which are diagnostic of an activated plant immune response. Deposition of the cell wall β-1,3-glucan polymer callose, identified and quantified following aniline blue staining of inoculated leaves, clearly lagged behind observed editing defects and dismantling of NDH complex (Figure 7E). Similarly, transcriptional activation of the defense related gene *PDF1.2* followed editing defect accumulation (Figure 7F). Furthermore, the inhibitory effect on NdhI accumulation was mirrored by *Botrytis cinerea* inoculation, another fungal pathogen (Figure 7G). The decay in NDH subunit content promoted by *B. cinerea* (Figure 7G, right graph) was notable, but not as progressive as observed in *P.*

cucumerina, presumably reflecting different infection styles for the two distinct fungal pathogens.

The rapid editing inhibition and the parallel dismantling of the NDH complex constitutes two early chloroplast responses to pathogens, evoking integration of these processes as part of the mechanism governing immune response activation. Therefore, we verified if editing inhibition and NDH complex destabilization could be similarly triggered by application of chitosan (2-amino-2-deoxy-(1-4)-β-D glucopiranan), a naturally-occurring pathogen-associated molecular pattern (PAMP) compound able to elicit plant innate immune responses similar to those activated by complex fungal pathogens [37]. Results showed strong and rapid (within 2 h) down-regulation of NDH subunit accumulation was promoted by the sole application of chitosan to wild type Arabidopsis seedlings (Figure 7H). Interestingly, we observed also an early and abrupt down-regulation of *OCP3*, *CRR21* and *PPRa* gene expression following chitosan treatment, which contrasted with the concurring high transcriptional activation of *MYB51* (Figure 7I), the latter a transcription factor required for PAMP-triggered callose deposition in Arabidopsis [38]. Since CRR21, OCP3 and PPRa are nuclear-encoded chloroplast factors required for an effective plant immune response (Figure 6), the results indicate the existence of a nuclear PAMP-mediated transcriptional regulation of NDH complex-related editing regulatory genes as an integral component of innate immunity. This thus represents an additional layer of control of chloroplast NDH complex activity interconnecting nuclei and chloroplasts. Moreover, electrophoregrams comparison of bulk sequencing of RT-PCR products, generated at different times after chitosan treatment, revealed that chitosan-induced NDH complex dismantling was also accompanied by an early editing inhibition at seven *ndhB* editing sites, and at three *ndhD* editing sites (Figure S7).

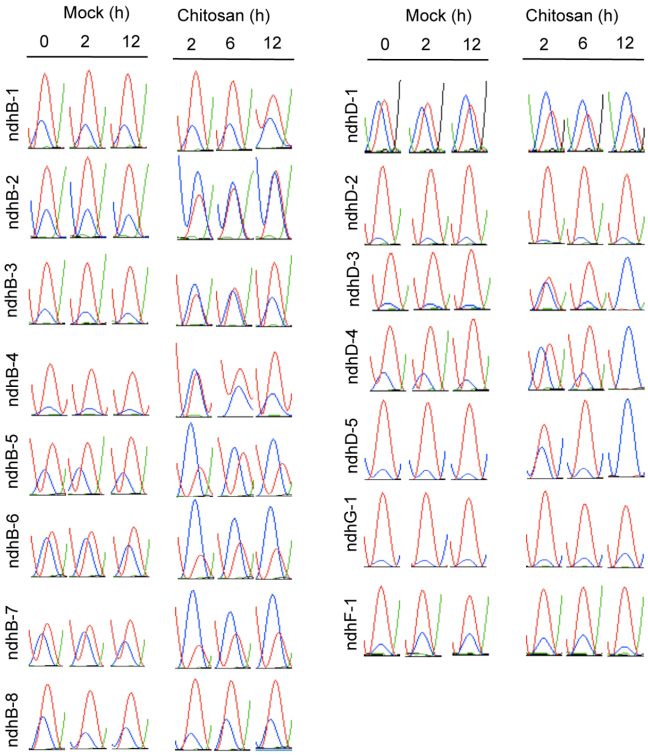


Figure S7. Sequencing electrophoregrams of nucleotide sequence of RT-PCR products obtained from Col-0 seedlings at the times indicated following mock or chitosan (10 µg/mL) treatment. The electrophoregrams show the C nucleotide either edited or not edited at the corresponding editing site of the corresponding transcript. Shown are editing sites for which chitosan exerts editing inhibition effect.

Therefore, these results provided additional support for the engagement of plastid RNA editing inhibition in plant immunity. Moreover, the observed rapid and transient dismantling of the NDH complex that follows perception of pathogenic cues suggests the engagement of a highly regulated proteolytic system in the chloroplast. The identification and characterization of such proteolytic system remains a challenge for the future.

DISCUSSION

This study provides new insights into the control of disease resistance in plants, and reinforces the importance of the chloroplasts in plant immunity. Our data identified OCP3 targeted to chloroplast, a finding that conceptually changed the previous assumption that OCP3 could function as a nuclear transcription factor. Furthermore, confocal microscopy revealed that OCP3 accumulated in plastids matching several PPR proteins. Moreover, *OCP3* was found to be closely co-expressed with a cluster of 9 genes encoding PPR proteins including *CRR21*. CRR21 is responsible for site 2 editing at *ndhD* transcript, and *ndhD* encodes the D subunit of the chloroplast NDH complex [13], a crucial component of the CEF machinery around PSI [11]. In *crr21* plants, NDH complex activity is impaired and CEF activity compromised [13], supporting a predominant post-trascriptional level of control. All these observations prompted us to hypothesize that OCP3 was involved in RNA editing in plastids. Therefore, we performed a comparative systematic study of the editing status of chloroplast transcripts between Col-0 and different *ocp3* mutants. This study revealed that OCP3-defective plants carry specific editing defects at ndhB-6, ndhB-4, ndhB-3, and ndhB-2 sites. The observation that OCP3 associates *in vivo* with the *ndhB* transcript, as revealed by RIP assays, reinforce the consideration that OCP3 contributes to control over the extent of *ndhB* transcript editing. However, OCP3 appears not to carry any structural motif resembling the conserved RNA recognition motif (RRM), not even the motifs characteristic of other proteins functioning as *trans*-factors essential for editing, such as those present in the large subclasses of the pentatricopeptide repeat (PPR)-containing family proteins [13,14]. This may suggest that the association of OCP3 with the *ndhB* RNA molecule may be likely indirect, presumably though the interaction with canonical RNA binding proteins recognizing appropriate *cis*-elements present in the *ndhB* RNA molecule such as those RNA-binding proteins mentioned above. Therefore, OCP3 may serve a regulatory role on the editing apparatus by regulating and/or adjusting the editing extent of the *ndhB* transcript according to external environmental cues. This appears to be the case also for other described editing accessory proteins such as the recently identified multiple

organellar RNA editing factor (MORF) and members of the RNA-editing interacting protein (RIP) family [39,40].

ndhB encodes the NDH complex B subunit, therefore we next hypothesized that the absence of a functional OCP3 protein should result in a defective NDH complex. Results indicated that the observed alterations in *ndhB* editing in *ocp3* plants affected NDH activity but not NDH complex stability. This is a feature also found in other editing-related mutants (i.e. *crr21*). However, in other cases, the defective gene results in lack of NDH complex accumulation, as seen when editing defects impedes translation initiation of NdhD subunit (i.e. *crr4*, [10]) or when appropriate maturation of *ndhB* transcript is blocked (i.e. *crr2*, [35]). The editing defects in *ocp3* plants resulted in an inactive NDH complex that compromised normal CEF around PSI. We therefore concluded that OCP3 is an integral plastidial factor required for fine-tuning CEF around PSI, and this control is exerted post-transcriptionally through the regulation of *ndhB* transcript editing. This finding has important consequences as it represents the first evidence interfacing plant immunity, RNA editing and CEF. Therefore, one can propose that when OCP3 fails, as occurs in *ocp3* plants, then accurate *ndhB* transcript editing is impeded, and in turn NDH complex is altered and eventually CEF inhibited. Since in chloroplast the NDH complex is considered to alleviate various oxidative stresses [41,42], it can be speculated that a defective CEF pathway could generate ROS locally that eventually may resulted in disease resistance activation. Since *ocp3* plants carry constitutive enhanced production of ROS species, particularly H_2O_2, and also show constitutive expression of ROS-inducible genes [25, 28], and *OCP3* gene expression was rapidly down regulated following fungal attack [25], we reasoned that editing will fail, and consequently NDH impaired, when a plant encounters a pathogen. This led us to find that Arabidopsis respond to attempted *P.cucumerina* infections by activating a rapid mechanism of editing inhibition which affected the 8 major editing sites in *ndhB*, therefore including those requiring OCP3, plus an additional editing site at *ndhD*. This suggests involvement of other factors functioning as pathogen-sensitive regulators of the editing process. In fact, a similar cause-effect relationship was observed in *crr2*, *crr21 and ppra* plants, which exhibited the same response as *ocp3*

plants when inoculated with *P. cucumerina*. Their characteristic heightened disease resistance was accompanied by increased callose deposition in response to fungal infection, evoking activation of a mechanism for priming of callose deposition in these mutants similar to that previously discovered in *ocp3* plants [26].

In wild type plants, in addition to the pathogen-induced editing inhibition of *ndhB* transcripts, we observed the NDH complex becaming rapidly destabilized and therefore dismantled, presumably by the action of chloroplast proteases. Therefore, either NDH complex activity and/or stability constitute distinct hallmarks of the plant´s defense response to fungal pathogens. Whether or not editing inhibition and NDH complex stability are linked processes or rather represent independent processes remains unknown and is a challenging issue for future research. Furthermore, strong repression of *OCP3* and *CRR21* gene expression, severe editing inhibition of *ndhB* and *ndhD* transcripts, and NDH destabilization were also rapidly triggered by the sole application of chitosan, which functions as a PAMP mimicking fungal structures. Consequently, editing inhibition and dismantling of the NDH complex appeared definitively engaged during activation of innate immunity. Therefore, when appropriately and timely activated following pathogen perception, the mechanisms leading to alteration of the NDH complex in the chloroplast should serve to set in motion a signaling process leading to an effective defense response to halt the advance of the pathogen.

Cumulatively, these observations reinforced the idea that maintaining NDH complex integrity is pivotal to normal CEF functioning during photosynthesis, however its timely inhibition following pathogen attack is fundamental for plant immunity. Therefore, modulation of the NDH complex activity must be under a delicate balance, requiring precise but flexible control. The breadth of our data indicate control occurs at the RNA editing level, a process where the described proteins ultimately serve as sensors regulating the rate of NDH complex activity. Therefore, OCP3, and presumably those PPRs and accessory proteins mediating editing extent of NDH complex subunits, exhibit a Janus-faced function, serving reciprocally as negative regulator of plant immunity and as positive regulator of CEF during oxygenic photosynthesis.

MATERIALS AND METHODS

Plants growth conditions

Arabidopsis thaliana plants were grown in a growth chamber (19-23ºC, 85% relative humidity, 100 mEm^{-2} sec^{-1} fluorescent illumination) on a 10-hr-light and 14-hr-dark cycle. All mutants are in Col-0 background.

Gene constructs and transgenic lines

For the *OCP3-GFP, -YFP and -mCHERRY* constructs, the *OCP3* full lenght cDNA was amplified by PCR using *Pfu* DNA polymerase (Stratagene, San Diego, CA) and specific primers including Gateway adapters, and recombined into pDONR221/207 using BP ClonaseMixII kit (Invitrogen). After sequencing, all constructs were recombined into pEarleyGate101 destination vector using LR ClonaseMixII kit (Invitrogen) and introduced into *ocp3* plants for complementation analysis or when indicated in Col-0 via *Agrobacterium* transformation. Cloning of the different ORFs employed in the present work and their fusion with the indicated fluorescent tag was done is a similar way. List of primers used for cloning purposes is provided in Supplementary information.

Chlorophyll Fluorescence Analysis

Chlorophyll fluorescence was measured using a MINI-pulse-amplitude modulation portable chlorophyll fluorometer (Dual-PAM-100, Walz, Effeltrich, Germany). The transient increase in chlorophyll fluorescence after turning off actinic light (AL) was monitored as described [12].

Confocal laser-scanning microscopy

Plant tissue was observed with a Leica TCS LS spectral confocal microscope using and HCX PL APO x40/1.25-0.75 oil CS objective. GFP- or YFP-derived fluorescence was monitored by excitation with 488- and 514-nm argon laser lines,

respectively, and emission was visualized with a 30-nm-width band-pass window centered at 515 nm. When RFP and CHERRY were used, excitation was performed by means of a 543-nm green-neon laser line, and fluorescence emission was collected at 695 to 630 nm .

OCP3 amiRNAs

The artificial microRNA designer web WMD3 (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) was employed for designing the 21 mer amiRNA sequence specific for OCP3 (At5g11270) and for subsequent cloning and amplifications protocols. The target region selected in *OCP3* was 5-´GCGTCGTAAAACTAGTATTAA-3 (positions 625 to 645) and the 4 oligonucleotide sequences used to engineer the artificial miRNA into the endogenous miR319a precursor by site-directed mutagenesis were:

I miR-s TTAATACTAGTTTTACGGCGCtctctctttgtattccaa,

II miR-a GCGCCGTAAAACTAGTATTAAtcaaagagaatcaatgatc,

III miR*s GCACCGTAAAACTTGTATTATtcacaggtcgtgatatgat,

IV miR*a ATAATACAAGTTTTACGGTGCtctacatatatattcctaa,

As a template for the PCRs the pRS300 was used. The amiRNA sequence was cloned behind a 35S gene promoter and the binary vector used to transform Arabidopsis Col-0 plants. Eight independent tramsformants were initially selected and two homozygous lines showing remarkable reduced expression (amiRNA-2 and amiRNA-3) of *OCP3* were selected for further studies.

RNA extraction, RT, qPCR, HRM and PPE

Total RNA was extracted using TRIzol® reagent (Invitrogen) following the manufacturer's recommendations and further purified by lithium chloride precipitation. For reverse transcription, the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas Life Sciences) was used. Quantitative PCR (qPCR) amplifications and measurements were performed using an ABI PRISM 7000 sequence detection system, and SYBR-Green (Perkin-Elmer Applied Biosystems). *ACTIN2/8* was chosen as the reference gene. All 34 known Arabidopsis chloroplast

RNA editing C targets were assaed by high resolution melt (HRM) as described [7]. Chloroplast RNA editing sites were assayed by RT-PCR bulk sequencing using similar set of primers. Primers for amplicons covering each of the 34 editing sites present in plastids are listed below in Supplemental information. Poison primer extension (PPE) analysis on chloroplast sites were conducted as described [43] and sequence of the corresponding fluorescent primers used are listed below in Supplemental information.

Transient expression in protoplasts

Protoplasts isolation and transfection protocol with the different gene constructs was as described [44].

Western blots

Protein crude extracts were prepared by homogenizing ground frozen leaf material with Tris-buffered saline (TBS) supplemented with 5mM DTT, protease inhibitor cocktail (Sigma-Aldrich). Protein concentration was measured using Bradford reagent; unless otherwised indicated 20 µg of total protein was separated by SDS-PAGE (12% acrylamide w/v) and transferred to nitrocellulose filters. The filter was stained with Ponceau-S after transfer, and used as a loading control. Unless otherwise indicated, immunoblots were incubated with the indicated primary antibodies at the appropriate dilution and developed by chemiluminescence using an anti-IgG peroxidase antibody (Roche) at a 1:1000 dilution and Western Lighting plus-ECL substrate (Perkin-Elmer).

N-terminal sequencing

Protein samples resolved by SDS-PAGE were blotted to PVDF membranes and the band of interest identified by Ponceau staining. The band sector corresponding to OCP3 was recovered and subjected to five/six cycles of automated microsequencing by sequential Edman degradation in an Applied Biosystems, Procise 494.

Botrytis cinerea and *Plectosphaerella cucumerina* bioassays

In both *B. cinerea* and *P. cucumerina* infections, five-week-old plants were inoculated as described [25,26], with a suspension of fungal spores of $2.5x10^4$ and $5x10^6$ spores/mL respectively. The challenged plants were maintained at 100% relative humidity. Disease symptoms were evaluated by determining the lesion diameter of at least 50 lesions 3 or 12 days after inoculation. For pathogen-induced callose deposition analyses, infected leaves were stained with aniline blue and callose deposition quantifications were perfomed as described by Garcia-Andrade *et al*. [26].

Chitosan treatments

Approximately 15 sterilized Col-0 seeds were sown per well in sterile 12-wells plates, containing filter-sterilized MS mediums without Gamborg's vitamins and with 0,5% of sucrose. Seedlings were cultivated under standard growth conditions (15 h day cycle; 20ºC /17ºC) with a light intensity of 150 µM/m2/s. After 7 days, the growth medium was replaced by fresh MS medium. One day later, plants were mocked or challenged with chitosan at the final concentration of 10 µg/mL in the growth medium, and at the indicated times the samples were collected and immediately frozen in liquid nitrogen.

Chloroplast fractionation.

Fractionation of total chloroplasts preparations from full expanded Arabidopsis leaves into stromal, thylakoids and membrane envelope was performed as described [45]. Each suborganellar fraction was identified and validated by developing Western blots with anti-BCCP (stroma), anti-NIP (thylakoids) and anti-OEP21 (membrane envelope) antibodies. Antibodies were obtained from Uniplastomic (Gieres, France).

RNA Immunoprecipitation (RIP) Followed by RT-PCR

RIP assays were performed as described [46] with minor modifications. Essentially, 2 g of leaf tissue from Arabidopsis plants (4 weeks old plants) were ground to a fine powder with a mortar and pestle in liquid nitrogen and homogenized in 12.5 mL/g

lysis buffer (50 mM Tris-HCl, pH 7.4, 2.5 mM $MgCl_2$, 100 mM KCl, 0.1% Nonidet P-40, 1 µg/mL leupeptin, 1 µg/mL aprotonin, 0.5 mM phenylmethylsulfonyl fluoride, one tablet of Complete proteinase inhibitor tablet (Roche), and 50 units/mL RNase OUT (Invitrogen). Cell debris was pelleted by centrifugation for 5 min at 12,000 rcf at 4°C. Clarified lysates were incubated with 4 µg/mL of anti-HA ntibody (Roche) for 15 min at 4°C and then with 100 µL of Protein-A agarose (Roche) per milliliter for 30 min at 4°C. Beads were washed six times for 10 min with lysis buffer at 4°C and then divided for protein and RNA analysis. RNAs were recovered by incubating the beads in 0.5 volumes of proteinase K buffer (0.1 M Tris-HCl, pH 7.4, 10 mM EDTA, 300 mM NaCl, 2% SDS, and 1 µg/µL proteinase K (Roche)) for 15 min at 65°C, extraction with saturated phenol, phenol:chloroform:isoamyl alcohol and chloroform, and ethanol precipitation. For RT-PCR assays, 1 µg of total RNA was used for the input fraction, and 20% of the RNA immunoprecipitate was used for the immunoprecipitation. PCR to amplify fragments corresponding to *ndhB* and *ndhD* cDNAs was done using specific oligos, CHLORO 187 FW/RV and CHLORO 212 FW/RV, respectively, as listed in Supplememtal material. For protein blot assays, 10 µL of clarified eluate was loaded for the input fraction, and 3% of the immunoprecipitated beads was used for the immunoprecipitation. OCP3:GFP:HA was detected by immunoblotting and chemiluminescence using and anti-GFP peroxidase antibody (Roche) at a 1:1000 dilution and Western Lighting plus-ECL substrate (Perkin-Elmer).

T-DNA Arabidopsis mutants

Homozygous lines of *ppra* and *pprb* T-DNA insertion mutants were identified by PCR using primers listed in Supplemental information.

ACKNOWLEDGEMENTS

We acknowledge Alice Barkan for anti-PetD antibodies, and Peter Nixon for anti-NdhI and anti-NdhJ antibodies. We also appreciate the assistance of Mª Rosa Ponce and J.L. Micol in preparing the amiRNA lines, and Anna Marika Lindahl and Belén

Naranjo for their help in determining chlorophyll fluorescence parameters.

REFERENCES

1. **Li HM, Chiu C-C** (2010) Protein transport into chloroplasts. Annu Rev Plant Biol 61: 157–180.

2. **Timmis JN, Ayliffe MA, Huang CY, Martin W** (2004). Endosymbiotic gene transfer: Organelle genomes forge eukaryotic chromosomes. Nat Rev Genet 5: 123–135.

3. **Soll J, Schleiff E** (2004). Protein import into chloroplasts. Nat Rev Mol Cell Biol 5: 198–208.

4. **Schmitz-Linneweber C, Small I** (2008). Pentatricopeptide repeat proteins - a socket set for organelle gene expression. Trends. Plant. Sci.13: 663-670.

5. **Kroeger TS, Watkins KP, Friso G, van Wijk KJ, Barkan A** (2009). A plant-specific RNA-binding domain revealed through analysis of chloroplast group II intron splicing. Proc Natl Acad Sci USA 106: 4537–4542.

6. **Shikanai T** (2006). RNA editing in plant organelles: machinery, physiological function and evolution. Cell Mol Life Sci 63: 698-708.

7. **Chateigner-Boutin AL, Small I** (2007). A rapid high-throughput method for the detection and quantification of RNA editing based on high-resolution melting of amplicons. Nucleic Acids Res 35: e114.

8. **O'Toole N, Hattori M, Andrés C, Iida K, Lurin C, Schmitz-Linneweber C, et al.** (2008). On the expansion of the pentatricopeptide repeat gene family in plants. Mol Biol Evol 25: 1120–1128.

9. **Fujii S, Small I** (2011). The evolution of RNA editing and pentatricopeptide repeat genes. New Phytol 191: 37-47.

10. **Kotera E, Tasaka M, Shikanai T** (2005). A pentatricopeptide repeat protein is essential for RNA editing in chloroplasts. Nature 433:326–330.

11. **Shikanai, T** (2007). Cyclic electron transport around photosystem I: Genetic approaches. Annu Rev Plant Biol 58: 199-217.

12. **Shikanai T, Endo T, Hashimoto T, Yamada Y, Asada K, Yokota A** (1998). Directed disruption of the tobacco *ndhB* gene impaired cyclic electron flow around photosystem I. Proc Natl Acad Sci USA 95: 9705–9709.

13. **Okuda K, Myouga F, Motohashi R, Shinozaki K, Shikanai T** (2007). Conserved domain structure of pentatricopeptide repeat proteins involved in chloroplast RNA editing. Proc Natl Acad Sci USA 104: 8178–8183.

14. **Barkan A, Rojas M, Fujii S, Yap A, Chong YS, Bond CS, Small I** (2012). A combinatorial amino acid code for RNA recognition by pentatricopeptide repeat proteins. PLoS Genetics 8(8):e1002910.

15. **Grant MR, Jones JD** (2009). Hormone (dis)harmony moulds plant health and disease. Science 324: 750–75.

16. **Gil MJ, Coego A, Mauch-Mani B, Jordá L, Vera P** (2005). The Arabidopsis *csb3* mutant reveals a regulatory link between salicylic acid- mediated disease resistance and the methyl-erythritol 4-phosphate pathway. Plant J 44: 155–166.

17. **Xiao Y, Savchenko T, Baidoo EEK, Chehab WE, Hayden DM, Tolstikov V, Corwin JA, Kliebenstein DJ, Keasling JD, Dehesh K** (2012). Retrograde signaling by the plastidial metabolite MEcPP regulates expression of nuclear stress-response genes. Cell 149: 1525-1535.

18. **Nomura H, Komori T, Uemura S, Kanda Y, Shimotani K, Nakai K, et al**. (2012*)*. Chloropast-mediated activation of plant immune signaling in Arabidopsis. Nature Communications 3, doi:10.1038/ncomms1926.

19. **Laloi C, Stachowiak M, Pers-Kamczyc E, Warzych E, Murgia I, Apel K** (2007). Cross-talk between singlet oxygen- and hydrogen peroxide-dependent signalling of stress responses in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 104: 672–677.

20. **Terashima M, Petroutsos D, Hüdig M, Tolstygina I, Trompelt K, et al** (2012). Calcium-dependent regulation of cyclic photosynthetic electron transfer by a CAS, ANR1, and PGRL1 complex. Proc Natl Acad Sci USA 109: 17717–17722.

21. **Krieger-Liszkay A, Fufezan C, Trebst A** (2008). Singlet oxygen production in photosystem II and related protection mechanism. Photosynth Res 98:551-64.

22. **Müller P, Li X-P, Niyogi KK** (2001). Non-photochemical quenching. A response to excess light energy. Plant Physiol 125: 1558-1566.

23. **Göhre V, Jones AME, Sklenář J, Robatzek S, Weber APM** (2012). Molecular crosstalk between PAMP-triggered immunity and photosynthesis. Mol Plant-Microbe Interac 25: 1083-1092.

24. **Zurbriggen MD, Carrillo N, Tognetti VB, Melzer M, Peisker M, Hause B,**

Hajirezaei M-R (2009). Chloroplast-generated reactive oxygen species play a major role in localised cell death during the non-host interaction between tobacco and *Xanthomonas campestris* pv. *vesicatoria*. Plant J 60: 962-73.

25. **Coego A, Ramirez V, Gil MJ, Flors V, Mauch-Mani B, Vera P** (2005). An Arabidopsis homeodomain transcription factor, OVEREXPRESSOR OF CATIONIC PEROXIDASE 3, mediates resistance to infection by necrotrophic pathogens. Plant Cell 17**:** 2123-2137.

26. **García-Andrade J, Ramírez V, Flors V, Vera P** (2011). Arabidopsis *ocp3* mutant reveals a mechanism linking ABA and JA to pathogen-induced callose deposition. Plant J 67: 783-794.

27. Ramírez **R, Van der Ent S, García-Andrade J, Coego A, Pieterse CMJ, Vera P** (2010). OCP3 is an important modulator of NPR1-mediated jasmonic acid-dependent induced defenses in *Arabidopsis.* BMC Plant Biol 10: 199.

28. **Coego A, Ramirez V, Ellul P, Mayda E, Vera P** (2005). The H_2O_2-regulated Ep5C gene encodes a peroxidase required for bacterial speck susceptibility in tomato. Plant J 42**:** 283-293.

29. **Vidi P, Kessler F, Brehelin C** (2007). Plastoglobules: a new address for targeting recombinant proteins in the chloroplast. BMC Biotechnol 7, 4.

30. **Terasawa K, Sato N** (2009). Plastid localization of the PEND protein is mediated by a noncanonical transit peptide. FEBS J 276: 1709-1719.

31. **Melonek J, Mulisch M, Schmitz-Linneweber C, Grabowski E, Hensel G, Krupinska, K** (2010). Whirly1 in chloroplasts associates with intron containing RNAs and rarely co-localizes with nucleoids. Planta 232: 471–481.

32. **Usadel B, Obayashi T, Mutwil M, Giorgi FM, Bassel GW, Tanimoto M, Chow A, Steinhauser D, Persson S, Provart NJ** (2009). Co-expression tools for plant biology: opportunities for hypothesis generation and caveats. Plant Cell Environ 32: 1633–1651.

33. **Mutwil M, Klie S, Tohge T, Giorgi FM, Wilkins O, Campbell MM, Fernie AR, Usadel B, Nikoloski Z, Persson S** (2011). PlaNet: Combined Sequence and expression comparisons across plant networks derived from seven species. Plant Cell 23: 895-910.

34. **Peng L, Shimizu H, Shikanai T** (2008). The chloroplast NAD(P)H dehydrogenase complex interacts with photosystem I in Arabidopsis. J Biol Chem 83: 34873–34879.

35. **Hashimoto M, Endo T, Peltier G, Tasaka M, Shikanai T** (2003). A nucleus-

encoded factor, CRR2, is essential for the expression of chloroplast *ndhB* in Arabidopsis. Plant J 36: 541–549.

36. **Suorsa M, Sirpiö S, Aro E-M** (2009). Towards characterization of the chloroplast NAD(P)H dehydrogenase complex. Mol Plant 2: 1127–1140.

37. **Doares SH, Syrovets T, Weiler EW, Ryan CA** (1995).Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. Proc Natl Acad Sci USA 92: 4095– 4098.

38. **Clay NK, Adio AM, Denoux C, Jander G, Ausubel FM** (2009). Glucosinolate metabolites required for an Arabidopsis innate immune response. Science 323: 95–101.

39. **Takenaka M, Zehrmann A, Verbitskiy D, Kugelmann M, Härtel B, Brennicke A** (2012). Multiple organellar RNA editing factor (MORF) family proteins are required for RNA editing in mitochondria and plastids in plants. Proc Natl Acad Sci USA 109: 5104-5109.

40. **Bentolila S, Heller WP, Sun T, Babina AM, Friso J, van Wijk KJ, Hanson MR** (2012). RIP1, a member of an Arabidopsis protein family interacts with the protein RARE1 and broadly affects RNA editing. Proc Natl Acad Sci USA 109(22):E1453-E1461.

41. **Endo T, Shikanai T, Takabayashi A, Asada K, Sato F** (1999). The role of chloroplastic NAD(P)H dehydrogenase in photoprotection. FEBS Lett 457: 5-8.

42. **Wang P, Duan W, Takabayashi A, Endo T, Shikanai T, Ye J-Y, Mi, H** (2006). Chloroplastic NAD(P)H dehydrogenase in tobacco leaves functions in alleviation of oxidative damage caused by temperature stress. Plant Physiol 141: 465-474.

43. **Bentolila S, Knight, WE, Hanson MR** (2010). Natural variation in Arabidopsis leads to the identification of REME1, a pentatricopeptide repeat-DYW protein controlling the editing of mitochondrial transcripts. Plant Physiol 154: 1966–1982.

44. **Martínez F, Sardanyés J, Elena SF, Darós J-A** (2011). Dynamics of a plant RNA virus intracellular accumulation: stamping machine vs. geometric replication. Genetics 188: 637-646.

45. **Hall M, Mishra Y, Schröder WP** (2011). Preparation of stroma, thylakoid membrane, and lumen fractions from Arabidopsis thaliana chloroplasts for proteomic analysis. Methods Mol Biol 775: 207-222.

46. **Carbonell A, Fahlgren N, Garcia-Ruiz H, Gilbert KB, Montgomery TA, Nguyen T, Cuperus, JT, Carrington JC** (2012). Functional analysis of three

Arabidopsis ARGONAUTES using slicer-defective mutants. Plant Cell 24:3613-3629.

Capítulo III

Dual antagonistic role of ABA in plant immunity is mediated through the PYR1 receptor

Javier García-Andrade, Beatriz González, Miguel Gonzalez-Guzman, Pedro L. Rodriguez and Pablo Vera*

Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia-C.S.I.C, Ciudad Politécnica de la Innovación, Edificio 8E, Valencia, Spain.

*Corresponding author: Pablo Vera: E-mail: vera@ibmcp.upv.es

ABSTRACT

An activation of immune responses following pathogen attack requires a fine tuning of the regulation of several plant hormones. We identified that PYR1, a member of the family of PYR/PYL/RCAR abscisic acid (ABA) receptors, specifically perceives ABA during biotic stress and initiates downstream signaling mediated by ABA-activated SnRK2 protein kinases. PYR1-mediated signaling exerts a damping effect on basal defense and salicylic acid (SA)-mediated signaling, which is vital for resistance to biotrophic pathogens. Moreover, PYR1 exerts, simultaneously, a positive control over jasmonic acid (JA)-mediated signaling, which is required for resistance to necrotrophic pathogens. Hence PYR1-mediated signaling exerts a control on a *priori* established hormonal cross-talk between SA and JA thereby redirecting defense outputs. Defects in ABA/PYR1 signaling activate plant defenses by poising SA-responsive genes for enhanced expression, through chromatin remodeling, and by enhancing activation of MAP kinases following pathogen attack. Our results also point to an ABA/PYR1-mediated epigenetic control of plant immunity. In summary, PYR1 perception of ABA represents a regulatory node in immunity, modulating basal defense and induced resistance.

INTRODUCTION

To prevent microbial growth, plants rely on efficient resistance mechanisms that involve multiple and complex signaling networks to orchestrate different types of plant-inducible and durable defenses. Induced resistance (IR), or cross-protection, is characterized by heightened activation of the innate immune response state as a result of a pre-exposure biotic stress, exhibits memory characteristics, and appears evolutionarily conserved even outside the plant kingdom. Netea *et al.* [1] coined the term "trained immunity" to differentiate it from "innate immunity" (which is induced only secondarily in hosts that have previously encountered a primary infection), or from "adaptive immunity'' (which is specifically induced through T and B cells). Relevant in IR responses in plants is the observation that defense genes respond to a pathogenic stimulus in a more rapid and robust manner, thus revealing a ''priming'' phenomenon. Priming has long been known as a component of IR responses in plants [2,3] and mammals [4–6], and more recently in invertebrates, which like plants lack adaptive immunity [7]. Recent studies have identified different molecular components, common to basal defense mechanisms, that activate and maintain the primed immune state in Arabidopsis. Pre-stress deposition of a mitogen-activated protein kinase (MAPK) cascade which results in a stronger activation of MAPKs in primed plants after exposure to the challenge of a biotic stressor is linked to the enhancement of defense gene expression characteristics of primed plants [8]. Chromatin remodeling and covalent histone modifications of defense related genes are critical for priming plants to induce a full defense response during IR [9-13], thus histone memory may be used for information storage in plant stress responses [14]. RNA Polymerase V is an enzyme critical in the epigenetic RNA-directed DNA methylation (RdDM) pathway that regulates both DNA methylation and histone modifications [15] and is key in regulating plant immunity [10]. RNA Polymerase V defective mutants carry a constitutive priming phenotype, on which defense genes are poised for enhanced activation via the deposition of histone activation marks (i.e., H3K4me3 and H3K9ac) in their promoters [10], demonstrating the importance of epigenetic control in plant immunity and IR

regulation. The activation of the *SBT3.3* gene, and deposition of the encoded subtilase protein in the plant extracellular matrix, also contributed to immune priming [13]. However, it remains poorly understood how IR is connected to various hormone combinations and distinct signaling pathways that are activated upon pathogen perception.

Pathogen recognition triggers several cellular responses that precede the accumulation of two major defense hormones: salicylic acid (SA) and jasmonic acid (JA). SA is essential for establishing resistance to many virulent biotrophic pathogens, while JA tends to be associated with resistance to necrotrophic pathogens [16]. Interactions occur within and between these hormone signaling networks, resulting in overall antagonism between SA and JA signaling [17-19]. In Arabidopsis, JA levels decline soon after SA begins to accumulate [20]; this therefore suggests that, in response to a pathogen that can induce synthesis of both SA and JA, cross-talk is used by the plant to adjust the response in favor of the more effective pathway (i.e., the SA-mediated pathway). Therefore, tradeoffs between plant defenses against pathogens with different lifestyles must be strictly regulated [21], implying the fine-tuned deployment of conserved defense signals and effectors in different plant-pathogen interactions. However, the mechanism controlling the fine-tuning of hormonal cross-talk during plant-pathogen interactions remains poorly understood.

Various studies have demonstrated that ABA is widely involved in plant responses to biotic stresses caused by a broad range of plant pathogens [22-24]. However, ABA effect varies in different pathosystems, being the outcome influenced by the infection biology. In general, ABA biosynthesis is required for effective disease resistance against necrotrophic pathogens [25-27], whereas resistance to (hemi)biotrophic pathogens is negatively regulated by ABA, with ABA-deficient mutants showing resistance enhancement [24,28,29]. Therefore, endogenous ABA synergizes with JA, and exhibits a complex antagonistic relationship with SA during disease development [17,18]. However, the specific components of the ABA signaling apparatus and the specific mechanisms that exploit the positive and negative responses of ABA during immune responses to

specific plant-pathogen interactions remains unknown. Therefore, understanding the regulatory system of ABA responses during activation of immune responses is critical for improving agricultural issues related to disease resistance.

Three major protein families form the core ABA signaling pathway; (i) the soluble ABA receptors, which are 14 members of pyrabactin resistance 1 (PYR1) and PYR1-like (PYL) proteins, also known as regulatory component of ABA receptors (RCAR) family and collectively referred to as PYR/PYL/RCAR, (ii) group A of type 2C protein phosphatases (PP2Cs), and (iii) SNF1-related protein kinases (SnRKs) subfamily 2 (SnRK2s), namely SnRK2.2, 2.3 and 2.6 [30-33]. In the absence of ABA, PP2Cs dephosphorylate and inactivate SnRK2s, repressing ABA-dependent responses [34,35]. When ABA concentration increases in response to stressing conditions or developmental cues, ABA binds to receptors of the PYR/PYL/RCAR family, which leads to formation of ternary complexes with PP2Cs, thereby inactivating them [36-38]. This results in activation of SnRK2s, which subsequently phosphorylate a myriad of substrate protein [39, 40].

Here, we identify key components of the core ABA signaling pathway involved in plant immune responses.

RESULTS

The SnRK2s protein kinases are engaged in disease resistance to the necrotrophic fungal pathogen *Plectosphaerella cucumerina*

Liquid chromatography-mass spectrometry (LC-MS) showed marked accumulation of ABA in leaves of Arabidopsis plants at 72 h after drop inoculation with a spore suspension of the fungal necrotroph *Plectosphaerella cucumerina* (Fig. 1C). ABA enhancement supported the up-regulation of *ABI4* gene expression, an ABA-responsive gene encoding a transcription factor required for callose deposition [25] (Fig. 1D). Therefore, ABA biosynthesis and signaling was triggered by *P. cucumerina* infection in Col-0 plants. The ABA-mediated activation of three monomeric SnRK2s (i.e., SnRK2.2, -2.3, and -2.6) is central to ABA signaling [41],

so we investigated whether SnRK2s are engaged in Arabidopsis defense responses towards *P. cucumerina.* Transgenic lines overexpressing HA-tagged SnRK2.6 (SnRK2.6-HA/OE) and SnRK2.2 (SnRK2.2-HA/OE) were inoculated with *P. cucumerina* or mock treated, and leaf samples were collected at 0, 24 and 48 h post-inoculation (h.p.i.). Immunoprecipitation of SnRK2.2-HA and SnRK2.6-HA with an anti-HA antibody, and the subsequent kinase assay of the immunoprecipate by determining the incorporation of ^{32}P to purified ABF2 protein, revealed two- and three-fold enhancement for SnRK2.6 and SnRK2.2 kinase activity, respectively, following fungal inoculation (Fig. 1A-B). For both kinases, enhanced activity occurred at 24 h.p.i., and activation was sustained at 48 h.p.i.. Therefore, ABA-activated SnRK2s were actively engaged in response to *P. cucumerina* infection.

We then investigated whether gain-of-function or loss-of-function in SnRK2s altered disease resistance to *P. cucumerina*. Symptoms of fungal disease appear in the form of necrotic lesions which are routinely measured following inoculation and are used to quantify the degree of plant susceptibility [10,27,42]. Inoculation of transgenic plants individually overexpressing (OE) SnRK2.2, -2.3, and -2.6 revealed no significant variation in disease susceptibility towards *P. cucumerina* when compared to Col-0 plants (Fig. 1E); thus, endogenous SnRK2s levels are sufficient to achieve pathogen-triggered ABA signaling. Although functional redundancy between SnRK2.2 and SnRK2.3 exists, functional segregation between SnRK2.6 and SnRK2.2/2.3 has been described [43]. Therefore, for loss-of-function studies we inoculated a *snrk2.2/2.3* double mutant and the single *snrk2.6* mutant with *P. cucumerina* and recorded disease resistance. The triple *snrk2.2/2.3/2.6* mutant, which is drastically affected in plant growth [41], was not compatible with the pathogenic assay and was therefore not used in the present study. Figure 1F-G shows that *snrk2.2/2.3* and *snrk2.6* plant resistance to *P. cucumerina* is severely compromised, indicating that these SnRK2s are required for resistance. An ABA deficient mutant (i.e., *aba2*) was similarly affected in disease resistance to *P. cucumerina* (Fig. 2A), supporting previous studies showing that ABA promotes enhanced resistance to this fungal pathogen [25,27,44]. Thus, the

pathogen-induced ABA increase and concurrent activation of SnRK2s positively regulates disease resistance to *P. cucumerina*.

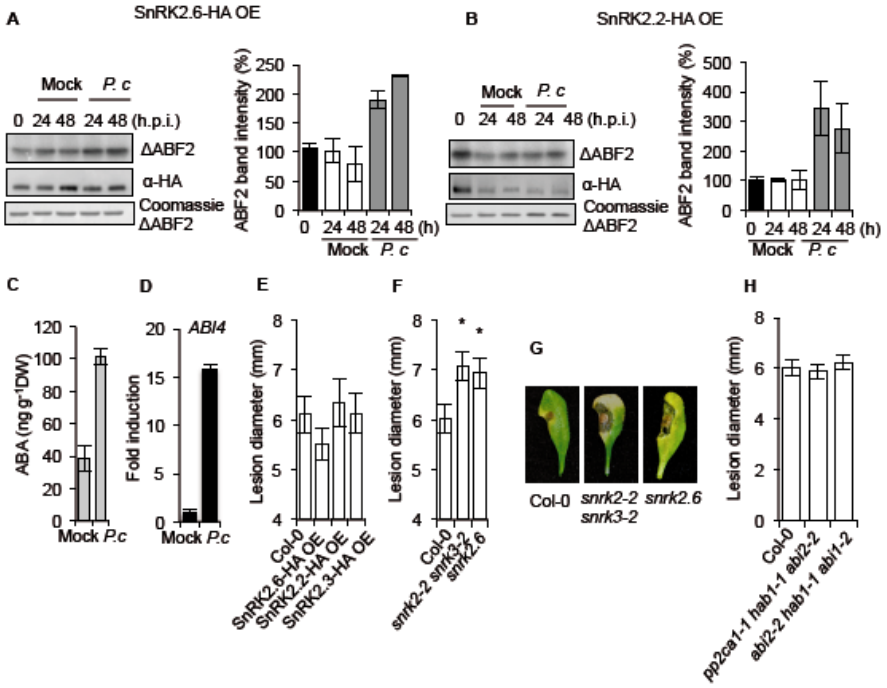


Figure 1. Participation of SnRK2s kinases in the response of Arabidopsis plants to infection by the fungal pathogen *P. cucumerina*. (**A-B**) *P. cucumerina*-mediated activation SnRK2.6 (A) and SnRK2.2 (B). Transgenic Arabidopsis plants expressing HA-tagged versions of the kinases were inoculated with *P. cucumerina*, or were mocked, and leaf samples taken at 0, 24 and 48 h.p.i., and protein extracts immunoprecipitated with anti-HA antibodies. The immunoprecipitates were incubated with a His-ABF2 fragment (Gly73 to Gln 119; ΔABF2) in the presence of [γ-^{32}P]ATP and proteins resolved by SDS-PAGE. Bands corresponding to ΔABF2 fragments and to SnRK2.6 and SnRK2.2 kinases are indicated. Radioactivities of ΔABF2 fragment bands were measured with a phosphoimager and values plotted on the graphs shown at the ritgh of the figures. Error bars indicate S.E.M.; n=3. (**C**) ABA accumulation determined in mock and *P. cucumerina* infected Col-0 plants. (**D**) RT-qPCR of *ABI4* in mock and in *P. cucumerina*-infected Col-0. (**E**) Disease resistance towards *P. cucumerina* of transgenic plants over-expressing SnRK2.6, SnRK2.2 and SnRK2.3 in comparison to Col-0. (F) Disease resistance towards *P. cucumerina* in the double *snrk2.2 snrk2.3* mutant and in *snrk2.6* mutant plants. (**G**) Representative leaves from each genotype at 12 days following inoculation with *P. cucumerina*. (**H**) Disease resistance towards *P. cucumerina* in the triple PP2C mutants *pp2ca1 1hab1 1abi2-2* and *abi2-2 hab1 abi1-2*. For the bioassays with *P. cucumerina* lesion diameter of 20 plants per genotype and four leaves per plant were determined 12 d following inoculation with *P. cucumerina*. Data points represent average lesion size ± SE of measurements. An ANOVA was conducted to assess significant differences in disease symptoms, with a priori $P < 0.05$ level of significance; the asteriks above the bars indicate statistically significant differences.

Redundancy of group A PP2Cs in mediating resistance to *P. cucumerina*

ABA signaling mediated through SnRK2s is negatively regulated by clade A protein phosphatase type 2C (PP2C), particularly by ABI1, ABI2, PP2CA/AHG3, AHG1, HAB1 and HAB2 (see [45], and references therein) Therefore, clade A PP2Cs might negatively regulate ABA and SnRK2s-mediated disease resistance to *P. cucumerina*. Because of the demonstrated redundancy existing for these PP2Cs, combined inactivation of selected groups of these phosphatases is required to determine functionality. We combined loss-of-function mutations in ABI1, ABI2, HAB1, and PP2CA genes to determine their contribution to ABA-mediated disease resistance. Different combinations of mutations were used with two triple mutants, *pp2ca1-1;hab1-1;abi2-2* and *abi2-2;hab1-1;abi1-2*, which represent four of the 9 closely related group A PP2Cs. Both multilocus mutants showed an extreme response to exogenous ABA, partial constitutive response to endogenous ABA, and partial constitutive activation of SnRK2s [38,39,45]. Inoculation of both triple mutants with *P. cucumerina* showed no defective disease resistance (Fig. 1H). This result suggests that the demonstrated redundancy of PP2Cs masks the manifestation of a clear phenotype upon pathogen inoculation. Additionally, ABA response in triple *pp2c* mutants is partially equivalent to that of lines OE SnRK2s, which did not show altered disease resistance to the pathogen (Fig. 1E). It is also possible that other members of the large PP2C family, represented by 76 homologous genes [46], are key for resistance to *P. cucumerina*. This interpretation is supported by a recent study showing that a distinct PP2C member (i.e., AtDBP1 [47]) is required for other aspects of plant immunity [48].

Requirement of the PYR1 receptor for disease resistance to *P. cucumerina*

We next investigated which one of 14 soluble PYR/PYL/RCAR receptors perceives the ABA produced during *P. cucumerina* infection. Partial functional redundancy of ABA receptors has been demonstrated by genetic analysis; however, PYL8 plays a non-redundant role to regulate root sensitivity to ABA [49,50]. We characterized

disease resistance to *P. cucumerina* in a series of multilocus mutants from different PYR/PYL receptors. The triple *pyl4;pyl5;pyl8*, *pyr1;pyl4;pyl8,* and *pyr1;pyl4;pyl5* mutants, and the quadruple *pyr1;pyl1;pyl2;pyl4* mutant, representing the highest genetic impairment in PYR/PYL function without affecting plant growth [38], were inoculated with *P. cucumerina* and their impact on disease resistance was compared to *aba2-1* (which enhances susceptibility [27]), to *ocp3-1* (which enhances resistance [42]) and Col-0 plants. The two triple mutants incorporating the *pyr1* mutation (i.e., *pyr1;pyl4;pyl8* and *pyr1;pyl4;pyl5*) exhibited noticeably enhanced disease susceptibility to *P. cucumerina* (Figure 2A), which was of a magnitude similar to that observed in *aba2-1* plants. Conversely, the disease resistance of the triple *pyl4;pyl5;pyl8* mutant was unaltered compared to Col-0 plants. The quadruple mutant (also containing the *pyr1* mutation) enhanced disease susceptibility to *P. cucumerina*. The quadruple mutant included a mutation in the PYL2 receptor (i.e., *pyl2* mutation), which is in a Landsberg background and highly linked to the *Erecta* locus, and since the latter locus is described to be involved in perception of fungal pathogens [51], we did not characterize this mutant further.

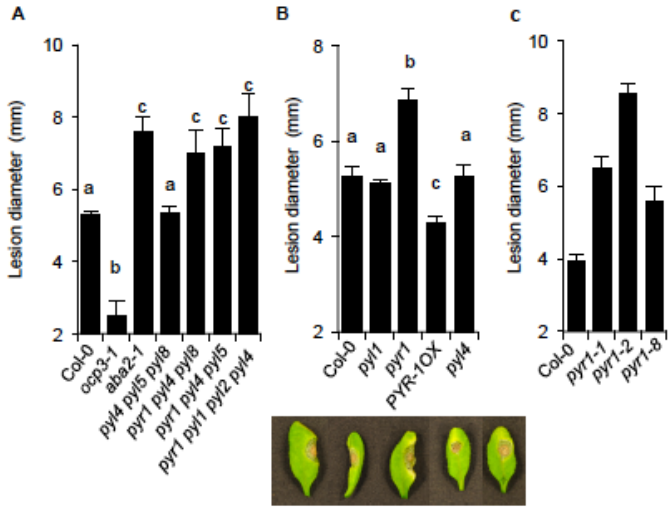


Figure 2. PYR1 is required for disease resistance towards *P. cucumerina*. (**A**) Disease resistance towards *P.cucumerina* in Col-0, the resistant *ocp3-1 mutant*, the susceptible *aba2-1* and the triple and cuadruple multilocus mutants *pyl4 pyl5 pyl8*, *pyr1 pyl4 pyl8, pyr1 pyl4 pyl5*, and *pyr1 pyl1 pyl2 pyl4.* (**B**) Disease resistance in single *pyl1*, *pyr1* and *pyl4* mutants, in a transgenic line

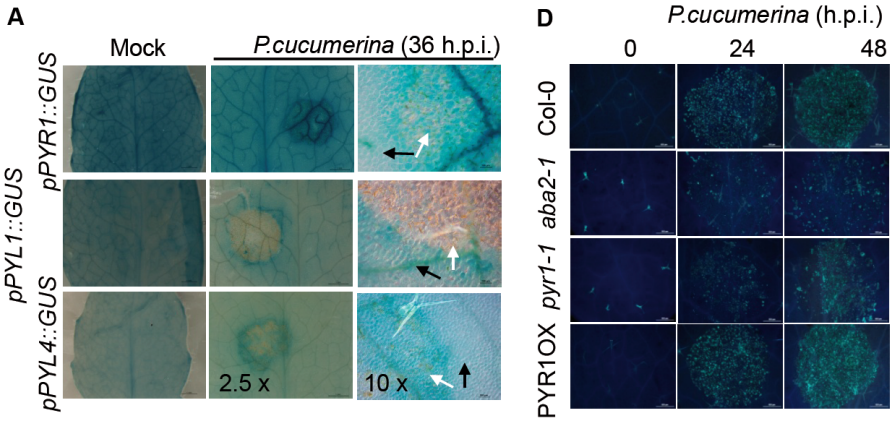
overexpressing PYR1 (PYR1-OE) and in Col-0. Below the graph representative leaves from each genotype are shown at 12 days following inoculation with *P. cucumerina*. (**C**) Comparative disease resistance towards *P. cucumerina* among the allelic *pyr1-1*, *pyr1-2* and *pyr1-8* mutants. Data points represent average lesion size ± SE of measurements. An ANOVA was conducted to assess significant differences in disease symptoms ($P < 0.05$); the letters above the bars indicate different homogeneous groups with statistically significant differences.

The results showed that the PYR1 receptor is pivotal for eliciting ABA-mediated defense responses towards *P. cucumerina*. However, it might occur that the presence of additional *pyl* loci was required to reveal the *pyr1* phenotype in pathogen response. Therefore, the specificity of PYR1 at eliciting plant immune responses was further tested by assaying the single *pyr1-1* mutant. The individual *pyr1-1* mutant had has a compromised disease resistance phenotype (Fig. 2B), contrasting to other single *pyl* mutants (e.g., *pyl1*, *pyl4*) for which resistance to the fungus remained intact. Moreover, the overexpression of the PYR1 receptor in transgenic Arabidopsis (PYR1-OE line) conferred significant enhancement of disease resistance to the fungus (Fig 2B). Different mutant alleles, predicted to produce a variety of defects in PYR1 [38], consistently compromised disease resistance to *P. cucumerina*, with different disability (Fig. 2C). The *pyr1-2* mutant allele (Ser152Leu) had the strongest phenotype, showing its importance for normal PYR1 function. The *pyr1-1* (Gln169STOP) and *pyr1-8* (Gly107Glu) mutant alleles had similar conspicuous phenotypes when compared to Col-0, but were discernible when compare to *pyr1-2* (Fig. 2C). These results support PYR1 as the receptor that positively promotes ABA-dependent plant immunity against *P. cucumerina*.

Local induction of *PYR1* gene expression by *P. cucumerina*.

We next investigated whether transcriptional reprograming occurs to enhance *PYR1* expression upon pathogen inoculation. Transgenic plants expressing the promoter of *PYR1* gene fused to the β-glucuronidase GUS reporter gene (*pPYR1::GUS*) [49] were used to detect potential *P. cucumerina*-mediated activation of *PYR1*. Transgenic lines carrying the *pPYL1::GUS* and *pPYL4::GUS* gene construct were also assayed to determine specificity. Local infection, i.e., by drop inoculation on the upper leaf surface with a *P. cucumerina* spore suspension, of transgenic

pPYR1::*GUS* plants revealed early transcriptional activation of *PYR* triggered by the pathogen (Fig. 3A). *PYR1* induction mostly occurred within the vascular bundles of the primary and secondary veins of the *P. cucumerina*-inoculated leaf sectors. This highly localized induced expression pattern was specific to *PYR1*, because neither *PYL1* nor *PYL4* genes were transcriptionally activated under similar circumstances (Fig. 3A). Local induction of *pPYR1::GUS* by *P. cucumerina* inoculation concurred with local synthesis and deposition of callose, easily detected at 36 h.p.i., as revealed by staining with aniline blue (Fig. 3B), and at later stages was followed by cell deterioration and death, which started to be seen at 36 h.p.i. as revealed by trypan blue staining (Fig. 3C). These microscopy markers demarcated inoculated tissue sectors in advance to the appearance of visible necrosis and served to delimit local transcriptional responses. Moreover, callose depostion was compromised in *pyr1-1* and *aba2-1* mutants following fungal infection (Fig. 3D-E), thus supporting the participation of ABA and PYR1 in this local process.



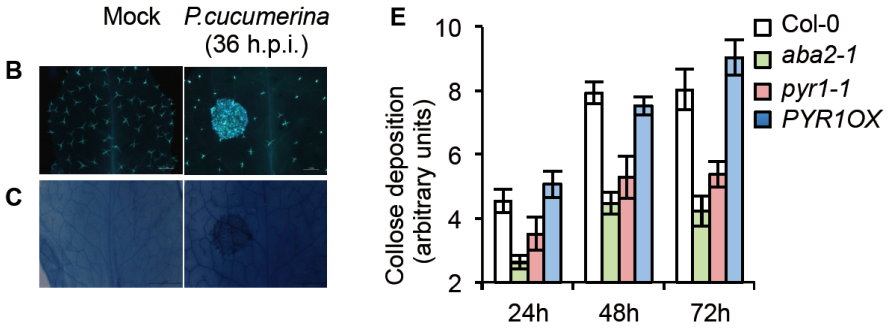


Figure 3. Local activation of *PYR1* gene expression at pathogen inoculation sites, and requirement of PYR1 for pathogen-induced callose deposition. (**A**) Comparative histochemical analysis of GUS activity in rosette leaves from transgenic plants carrying *pPYR1::GUS*, *pPYL1::GUS*, and *pPYL4::GUS* gene construct and that were either mocked or inoculated *P. cucumerina*. Leaves were stained for GUS activity at 36 h.p.i. The left panel corresponds to mocked plants. The central and right panels correspond to enlargements of the inoculated leaf sectors. Black arrow points towards leaf tissues proximal to the inoculation point, and white arrows denote tissues that directly received the spore inoculum. Note that *pPYR1::GUS* is heavily induced in leaf veins within the inoculated sector. (**B**) Characteristic spore-inoculated leaf sector, similar to those shown in A, stained with aniline blue to detect pathogen-induced callose deposition (top panel), or with trypan blue (lower panel) to identifiy incipient cell deterioration due to fungal infection at 36 h.p.i. (**C**) Aniline blue staining and epifluorescence microscopy was applied to visualize callose accumulation. Micrographs indicate *P. cucumerina* inoculation and infection site in the different Arabidopsis genotypes at 0 h.p.i (right panel), at 24 h.p.i. (central panel), and at 48 h.p.i. (right panel). (**D**) The number of yellows pixels (corresponding to pathogen-induced callose) per million on digital photographs of infected leaves were used as a means to express arbitrary units (i.e. to quantify the image) at the indicated times. Bars represent mean ± SD, n = 15 independent replicates.

Pathogen specificity of PYR1 signaling

Plant defense mechanisms against necrotrophic pathogens are complex, exhibiting diverse effectiveness against pathogens with different lifestyle. We investigated whether PYR1 is also required for immune activation to other pathogens. Figure 4A shows that effective resistance to *Alternaria brassicicola*, a necrotrophic fungal pathogen and causal agent of black spot disease in Brassica species, was similarly compromised in *aba2* and *pyr1* plants compared to Col-0, *pyl1* and *pyl4* plants. The enhancement of necrosis in *A. brassicicola*-inoculated leaves of *pyr1* plants compared to Col-0 plants (Supplemental Fig. S1), demonstrates the high susceptibility of the *pyr1* mutant to *A. brassicicola*. This result supports the importance of PYR1-mediated perception of ABA for mounting effective defense responses towards necrotrophs.

In contrast, resistance to *Botrytis cinerea*, another necrotrophic fungal pathogen and the causative agent of grey mold disease, was enhanced in *pyr1* and *aba2* plants, and, to a lesser extent, *pyl4* plants. These different responses support previous studies showing that ABA functions as a negative regulator of the defense program towards *B. cinerea* through poorly understood mechanisms that may involve H_2O_2-dependent defense in the epidermis and cuticle [52-54]. Thus, these different phenotypes reflect a certain degree of pathogen-specificity in the requirement of ABA and PYR1 to positively regulate defense activation against necrotrophic pathogens. This phenomenon adds further complexity to the documented role of ABA in repressing plant immunity against (hemi)biotrophic pathogens [17,22-24]. We next asked whether the negative role of ABA in plant immunity against (hemi)biotrophic pathogens could similarly be funneled through PYR1. If so, we would expect resistance enhancement in *pyr1* plants. *pyr*1 plants were inoculated by leaf infiltration with the bacterial pathogen *Pseudomonas syringae* DC3000, and the rate of bacterial growth in the inoculated leaves was determined at 3 days post inoculation in comparison to *aba2*, *pyl1*, *pyl4,* and Col-0 plants. Figure 4C shows that bacterial growth was reduced 10-fold in both *aba2* and *pyr1* mutants compared to Col-0, *pyl1* and *pyl4*. This result confirms the negative role of ABA in resistance towards *P.syringae* DC3000, and demonstrates the specific requirement of PYR1 for the negative role of ABA during this plant-pathogen interaction. Moreover, pre-treatment of Col-0 with 150 µM ABA, applied by drenching, predictably provoked disease susceptibility enhancement to *P.syringae* DC3000 (Fig. 4D), denoting a damping effect of ABA on SA signaling. This ABA-mediated enhancement in susceptibility to *P.syringae* DC3000 did not occurred in *pyr1-2* plants whose enhanced resistance was not altered by the hormone (Fig. 4D).

Therefore, our results indicate the dual antagonistic role of ABA in plant immunity is mediated through the PYR1 receptor, which reciprocally activates and represses immune responses towards necrotrophic and biotrophic pathogens, respectively.

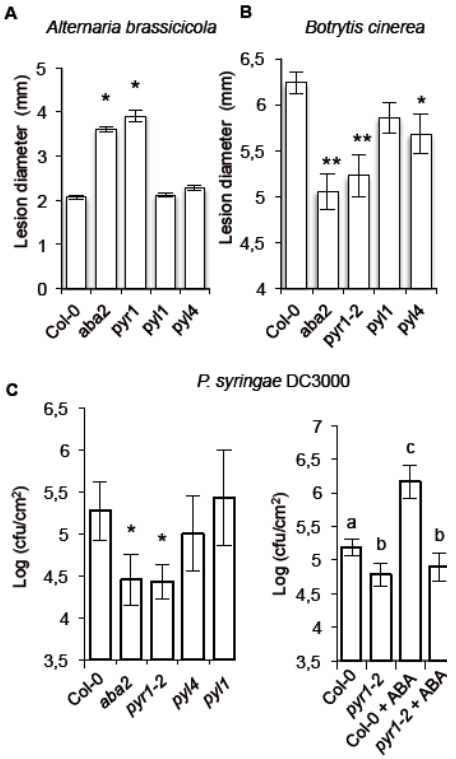
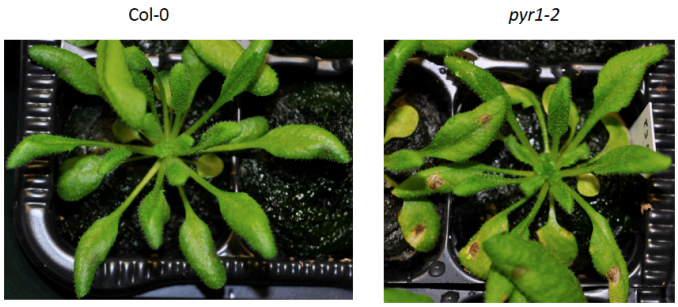


Figure 4. Dissimilar responses of *pyr1* plants to infection by *Alternaria brassicicola*, *Botrytis cinerea* and *Pseudomonas syringae DC3000*. Col-0, *aba2-1*, *pyr1-2*, *pyl1* and *pyl4* mutants were inoculated with the indicated pathogens and their disease responses recorded. (**A**) Resistance response to virulent *A. brassicicola*. Lesion size was measured 8 days after inoculation (d.p.i). (**B**) Resistance response to virulent *B. cinerea*. Lesion size was measured 3 days after inoculation (d.p.i). Data points represent average lesion size ± SE of measurements. For both fungal pathogens lesion diameter of 25 plants per genotype and four leaves per plant were used. (**C-D**) Growth of *P. syringae* DC3000 was measured at 3 d.p.i. Error bars represent standard deviation (n=12). An ANOVA was conducted to assess significant differences in disease symptoms, with a priori $P < 0.05$ level of significance; the asteriks above the bars indicate different homogeneous groups with statistically significant differences.



Supplementary Figure S1. Comparison of Col-0 and *pyr1-2* plants upon inoculation with *Altenaria brassicicola*. Note the enhanced susceptibility of *pyr1-2* plants to the fungi, with appearance of necrotic lesions on the inoculated leaves at the time (8 d.p.i) when Col-0 plants do not manifest severe symptoms of fungal infection, hence denoting the enhanced susceptibility of *pyr1* plants to *A. brassicicola*.

SA–responsive defense genes are activated in PYR1 defective mutants

The SA and JA signal pathways cross communicate and are under an antagonistic equilibrium, providing plants with the capacity to finely tune the induced defense

responses, leading to the inhibition of the JA pathway when the SA pathway is facilitated [19-21]. The interaction between the SA and JA pathways serves to optimize responses to a specific type of pathogenic insult. Thus, we investigated whether *pyr1* and *aba2* plants carry constitutive elevated expression of SA-responsive genes, which may explain the observed enhanced resistance to *P.syringae* DC3000 (Fig. 4C). The accumulation of *PR-1* and *PR-2* transcript, which are classical SA- and pathogen-responsive genes, was examined by RT-qPCR in *pyr1* and *aba2* plants compared to Col-0. In addition, we examined *PR-4* and *PR-5,* which are also pathogen-responsive genes, but are simultaneously influenced by SA and JA [55]. The level of transcript accumulation for these four genes was also evaluated in *pyl1* and *pyl4* mutants, which served as additional controls. Figure 5A shows that *pyr1* and *aba2* plants carry constitutive elevated levels of *PR-1* and *PR-2* transcripts compared to Col-0. There was no constitutive expression of *PR-1* and *PR-2* in *pyl1*, while *PR-2* was minimally expressed in *pyl4* plants. Conversely, the constitutive levels of transcript accumulation for *PR-4* and *PR-5* occurring in Col-0 were repressed in *pyr1* and *aba2* plants, and only partially enhanced in *pyl1* plants (Fig. 5A). Enhanced expression of *PR-1* and *PR-2* and the concerted repression of *PR-4* and *PR-5* was corroborated by the *pyr1* allelic series, with the *pyr1-2* allele strongly activating *PR-1* and *-2*, while concurrently repressing *PR-4* and *-5* (Fig. 5B). Thus, the ABA/PYR1 module may function as an integration node regulating distinct branches of defenses. The constitutive activation of *PR1-* and *-2* and concomitant repression of *PR-4* and *-5* in *pyr1* plants supported the enhanced accumulation of both free and conjugated SA, which was accompanied by the concomitant reduction of JA content in the mutant compared to Col-0 (Fig. 5C-D). Concurrent SA enhancement and JA reduction in healthy *pyr1* plants may partly explain the observed opposed disease resistance phenotypes of the mutant when confronted to biotrophic and necrotrophic pathogens. Interestingly, *pyr1* plants also showed enhance accumulation of camalexin (Fig. 5E), which is a metabolite that is essential for the resistance of Arabidopsis to *B. cinerea* [56]. This phenomenon may explain why *pyr1* plants showed enhanced resistance to this particular necrotrophic pathogen, even in the presence of reduced levels of JA.

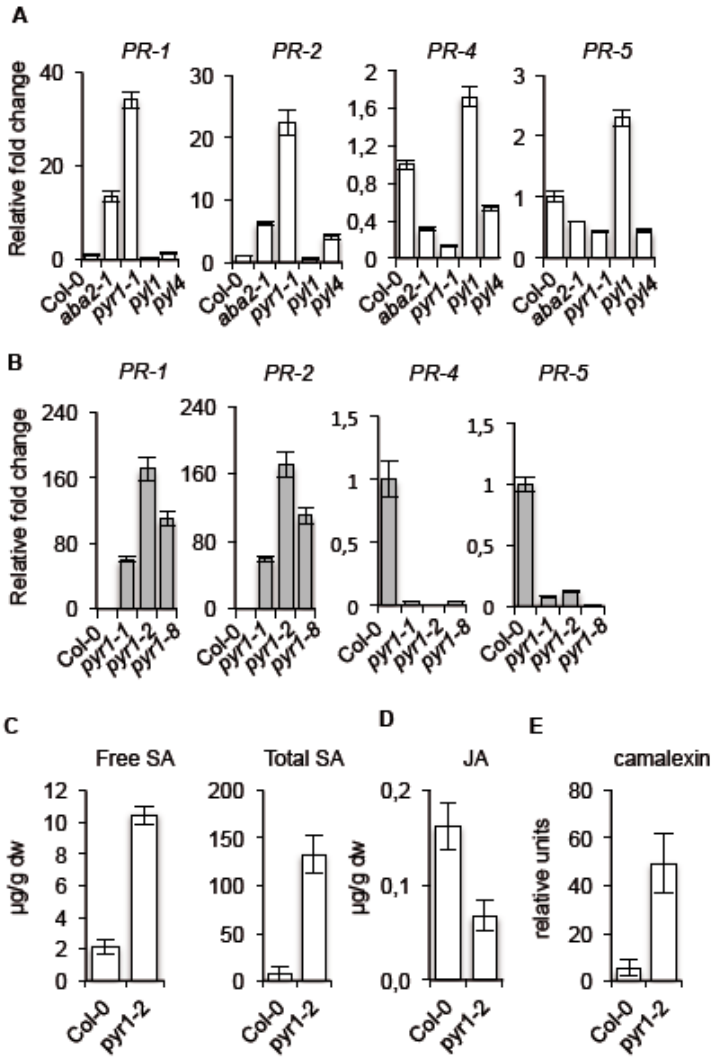


Figure 5. Expression of SA-responsive genes in *pyr1* and *aba2* mutants. (**A-B**) RT-qPCR analysis showing constitutive expression levels of *PR-1*, *PR-2*, *PR-4* and *PR-5* genes in (**A**) Col-0, *aba2-1*, *pyr1-1*, *pyl1* and *pyl4* plants, and (**B**) their comparative expression levels in the allelic *pyr1-1*, *pyr1-2* and *pyr1-8* mutants. Data represent mean ± SD; n = 3 replicates. Expression was normalized to the constitutive *ACT2/8* gene, then to expression in Col-0 plants. (**C**-**E**) Accumulation of free SA, total SA, total JA and camalexin in Col-0 and *pyr1-2* plants. Data represent the average of three biological replicates.

Enhanced activation of MPK kinases in *pyr1* plants

We investigated whether the enhanced resistance to *P. syringae* DC3000 observed in *pyr1* plants is associated to elevated mitogen-activated protein kinases (MAPKs) activation, which is linked to the activation of immune responses following pathogen perception. We employed an antibody recognizing the phosphorylated residues within the MAPK activation loop (i.e., the pTEpY motif). Western blot analysis of protein extracts derived from healthy Col-0 and *pyr1* plants showed positive immunoreactive signals in two polypeptides corresponding to MPK6 and MPK3 [8] (Fig. 6). Following densitometric scanning of Western blots, the MPK3 immunoreactive band was more intense in the *pyr1* line compared to Col-0 control line of non-inoculated plants. Inoculation with *P.syringae* DC3000 promoted further activation-associated dual TEY phosphorylation of MPKs, which was noticeably higher for MPK3 in *pyr1* compared to Col-0 plants at 24 h.p.i. (Fig. 6). At the latter stages of infection (i.e., 48 h.p.i.), MPK activation was similar in Col-0 and *pyr1* plants. This increase in MPK phosphorylation is independent of the increase in MPK6 acumulation, so we observed the same level of accumulation in all samples. Therefore, MPK activation may be prone to activation in plants defective of ABA perception through the PYR1 receptor. Indeed, partial pre-activation of MPK was reflected in detectable PR-1 protein accumulating in *pyr1* plants at time zero (Fig. 6). This result is in agreement with the higher expression level of the *PR-1* gene determined by RT-qPCR (Fig. 5A-B). Interestingly, inoculation with *P.syringae* DC3000 promoted the further accumulation of the PR-1 protein, which progressively increased over time to a much higher level in the *pyr1* mutant compared to Col-0 plants (Fig. 6).

Thus, we hypothesize that the lack of ABA perception through the PYR1 receptor de-represses a pathway that triggers a MPKs activation and downstream defense gene reprogramming, even in the absence of a pathogenic insult. Activated cell signalling may be ready for the enhanced induction of this defense pathway following pathogen infection, which, in turn, may explain why *aba* and *pyr1* plants exhibit enhanced disease resistance to *P.syringae* DC3000. These observations support that ABA and PYR1 function as a repressor module of SA-mediated onset

and maintenance of induce resistance to biotrophic pathogens.

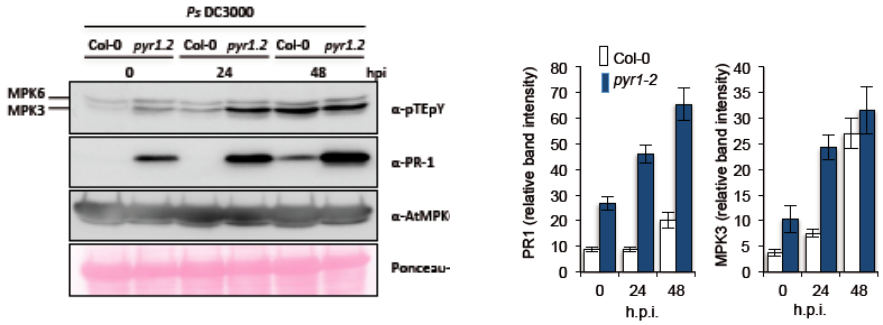


Figure 6. Loss of PYR1 function confers enhanced mitogen-activated kinase activation and PR-1 protein accumulation following *P. syringae* DC3000 infection. (**A**) Western blot with anti-pTEpY, anti-PR-1 and and anti-MPK6 antibodies of crude protein extracts derived from Col-0, *pyr1-2* plants at 0, 24, and 48 h.p.i with *P. syringae* DC3000. Equal protein loading was check by Ponceau-S staining of the nitrocellulose filter. MPK6 and MPK3 migrating bands are indicated on the right. The experiments were repeated three times with similar results. (**B**) Scan quantification of protein bands corresponding to MPK3 and PR-1. Data represent the mean ± SD; n = 3 replicates.

SA-mediated defense genes are activated through chromatin remodeling in *pyr1* plants

Our results indicate that ABA/PYR1 has a negative role in the defense mechanisms implicated in setting induced resistance. Thus, we investigated whether other markers diagnostic of plant defense common to primed immune status, apart from the observed pre-stress activation of MPKs (Fig. 6), are similarly activated in *pyr1* plants. The expression of the extracellular subtilase *SBT3.3* gene has been recently described to be a switch for poising SA-related genes and the activation of immune priming [13]. Moreover, constitutive *SBT3.3* expression, MPK activation and readied SA-related genes convey in plants defective in the RNA-directed DNA methylation (RdDM) pathway, such as mutants defective in RNA Pol V (i.e. *nrpd2*), which control immune priming in Arabidopsis [10]. Consequently, the expression level of genes encoding SBT3.3 and either of the two subunits of RNA Pol V (i.e., NRPD2 and NRPE1) were determined by RT-qPCR to determine the plant immune status in *pyr1* mutants. Figure 7A shows the constitutive up-regulation of *SBT3.3*

and concurrent down-regulation of *NRPD2* in *pyr1* plants, congruent with activation of basal immunity in this mutant. Down-regulation was specific for *NRPD2*, encoding the second large subunit of Pol V, because expression of the gene encoding the large NRPE1 subunit appears to exhibit minimal or no variation in *pyr1* plants (Fig. 7A).

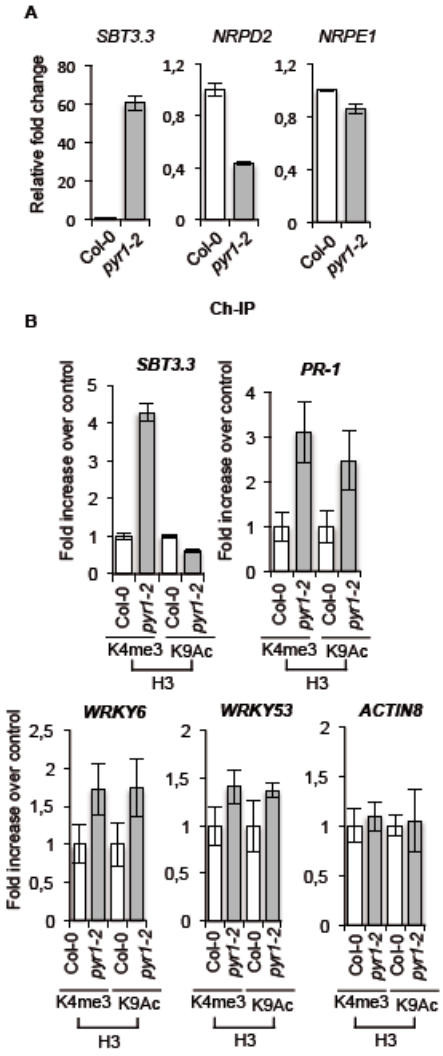


Figure 7. Loss of PYR1 function provokes setting of hallmarks characteristic of immune priming activation. (**A**) Comparative RT-qPCR of *SBT3.3*, *NRPD2* and *NRPE1* transcript levels between healthy Col-0 and *pyr1-2* plants. Expression was normalized to the constitutive *ACT2/8* gene, then to expression in Col-0 plants. (**B**) Chromatin immunoprecipitation (ChIP) and comparison between Col-0 and *pyr1-2* plants of level of histone H3 Lys4 trimethylation (H3K4me3) and histone H3 Lys9 acetylation (H3K9ac) on the *SBT3.3*, *PR-1*, *WRKY6* and *WRKY53* gene promoters as present in leaf samples. The setting of histone marks in *ACTIN2* was used as an internal control. Data are standardized for Col-0 histone modification levels. Data represent the mean ± SD; n = 3 biological replicates.

Under both circumstances, e.g. in plants defective in RdDM-mediated

epigenetic control and in plants constitutively expressing the SBT3.3 subtilase, immune priming is activated with chromatin histone activation marks being enriched in SA-related gene promoters and in the promoter region of the *SBT3.3* gene [10,13]. Therefore, the histone mark setting in these genes supports immune priming [9,10,13]. Thus, we hypothesized that SA-related defense genes and *SBT3.3* in *pyr1-2* plants are poised for enhanced expression by differential histone modification. We used chromatin immunoprecipitation (ChIP) to analyze H3 Lys4 trimethylation (H3K4me3) and H3 Lys9 acetylation (H3K9ac) on the *SBT3.3* and *PR-1* gene promoter regions in *pyr1* and Col-0 plants. We also examined the genes encoding WRKY6 and WRKY53, transcriptional regulators of SA-defense genes. Fig. 7B shows that H3K4me3 marks in the *SBT3.3* promoter region notably increased in *pyr1*plants compared to Col-0 plants. However, H3K9ac marks in the *SBT3.3* gene promoter did not increase in the mutant compared to Col-0 (Fig. 7B), supporting previous descriptions of plants constitutively expressing primed immunity [9,10,13]. On the *PR1* promoter, both H3K4me3 and H3K9ac activation marks increased three- and two-fold, respectively, in *pyr1* plants compared to Col-0 (Fig. 7B). The histone activation marks on *PR-1* also moderately increased in the *WRKY6* and *WRKY53* promoters of *pyr1* plants compared to Col-0 plants (Fig. 7B), supporting an activation of plant defense responses similar to immune priming activation. The setting of histone marks in *pyr1* plants remained unchanged in the *ACTIN2* gene promoter, which was used as the control (Fig. 7B). Therefore, chromatin activation marks proliferate in the promoter regions of the priming regulatory gene *SBT3.3* and the SA-responsive genes in *pyr1* plants. This phenomenon might increase DNA accessibility or increase the provision of docking sites for gene activators. These responses would explain why the PR-1 protein shows constitutive and enhanced accumulation in *pyr1* plants following pathogen inoculation (Fig. 6). Our results indicate that ABA and its PYR1-mediated perception represent novel integral components of a signalling process that represses induced resistance through the immune priming setting.

DISCUSSION

Despite the demonstrated role of ABA on the final outcome of immune responses, the specific components of the ABA signaling apparatus and the specific mechanisms that exploit ABA to positively and negatively influence immune responses to specific plant-pathogen interactions has remained largely unknown. Here, we showed that SnRK2s kinases are actively engaged in activating resistance towards *P. cucumerina,* whereas the loss-of-function of any of the three individual SnRK2s compromised this resistance. Furthermore, we demonstrated that PYR1 is pivotal in mediating resistance to *P. cucumerina* and plays a positive role in disease resistance to *P. cucumerina* since overexpression of PYR1 (i.e. *PYR1-OE* transgenic line) conferred significantly enhanced resistance to the fungus. This conclusion was reinforced by the observation that resistance to *P. cucumerina* was severely compromised in three single mutant alleles of PYR1 (e.i. *pyr1-1*, *pyr1-2* and *pyr1-8*). These results indicate that the PYR1 receptor has functional specificity in perceiving ABA produced in response to *P. cucumerina* infection to activate SnRK2s and hence plant immunity. This study provides novel information about a specific ABA receptor mediating specific plant immune responses and the first to describe ABA-activated SnRK2s as cardinal components for plant resistance. This information helps construct a functional classification scheme of the different members of the PYR/PYL receptor family with respect to their downstream signaling pathways in a true biological context. An explanation for the specific role of PYR1 in pathogen response could be the selective and highly localized pathogen-induced expression of *PYR1* in vascular bundles (Fig. 3A). This expression pattern mirrors the expression of genes encoding ABA-biosynthetic enzymes [57-59]. Therefore, the synthesis of ABA and the pathogen-induced expression of *PYR1* spatially concur in the vasculature, supporting the hypothesis that vascular tissues function as an integrating node, triggering stress signaling that set in motion local and systemic immune response in the plant [60-62].

This study showed that resistance to *A. brassicicola* is also dependent on ABA and PYR1, reinforcing the importance of this signal pathway for activating immunity

against necrotrophs. In contrast, resistance to *B. cinerea* was enhanced in *aba2* and *pyr1* mutants. This result support previously documented ABA repression of the defense program in the presence of *B. cinerea* [52-54]. Unlike *P. cucumerina and A. brassicicola*, resistance to *B. cinerea* is independent of JA and SA signaling, requiring the phytoalexin camalexin [56]. More camalexin accumulates in *pyr1* plants, explaining the specific and contrasting phenotype of *pyr1* and ABA deficient mutants towards *B. cinerea*.

Contradictory role of ABA at controlling of disease resistance has also been observed for biotrophic pathogens, with plant resistance appearing to be negatively regulated by ABA, whereas resistance by ABA-deficient mutants is enhanced [24,28,29]. In addition, growth of the virulent bacteria *P.syringae* DC3000 is severely restricted in *pyr1* plants, as documented for *aba2* plants. These results demonstrate the Janus functions of PYR1 in disease resistance: repression of immunity against biotrophs and activation of immunity against necrotrophs. Consequently, PYR1 may regulate which of these two plant immune programs prevails. This hypothesis support previous observations of ABA as a hormone that interacts antagonistically or synergistically with the SA-JA backbone of the plant immune signaling network, redirecting defense outputs [63-66]. Yet, how does the ABA/PYR1 module interfere with immunity to drive simultaneously the repression and activation of the SA and JA defense pathways, respectively? Hormone cross talk allows different hormone signaling pathways to act antagonistically or synergistically, providing powerful regulatory potential to flexibly tailor the plant´s adaptive response to a range of environmental cues. Thus, cross talk between the SA and JA signaling pathways is an important regulatory mechanism of plant immunity [14,17,18]. Endogenous SA accumulation antagonizes JA-dependent defenses, prioritizing SA-dependent resistance over JA-dependent defense [67]. Our results showed the basal activation of the SA-dependent pathway in *pyr1* mutants, and that *pyr1* is insensitive to the damping effect of ABA on SA signaling. This finding supports previous work demonstrating the negative role of ABA on disease resistance to biotrophs, and that *P.syringae*-induced ABA levels in Col-0 suppress SA biosynthesis and action, enhancing susceptibility to this pathogen [24,64,68].

Therefore, the basal enhancement of SA in *pyr1* plants may antagonize JA-dependent resistance (i.e., towards *P. cucumerina* and *A. brassicicola*) facilitating fungal growth in the mutant. Thus, there is underlying complexity in the control of disease resistance by ABA. The positive effect of ABA at promoting JA-dependent defenses, and therefore resistance to necrotrophs, may be indirect: perception of pathogenic ABA by PYR1 dampens SA signaling, which stops JA pathway repression. This ABA and PYR1 modulated cross-talk regulation of SA/JA, may provide the plant with a powerful regulatory potential to boost its defenses according to the lifestyle of the attacker. This phenomenon may also explain why disease promoting biotrophic pathogens (e.g. *P.syringae* DC3000) have developed strategies to alter the host ABA physiology as part of the infection strategy [68].

How does ABA/PYR1-mediated signaling control SA-mediated defenses? Our results demonstrate that *pyr1* plants are ready for the enhanced activation of MPKs and the enhanced expression of SA defense-related genes. Thus, *pyr1* plants present a constitutive activation of SA dependent defenses, related with the mechanism described for immune priming activation. The signal components mediating this sensitized cell state in *pyr1* plants have been identified and analyzed in this study. Specifically: (1) basal activation of MPKs; (2) enhanced accumulation of the defense-related PR-1 protein; (3) repression of *NRPD2* gene expression, encoding the second largest subunit of RNA Pol V, inhibiting epigenetic RdDM mechanisms that negatively control the onset of defense priming; (4) activation of the SA-independent, but ROS-dependent, SBT3.3 subtilase; and (5) readying of SA-related genes and *SBT3.3* for enhanced expression by pertinent chromatin modifications though the setting of histone activation marks in their gene promoter. *pyr1* plants reinforce the molecular and disease resistance phenotypes of RdDM defective mutants, which exhibit simultaneous enhanced susceptibility and resistance to necrotrophs and biotrophs, respectively [10], supporting immune responses activation in *pyr1* plants. The fact that immune priming, and hence IR, is negatively regulated by the RdDM, and that in *pyr1* plants repression of plant defence are relieved, both observations unveil the importance of ABA/PYR1 as new element participating in an epigenetic mechanism of control of gene expression, at least for

characters related to plant immunity. Our results provide new insights about plant immunity mechanisms and how ABA controls disease resistance.

MATERIALS and METHODS

Plants growth conditions

Arabidopsis thaliana plants were grown in a growth chamber (19-23ºC, 85% relative humidity, 100 mEm^{-2} sec^{-1} fluorescent illumination) on a 10-hr-light and 14-hr-dark cycle. All mutants and transgenic plants are in Col-0 background; SnRK2.6-HA/OE and SnRK2.2-HA/OE were previously described [69,70]; *snrk2.2 snrk2.3* and *snrk2.6* were described in [39-41]; the triple mutants *pp2ca1-1 hab1-1 abi2-2* and *abi2-2 hab1-1 abi1-2* were described in [45]; *ocp3-1* and *aba2-1* mutants described in [27,42], and the triple *pyl4 pyl5 pyl8*, *pyr1 pyl4 pyl8,* and *pyr1 pyl4 pyl5* mutants, along with the quadruple *pyr1 pyl1 pyl2 pyl4* and single *pyl1*, *pyl4*, *pyr1-1, pyr1-2* and *pyr1-8* mutants were described in [38,49]. Transgenic lines carrying *pPYR1::GUS, pPYL1::GUS, pPYL4::GUS* were described previously [49].

Gene expression analysis.

Total RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer's recommendations and further purified by lithium chloride precipitation. For reverse transcription, the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas Life Sciences) was used. Quantitative PCR (qPCR) amplifications and measurements were performed using an ABI PRISM 7000 sequence detection system, and SYBR-Green (Perkin-Elmer Applied Biosystems). *ACTIN2/8* was chosen as the reference gene. The primers used for RT-qPCR experiments are provided in the supporting information file Text S1. RT-qPCR analyses were performed at least three times using sets of cDNA samples from independent experiments.

Inmunoprecipitation of HA–SnRKs and *in vitro* phosphorylation

HA- tagged SnRK2.2 and SnRK2.6 were inmunoprecipitated and used for *in vitro*

kinase assay as described previously [69].

Chromatin immunoprecipitation

Chromatin isolation and immunoprecipitation were performed as described [12,13]. Chip samples, derived from three biological replicates, were amplified in triplicate and measured by quantitative PCR using primers for *SBT3.3, PR-1, WRKY6*, *WRKY53* and *Actin2* as reported [10,13]. All ChIP experiments were performed in three independent biological replicates. The antibodies used for immunoprecipitation of modified histones from 2 g of leaf material were antiH3K4m3 (#07-473 Millipore) and antiH3K9ac (#07-352 Millipore).

Western blot

Protein crude extracts were prepared by homogenizing ground frozen leaf material with Tris-buffered saline (TBS) supplemented with 5mM DTT, protease inhibitor cocktail (Sigma-Aldrich), and protein phosphatase inhibitors (PhosStop, Roche). Protein concentration was measured using Bradford reagent; 25 µg of total protein was separated by SDS-PAGE (12% acrylamide w/v) and transferred to nitrocellulose filters. The filter was stained with Ponceau-S after transfer, and used as a loading control.

Pathogen assays

Pseudomonas syringae DC3000 was grown for two days and used to infect 5-week-old *Arabidopsis* leaves by infiltration and bacterial growth determined following [10,13]. Twelve samples were used for each data point and represented as the mean ± SD of log c.f.u./cm^2. For *B. cinerea*, *P. cucumerina* and *A. brassicicola* bioassays, 5-week-old plants were inoculated as described [25,26], with a suspension of fungal spores of $2.5x10^4$, $5x10^6$, and $5x10^6$ spores/mL, respectively. The challenged plants were maintained at 100% relative humidity. Disease symptoms were evaluated by determining the lesion diameter of at least 100 lesions at 3, 12, and 8 days after inoculation with *B. cinerea*, *P. cucumerina* and *A. brassicicola*, respectively. For pathogen-induced callose deposition analyses, infected leaves were stained with

aniline blue and callose deposition quantifications were performed as described by Garcia-Andrade *et al*. [42].

Determination of plant hormones and metabolites

ABA, JA, SA and camalexin levels were determined as described previously [27,44]

REFERENCES

1. **Netea, M. G., Quintin, J. & van der Meer, J. W. M**. Trained immunity: a memory for innate host defense. *Cell Host Microbe* **9**, 355–361 (2011).

2. **Kuc, J**. Translocated signals for plant immunization. *Ann. N. Y. Acad. Sci.* **494**, 221–223 (1987).

3. **Zimmerli, L., Jakab, G., Métraux, J. P. & Mauch-Mani, B**. Potentiation of pathogen-specific defense mechanisms in *Arabidopsis* by β-aminobutyric acid. *Proc. Natl. Acad. Sci. USA* **97**, 12920–12925 (2000).

4. **Hayes, M. P., Enterline, J. C., Gerrard, T. L. & Zoon, K. C**. Regulation of interferon production by human monocytes: requirements for priming for lipopolysaccharide- induced production. *J. Leukoc. Biol.* **50**, 176–181 (1991).

5. **Gifford, G. E. & Lohmann-Matthes, M. L.** Gamma interferon priming of mouse and human macrophages for induction of tumor necrosis factor production by bacterial lipopolysaccharide. *J. Natl. Cancer Inst.* **78**, 121–124 (1987).

6. **Koerner, T. J., Adams, D. O. & Hamilton, T**. Regulation of tumor necrosis factor (TNF) expression: Interferon-c enhances the accumulation of mRNA for TNF induced by lipopolysaccharide in murine peritoneal macrophages. *Cell Immunol.* **109**, 437–443 (1987).

7. **Pham, L. N., Dionne, M. S., Shirasu-Hiza, M. & Schneider, D. S**. A specific primed immune response in *Drosophila* is dependent on phagocytes. *PLoS Pathog.* **3**, e26 (2007).

8. **Beckers, G. J. M. *et al.*** Mitogen-activated protein kinases 3 and 6 are required for full priming of stress responses in *Arabidopsis thaliana*. *Plant Cell* **21**, 944–953 (2009).

9. **Jaskiewicz, M., Conrath, U. & Peterhänsel, C.** Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response. *EMBO Rep.*

12, 50–55 (2011).

10. **López, A., Ramírez, V., García-Andrade, J., Flors, V. & Vera, P**. The RNA silencing enzyme RNA Polymerase V is required for plant immunity. *PLoS Genet.* **7**, e1002434 (2011).

11. **Mosher, R. A., Durrant, W. E., Wang, D., Song, J. & Dong, X**. A comprehensive structure–function analysis of Arabidopsis SNI1 defines essential regions and transcriptional repressor activity. *Plant Cell* **18**, 1750–1765 (2006).

12. **Luna, E., Bruce, T. J. A., Roberts, M. R., Flors, V. & Ton, J**. Next-generation systemic acquired resistance. *Plant Physiol.* **158**, 844–853 (2012).

13. **Ramírez, V., López, A., Mauch-Mani, B., Gil, M. J. & Vera, P.** An extracellular subtilase switch for immune priming in Arabidopsis. *PLoS Pathog.* **9**, e1003445 (2013).

14. **Spoel, S. H. & Dong, X. How** do plants achieve immunity? Defence without specialized immune cells. *Nature Rev. Immunol.* **12**, 89–100 (2012).

15. **Law, J. A. & Jacobsen, S. E**. Molecular biology. Dynamic DNA methylation. *Science* **323**, 1568–1569 (2009).

16. **Glazebrook, J.** Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* **43**, 205-227 (2005).

17. **Grant, M. R. & Jones, J. D**. Hormone (dis)harmony moulds plant health and disease. *Science* **324**, 750–752 (2009).

18. **Pieterse, C. M. J., Van der Does, D., Zamioudis, C., Leon-Reyes, A. & Van Wees S. C. M**. Hormonal modulation of plant immunity. *Annu. Rev. Cell Dev. Biol.* **28**, 489–521 (2012).

19. **Thaler, J. S., Humphrey, P. T. & Whiteman, N. K**. Evolution of jasmonate and salicylate signal crosstalk. *Trends Plant Sci.* **17**, 260–270 (2012).

20. **Spoel, S. H.** ***et al.*** NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. *Plant Cell* **15**, 760–770 (2003).

21. **Spoel, S. H., Johnson, J. S. & Dong, X**. Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. *Proc. Natl. Acad. Sci. USA* **104**, 18842–18847 (2007).

22**. Mauch-Mani, B. & Mauch, F.** The role of abscisic acid in plant-pathogen interactions. *Curr. Opin. Plant Biol.* **8**, 409-414 (2005).

23. **Asselbergh, B., De Vleesschauwer, D. & Hofte, M**. Global switches and fine-tuning-ABA modulates plant pathogen defense. *Mol. Plant Microbe Interact.* **21**, 709–719 (2008).

24. **Fan, J., Hill, L., Crooks, C., Doerner, P. & Lamb, C**. Abscisic acid has a key role in modulating diverse plant–pathogen interactions. *Plant Physiol*. **150**, 1750-1761. (2009).

25. **Ton, J. & Mauch-Mani, B**. β-amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose. *Plant J.* **38**, 119–130 (2004).

26. **Adie, B. A. T. *et al***. ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in *Arabidopsis*. *Plant Cell* **19**, 1665–1681 (2007).

27. **García-Andrade, J. Ramírez, V., Flors, V. & Vera, P**. Arabidopsis *ocp3* mutant reveals a potentiation mechanism linking ABA and JA for pathogen-induced callose deposition. *Plant J*. **67**, 783-794 (2011).

28. **Mohr, P. & Cahill, D**. Suppression by ABA of salicylic acid and lignin accumulation and the expression of multiple genes, in *Arabidopsis* infected with *Pseudomonas syringae* pv. *tomato*. *Funct. Integr. Genomics* **7**, 181–191 (2007).

29. **Jensen, M. K. *et al.*** Transcriptional regulation by an NAC (NAM-ATAF1,2-CUC2) transcription factor attenuates ABA signalling for efficient basal defence towards *Blumeria graminis* f. sp *hordei* in Arabidopsis. *Plant J.* **56**, 867–880 (2008).

30. **Hubbard, K. E., Nishimura, N., Hitomi, K., Getzoff, E. D. & Schroeder, J.** I. Early abscisic acid signal transduction mechanisms: newly discovered components and newly emerging questions. *Genes Dev*. **24**, 1695–1708 (2010).

31. **Cutler, S. R., Rodriguez, P. L., Finkelstein, R. R. & Abrams, S. R**. Abscisic acid: emergence of a core signaling network. *Annu. Rev. Plant Biol*. **61**, 651–679 (2010).

32. **Klingler, J. P., Batelli, G. & Zhu, J. K.** ABA receptors: The START of a new paradigm in phytohormone signalling. *J. Exp. Bot*. **61**, 3199–3210 (2010).

33. **Raghavendra, A. S., Gonugunta, V. K., Christmann, A. & Grill E**. ABA perception and signalling. *Trends Plant Sci*. **15**, 395–401 (2010).

34. **Umezawa, T. *et al***. Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **106**, 17588–17593 (2009).

35. **Vlad, F. *et al.*** Protein phosphatases 2C regulate the activation of the Snf1-related kinase OST1 by abscisic acid in *Arabidopsis*. *Plant Cell* **21**, 3170–3184 (2009).

36. **Ma, Y. *et al.*** Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* **324**, 1064–1068 (2009).

37. **Park, S. Y. *et al.*** Abscisic acid inhibits PP2Cs via the PYR/PYL family of ABA-binding START proteins. *Science* **324**, 1068–1071 (2009).

38. **Fujii, H. *et al.*** *In vitro* reconstitution of an ABA signaling pathway. *Nature* **462**, 660–664 (2009).

39. **Umezawa, T. *et al.*** Genetics and phosphoproteomics reveal a protein phosphorylation network in the abscisic acid signaling pathway in Arabidopsis thaliana. *Sci. Signal.* **6**, rs8 (2013).

40. **Wang, P. *et al.*** Quantitative phosphoproteomics identifies SnRK2 protein kinase substrates and reveals the effectors of abscisic acid action. *Proc. Natl. Acad. Sci. USA* **110**, 11205-11210 (2013).

41. **Fujii, H. & Zhu, J.K**. (2009) Arabidopsis mutant deficient in 3 abscisic acid-activated protein kinases reveals critical roles in growth, reproduction, and stress. Proc. Natl. Acad. Sci. *USA* 106, 8380–8385 (2009).

42. **García-Andrade, J., Ramírez, V., López, A. & Vera P**. Mediated plastid RNA editing in plant immunity. *PLoS Pathog.* **9,** e1003713 (2013).

43. **Fujii, H. M., Verslues, P. E. & Zhu J. K**. Identification of two protein kinases required for abscisic acid regulation of seed germination, root growth and gene expression in *Arabidopsis*. *Plant Cell* **19**, 485–494 (2007).

44. **Flors, V., Ton, J., van Doorn, R., Jakab, G., Garcia-Agustin, P. & Mauch-Mani, B.** Interplay between JA, SA and ABA signalling during basal and induced resistance against *Pseudomonas syringae* and *Alternaria brassicicola*. *Plant J.* **54**, 81–92 (2008).

45. **Rubio, S. *et al.*** Triple loss of function of protein phosphatases type 2C leads to partial constitutive response to endogenous abscisic acid. *Plant Physiol*. **150**, 1345–1355 (2009).

46. **Schweighofer, A., Hirt, H. & Meskiene, I**. Plant PP2C phosphatases: emerging functions in stress signaling. *Trends Plant Sci*. **9**, 236–243 (2004).

47. **Carrasco, J. L., Ancillo, G., Mayda, E. & Vera, P**. A novel transcription factor

involved in plant defense endowed with protein phosphatase activity. *EMBO J.* **22**, 3376–3384 (2003).

48. **Carrasco, J. L. *et al***. Arabidopsis protein phosphatase DBP1 nucleates a protein network with a role in regulating plant defense. *PLoS One* **9**, e90734 (2014).

49. **Gonzalez-Guzman, M. *et al***. *Arabidopsis* PYR/PYL/RCAR receptors play a major role in quantitative regulation of stomatal aperture and transcriptional response to abscisic acid. *Plant Cell* **24**, 2483–2496 (2012).

50. **Antoni, R. *et al***. PYRABACTIN RESISTANCE1-LIKE8 plays an important role for the regulation of abscisic acid signaling in root. *Plant Physiol* **161**, 931-941 (2013).

51. **Llorente, F. *et al***. Repression of the auxin response pathway increases *Arabidopsis* susceptibility to necrotrophic fungi. *Molecular Plant* **1**, 496–509 (2008).

52. **Audenaert, K., De Meyer, G. B. & Höfte, M. M.** Abscisic acid determines basal susceptibility of tomato to *Botrytis cinerea* and suppresses salicylic acid-dependent signaling mechanisms. *Plant Physiol.* **128,** 491–501 (2002).

53. **Asselbergh, B. *et al***. Resistance to *Botrytis cinerea* in *sitiens*, an abscisic acid-deficient tomato mutant, involves timely production of hydrogen peroxide and cell wall modifications in the epidermis. *Plant Physiol.* **144**, 1863–1877 (2007).

54. **Serrano, M., Coluccia, F., Torres, M., L'Haridon, F. & Métraux, J. P**. The cuticle and plant defense to pathogens. *Front*. *Plant Sci.* **5**, 274 doi: 10.3389/fpls.2014.00274 (2014).

55. **Clarke, J. D., Volko, S. M., Ledford, H., Ausubel, F. M. & Dong, X. N.** Roles of salicylic acid, jasmonic acid, and ethylene in *cpr*-induced resistance in Arabidopsis. *Plant Cell* **12**, 2175–2190 (2000).

56. **Ferrari, S., Galletti, R., Denoux, C., De Lorenzo, G., Ausubel, F. M. & Dewdney J**. Resistance to *Botrytis cinerea* induced in Arabidopsis by elicitors is independent of salicylic acid, ethylene, or jasmonate signaling but requires *PHYTOALEXIN DEFICIENT3*. *Plant Physiol.* **144**, 367–379 (2007).

57. **Cheng, W.H. *et al.*** A unique short-chain dehydrogenase/reductase in Arabidopsis glucose signaling and abscisic acid biosynthesis and functions. *Plant Cell* **14**, 2723–2743 (2002).

58. **Koiwai, H., Nakaminami, K., Seo, M., Mitsuhashi, W., Toyomasu, T. & Koshiba, T**. Tissue-specific localization of an abscisic acid biosynthetic enzyme, AAO3, in Arabidopsis. *Plant Physiol.* **134**, 1697–1707 (2004).

59. **Endo, A. *et al.*** Drought induction of Arabidopsis 9-cis-epoxycarotenoid dioxygenase occurs in vascular parenchyma cells. *Plant Physiol*. **147,** 1984–1993 (2008).

60. **Endo, A., Koshiba, T., Kamiya, Y. & Nambara, E**. Vascular system is a node of systemic stress responses. *Plant Signal. Behav.* **3**, 1138–1140 (2008).

61. **Alvarez, M. E., Pennell, R. I., Meijer, P. J., Ishikawa, A., Dixon, R. A. & Lamb, C.** Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. *Cell* **92**, 773–784 (1998).

62. **Yu, A. *et al*.** Dynamics and biological relevance of DNA demethylation in *Arabidopsis* antibacterial defense. *Proc. Natl. Acad. Sci. USA* **110**, 2389–2394 (2013).

63. **Ton, J., Flors, V. & Mauch-Mani, B**. The multifaceted role of ABA in disease resistance. *Trends Plant Sci.* **14**, 310–317 (2009).

64. **de Torres-Zabala, M., Bennett, M. H., Truman, W. H. & Grant, M. R**. Antagonism between salicylic and abscisic acid reflects early host-pathogen conflict and moulds plant defence responses. *Plant J.* **59**, 375-386 (2009).

65. **De Vleesschauwer, D., Yang, Y. N., Cruz, C. V. & Höfte M**. Abscisic acid-induced resistance against the brown spot pathogen *Cochliobolus miyabeanus* in rice involves MAP kinase-mediated repression of ethylene signaling. *Plant Physiol.* **152**, 2036–2052 (2010).

66. **Jiang, C. J. *et al*.** Abscisic acid interacts antagonistically with salicylic acid signaling pathway in rice-*Magnaporthe grisea* interaction. *Mol. Plant Microbe Interact.* **23**, 791–798 (2010).

67**. Koornneef, A. & Pieterse, C. M**. Cross talk in defense signaling. *Plant Physiol.* **146**, 839–844 (2008).

68. **de Torres-Zabala, M. *et al.*** *Pseudomonas syringae* pv. *tomato* hijacks the *Arabidopsis* abscisic acid signalling pathway to cause disease. *EMBO J.* **26**, 1434–1443 (2007).

69. **Planes, M. D. *et al.*** A mechanism of growth inhibition by abscisic acid in germinating seeds of *Arabidopsis thaliana* based on inhibition of plasma membrane H^+-ATPase and decreased cytosolic pH, K^+, and anions. *J. Exp. Bot*. **66**, 813–825 (2014).

70. **Belin, C. *et al*.** Identification of features regulating OST1 kinase activity and OST1 function in guard cells. *Plant Physiol*. **141**, 1316–1327 (2006).

ACKNOWLEDGEMENTS

We acknowledge V. Flors for technical assistance in the analysis of plant metabolites and hormones. M.G.G. was funded by a JAE-DOC contract. Work in P.L.R. laboratory was supported by grant BIO2011-23446. P.V. acknowledges the Spanish MINECO for grant BFU2012 and the Generalitat Valenciana for grant Prometeo2014/020.

Competing Financial Interest

Authors declare no competing financial interest.

Author contributions

MGG and PLR performed SnRKs kinase assays and provided genetic resources. JGA and BG perform the rest of experiment shown in the manuscript. PV wrote the manuscript. All the authors participated and analyzed the data and supervised the written manuscript.

Discusión general

OCP3 ejerce un control negativo de la deposición de calosa dependiente tanto del JA como del ABA.

El trabajo realizado en la presente Tesis Doctoral persigue identificar la función de la proteína OCP3 y su implicación en la regulación de los mecanismos defensivos frente al ataque de patógenos, en concreto frente a hongos necrotrofos. En el primer capítulo nos hemos centrando en entender qué mecanismos implicados en la respuesta defensiva están directa o indirectamente regulados por la proteína OCP3. Para ello se utilizaron los hongos necrotrofos *B. cinerea* y *P. cucumerina* y se observó que las plantas *ocp3*, que previamente habían sido descritas ser resistentes a dichos patógenos (Coego *et al*., 2005; Ramirez *et al*., 2009; Ramírez *et al*., 2010), presentaban también una mayor y más rápida deposición de calosa en la zona de inoculación. Esta activación de la deposición de calosa es clave para frenar el ataque del patógeno ya que el doble mutante *ocp3 pmr4* pierde la activación de la deposición de calosa y revierte el fenotipo de resistencia. Por lo tanto, OCP3 jugaría un papel represor de la señalización que activa la deposición de calosa y la resistencia mediada por este polímero. En este sentido, existen una gran cantidad de moléculas que activan la síntesis y la deposición de calosa, entre las que cabe destacar las especies ROS, las cuales son capaces de activar la deposición de calosa inducida por Flg22 (Benitez-Alfonso *et al*., 2009; Luna *et al*., 2011). De hecho, hemos observado que las plantas mutantes *ocp3* depositan mayor cantidad de calosa tras un tratamiento con Flg22.

En cuanto a la regulación hormonal de los mecanismos de defensa de las plantas, Ton y Mauch-Mani (2004) sugirieron que el ABA tenía un papel relevante en la inducción de resistencia, así como en la activación de la defensa mediada por el BABA. En la presente Tesis, hemos podido comprobar que el ABA es determinante en la activación de mecanismos de defensa frente a *P. cucumerina*. En este sentido, las plantas *ocp3*, que presentan un notable incremento en la resistencia a dicho patógeno, presentan también un mayor contenido en ABA respecto a las plantas silvestres, lo que les podría conferir una ventaja para activar de manera más eficiente los mecanismos de defensa correspondientes y poder reducir el avance del patógeno.

Así pues, tanto el incremento del contenido de ABA, como la rápida e intensa deposición de calosa en respuesta al patógeno se pierden en el doble mutante *ocp3 aba2*.

Otra de las hormonas clave en la defensa es el JA (Glazebrook 2005), siendo el mutante *coi1* (insensible a JA) susceptible al ataque de patógenos tales como *P. irregulare* o *B.cinerea* (Adie *et al*. 2007; Zimmerli *et al*. 2001; Shah 2005). Las plantas mutantes *ocp3* presenta una expresión constitutiva del gen *PDF1.2*, marcador de la señalización mediada por JA (Coego *et al*., 2005). Además, el doble mutante *ocp3 coi1* presenta una supresión de la resistencia frente *B. cinerea* mediada por la mutación *ocp3* (Ramírez *et al*., 2009); y este hecho se ve acompañado de una reducción en la mayor y más rápida deposición de calosa observada en el mutante simple *ocp3*. Sin embargo, el mutante *coi1*, aún siendo susceptible, es capaz de activar la correcta deposición de calosa y en la misma extensión que las plantas silvestres. Ello indicaría que la proteína OCP3 ejerce un control negativo de la señalización que regula la deposición de calosa tras el ataque de un patógeno necrotorofo y en la que se ven involucradas tanto la señalización dependiente de JA como la dependiente de ABA.

Nuevo papel de la edición de RNA de proteínas del complejo NDH en la inmunidad vegetal.

Aunque datos iniciales hacían pensar que OCP3 podría tener un papel como represor de la transcripción nuclear (Coego *et al*., 2005), los resultados mostrados en el segundo capítulo de la presente Tesis dan un giro conceptual al descubrir que la proteína OCP3 se localiza en el cloroplasto. Tras utilizar diferentes marcadores subcelulares con el fin de entender en que contexto podría estar implicada OCP3, se vio que colocalizaba con proteínas cloroplásticas de la familia de las PPRs implicadas en la edición de RNA. Poniendo la atención en la regulación del gen *OCP3* vimos que se corregulaba con un grupo de genes entre los que estaban sobrerrepresentados genes de la familia de las PPRs, incluido *CRR21*. CRR21 al igual que CRR4, son dos PPR que están implicadas en la edición del transcrito *ndhD*

que codifica la subunidad D del complejo NDH del cloroplasto (Okuda *et al*., 2007; Kotera *et al*., 2005), lo que nos pudo dar una idea sobre la posible función de OCP3. Estos datos nos llevaron a proponer que OCP3 podría estar implicado en la regulación de RNAs cloroplásticos, por lo que realizamos un estudio comparativo de los sitios de edición descritos del cloroplasto. Este estudio reveló que plantas *ocp3* tienen alterada la edición de los sitios ndhB-6, ndhB-4, ndhB-3 y ndhB-2. A través de un ensayo RIP pudimos confirmar que OCP3 esta asociado al transcrito *ndhB*, lo que refuerza la idea de su implicación en la regulación del RNA. Sin embargo, OCP3 no contiene ningún dominio de interacción a RNA que permita agruparlo a ninguna de las familias de proteínas descritas implicadas en la edición de RNA (Takenaka *et al*., 2012; Bentolila *et al*., 2012). Esto sugeriría que la interacción con el RNA podría ser a través de un factor que desconocemos actualmente.

Las plantas mutantes *crr21*, al tener alterada la edición del transcrito *ndhD* presentan una pérdida en la actividad del complejo NDH lo que se traduce en una reducción de la actividad CEF. Por lo tanto, estos defectos en la edición observados en las plantas *ocp3* conllevarían supuestamente a una inactivación del complejo NDH y consecuentemente a una alteración en el CEF del PSI. Así pues, pudimos comprobar que una reducción de los niveles de OCP3, a través de diferentes alelos de *ocp3*, provoca una alteración en la edición del transcrito ndhB y esto a su vez genera una reducción de la actividad del complejo NDH y la correspondiente inhibición de la actividad CEF. Dado que el complejo NDH del cloroplasto está involucrado en disipar diferentes estreses oxidativos (Endo *et al*., 1999; Wang *et al*., 2006) se puede proponer que una reducción del CEF generaría un incremento de ROS a nivel local que desencadenaría la más pronta activación de mecanismos defensivos ante la agresión por un patógeno. En este sentido, las plantas mutantes *ocp3* presentan unos niveles elevados de ROS a nivel constitutivo y dado que la expresión del gen *OCP3* en plantas silvestres se reprime rápidamente tras la percepción de un patógeno, es posible sugerir la existencia de un nexo de unión entre el control de la edición de proteínas del complejo NDH, que regularía la actividad de éste último, y la respuesta de la planta frente al ataque de un patógeno. Así pues, otros mutantes que tengan

alterado, de igual manera, la actividad del complejo NDH deberían ser más resistentes al ataque del hongo. Esto se comprobó al inocular plantas mutantes *crr2* y *crr21*, deficientes en la edición del transcrito *ndhB* y *ndhD,* respetivamente (Hashimoto *et al*., 2003; Okuda *et al*., 2007). Ambos mutantes respondieron con un incremento tanto en la resistencia a patógenos como en la deposición de la calosa en los sitios de inoculación, fenocopiando a lo observado en *ocp3* (García-Andrade *et al*., 2011). Este hecho, sumado a la implicación descrita de las ROS en la activación de la deposición de calosa durante la respuesta inmune (Benitez-Alfonso *et al*., 2009; Luna *et al*., 2011) refuerza la idea de que el complejo NDH puede jugar un papel señalizador en la defensa frente a hongos necrotrofos. Con el fin de confirmar si existía una posible regulación del grado de edición del RNA de las subunidades cloroplásticas del complejo NDH, se evaluaron los mismos en plantas silvestres tras una infección con *P. cucumerina.* Los resultados mostraron que 8 de los sitios del gen *ndhB,* incluidos los regulados por OCP3, y uno de los sitios del gen *ndhD* estaban alterados. Esto nos hizo pensar que al igual que OCP3, otras proteínas implicadas en la regulación de la edición deberían ser moduladas por la presencia del patógeno. Analizamos la expresión de *CRR21*, *PPRa* y *OCP3* tras un tratamiento con quitosano y observamos una fuerte represión de su expresión para todos ellos, hecho que contrastaba con el incremento de *MYB51* descrito como un factor de transcripción requerido para la deposición de calosa inducida por PAMPs (Clay *et al*., 2009). Así pues, cabe pensar que la estabilidad del complejo NDH es clave en la generación de una señal requerida para la activación de la defensa frente a hongos necrotrofos. En los resultados se muestra como, tanto tras la inoculación con *P. cucumerina*, como tras un tratamiento con quitosano, se produce una alteración de la edición de los transcritos *ndhB* y *ndhD* y una desestabilización del complejo NDH. Sin embargo, en el estado actual de la investigación, no podemos asegurar que la desestabilización del complejo NDH sea una consecuencia directa de la alteración en la edición del RNA o que concurra la activación de un mecanismos de proteolisis del complejo NDH. En definitiva, la estabilidad del complejo NDH es esencial para el normal funcionamiento del CEF durante la fotosíntesis, sin embargo su inhibición es importante para la activación de la inmunidad vegetal tras la percepción de un

patógeno. Por lo tanto, la modulación de la actividad del complejo NDH deber ser finamente regulada, interviniendo en ello los mecanismos de edición de mRNAs y la coparticipación de OCP3 y otras proteínas PPR. El conjunto de los resultados presentados en el presente capitulo de la Tesis Doctoral, indicarían que tanto OCP3, PPRs, como otras proteínas involucradas en el editosoma de las subunidades del complejo NDH, juegan un papel dual ya que actúan como reguladores negativos de la inmunidad vegetal y reguladores positivos del flujo cíclico de electrones durante la fotosíntesis oxigénica.

La implicación del ABA en la defensa vegetal se da específicamente a través del receptor PYR1

A través del estudio de la implicación en la defensa de la proteína OCP3, en el primer capitulo de la Tesis, se ha podido comprobar que el ABA es determinante en la activación de mecanismos de defensa frente a *P. cucumerina*. El ABA tiene múltiples y diversas funciones en las plantas como la regulación de procesos del desarrollo y la respuesta a estreses bióticos (Fujita *et al*., 2006; Wasilewska *et al*., 2008). En concreto, tiene un papel positivo en la resistencia basal frente a hongos necrotrofos como son *A. brassicicola*, *P.irregulare* y *P. cucumerina* (Adie *et al*., 2007; Flors *et al*., 2008; García-Andrade *et al*., 2011). Sin embargo, la implicación del ABA en la defensa es un punto en controversia ya que en algunos trabajos se ha observado un efecto negativo de esta hormona sobre la resistencia (Audenaert *et al*., 2002; Anderson *et al*., 2004; Asselbergh *et al*., 2008; L'Haridon *et al*., 2011; Sanchez-Vallet *et al*., 2012). Esto podría estar relacionado con la edad de las plantas y los diferentes métodos de inoculación utilizados. En este capitulo de la Tesis se pretende profundizar en el papel que juega cada uno de los componentes del núcleo central de percepción del ABA en la defensa vegetal. Tras un incremento en la concentración de ABA se activan los receptores PYLs que tras unirse al ABA modifican su estructura terciaria favoreciendo la interacción con las PP2Cs para inhibir su actividad. De esta forma, tras generarse el complejo ABA-PYL-PP2C, se interrumpe el efecto negativo que las PPC2s ejercen sobre las SnRKs permitiendo que estas quinasas se autofosforilen y activen a los factores de transcripción del tipo

ABF, lo que conlleva a una activación de la señalización dependiente de ABA (Umezawa *et al.,* 2009; Vlad *et al.,* 2009; Mustilli *et al.,* 2002; Fujii *et al.,* 2007; Fujii *et al.,* 2009; Johnson *et al.,* 2002; Furihata *et al.,* 2006). Los resultados presentados muestran que las SnRK2s tienen un papel activo en la resistencia a *P. cucumerina* ya que se ha observado una activación de estas quinasas tras la percepción del patógeno y además, mutantes de pérdida de función comprometen la resistencia. Por otra parte, tras analizar una serie de cuádruples mutantes de receptores, vimos que aquellos que contenían la mutación en el receptor PYR1 presentaban un incremento en la susceptibilidad. Esta especificidad en el receptor se comprobó al utilizar tres alelos diferentes del mutante simple *pyr1*. Además, al utilizar una línea de ganancia de función para PYR1 los resultados mostraron que se incrementaba la resistencia frente al ataque del patógeno. Estos resultados demuestran que PYR1 tiene la capacidad de percibir el ABA sintetizado tras la percepción del patógeno (García-Andrade *et al*., 2011) y de de-reprimir a las SnRK2s, de forma que permite activar los mecanismos de defensa. Así pues, al igual que PYL8 que juega un papel específico en la percepción del ABA en raíz (Antoni *et al*., 2013), PYR1 tiene un papel relevante en la percepción de ABA para los procesos implicados en la inmunidad vegetal.

Dado que se ha descrito que los receptores de ABA presentan un patrón diferencial de expresión, quisimos comprobar si *PYR1* se comportaba de manera diferente a *PYL1* y *PYL4* en presencia de *P. cucumerina*. Este resultado mostró que en la zona de inoculación se incrementaba la expresión de *PYR1*, siendo mayor en los haces vasculares. Este patrón se asemeja al observado para genes implicados en la síntesis de ABA (Cheng *et al*., 2002; Koiwai *et al*., 2004; Endo *et al.*, 2008), lo que apoya la idea de que los tejidos vasculares tienen un papel integrador de las señales de estrés locales y sistémicas (Endo *et al*., 2008).

Con el fin de comprobar la especificidad del receptor PYR1, en cuanto al patosistema se refiere, realizamos ensayos con diferentes patógenos para los que ya había sido descrita una implicación del ABA tanto incrementando como reduciendo la resistencia de la plantas. Previamente se ha descrito que aplicaciones exógenas de

ABA inducen deposición de calosa en respuesta a la infección por *A. brassicicola*, lo que provoca un incremento en la resistencia frente a este patógeno (Flors *et al*., 2008). Por lo tanto evaluamos la implicación del receptor PYR1 en la activación de defensas frente a *A. brassicola*. El resultado mostró que tanto la síntesis como la percepción del ABA específicamente a través de PYR1 son claves para la resistencia frente a este patógeno. Por el contrario, el ABA juega un papel negativo en la resistencia frente al hongo *B. cinerea*, ya que tanto mutantes deficientes en la síntesis de ABA de tomate como de Arabidopsis son más resistentes que las plantas silvestres (Audenaert *et al*., 2002; L'Haridon *et al*., 2011). Nuestros resultados mostraron, que al igual que lo descrito por otros grupos, el ABA percibido específicamente por PYR1 reprime la activación de las defensa frente al patógeno *B. cinerea*. El diferente papel del ABA entre estos patógenos, podría deberse a que la resistencia frente a *P. cucumerina* y *A. brassicicola* requiere de la activación de las vías de señalización dependientes de JA y SA, mientras que la resistencia a *B. cinerea* requiere de la fitoalexina camalexina (Ferrari *et al*., 2007). Puesto que las plantas mutantes *pyr1* acumulan más cantidad de camalexina, podría explicar la diferencia en la respuesta de los mutantes *pyr1* y *aba2* frente a diferentes patógenos. Por otra parte, también ha sido descrito un efecto negativo del ABA en la resistencia frente a *Pseudomonas syringae* DC3000. Al igual que con los hongos necrotrofos anteriores, en nuestras condiciones experimentales pudimos reproducir el fenotipo de susceptibilidad ya descrito para mutantes de pérdida de función de genes de síntesis de ABA (de Torres-Zabala *et al*., 2009; Fan *et al*., 2009). Al utilizar este patógeno biotrofo con el mutante *pyr1* vimos que se incrementaba la resistencia, por lo que el ABA percibido específicamente a través de PYR1 reprime la activación de la defensas frente a *P. syringae* DC3000. Por lo tanto, se podría decir que PYR juega un papel represor de la inmunidad frente a patógenos biotrofos y un papel activador frente a patógenos necrotrofos. Así pues, podría ser que PYR1 interfiriera en el cruce entre la señalización dependiente de SA y la dependiente de JA. Nuestros resultados muestran una activación basal de la respuesta dependiente de SA en las plantas mutantes *pyr1*, por lo que en las plantas silvestres el ABA ejerce un efecto negativo sobre la señalización dependiente de SA. Esto lo comprobamos al observar

cómo en el mutante *pyr1* se suprimió el efecto negativo en la resistencia frente a *P. syringae* DC3000 ejercido por aplicaciones exógenas de ABA. Así pues, este incremento de SA en condiciones basales observado en el mutante *pyr1* podría antagonizar con la resistencia dependiente de JA favoreciendo el crecimiento de los hongos necrotrofos. Nuestros resultados muestran como en el mutante *pyr1* está activa la vía dependiente de SA, mostrando: (1) activación constitutiva de MPKs; (2) acumulación en condiciones basales así como tras la infección por el patógeno de una mayor acumulación de PR1; (3) represión en condiciones basales del gen *NRPD2*, lo que sugiere una inhibición del mecanismo de RdDM que regula negativamente la defensa de SA; (4) Activación constitutiva de la subtilasa SBT3.3; (5) modificaciones de cromatina a través de marcas de histonas de la región promotora de genes relacionados en la defensa a biotrofos. De esta forma, el ABA a través del receptor PYR1 y a través de un control epigenético, tendría un papel regulador de la inmunidad vegetal en función del estilo de vida del patógeno que infecte a la planta.

Conclusiones

Las investigaciones desarrolladas en esta Tesis Doctoral han permitido alcanzar las siguientes conclusiones:

1. La proteína OCP3 actúa como un regulador negativo de la deposición de calosa en respuesta a la infección producida por patógenos necrotrofos. Por otra parte, la calosa sintasa PMR4 es indispensable para manifestar el fenotipo de resistencia e incremento de la deposición de calosa observado en las plantas *ocp3*.
2. El ABA tiene un papel relevante en la activación de mecanismos de resistencia como la deposición de calosa, en respuesta a la infección por *P cucumerina*. Además, esta hormona es indispensable en la manifestación de los fenotipos observados en las plantas *ocp3*.
3. El JA percibido a través de COI1 no es requerido para la deposición de la calosa basal pero sí para el fenotipo observado en las plantas *ocp3*. Por lo que podemos proponer dos vías diferenciadas en cuanto a la deposición de calosa.
4. La inducción de resistencia mediada por BABA, se da de una manera independiente a OCP3. Sin embargo, ambas requieren de ABA y de la calosa sintasa PMR4.
5. Aunque incialmente se propuso que la proteína OCP3 tenía una localización nuclear, atualemente podemos decir que se localiza en el cloroplasto junto a proteínas de la familia de las pentatricopeptidos (PPRs).
6. La proteína OCP3 se coexpresa, principalmente, con proteínas implicadas en el metabolismo del RNA cloroplástico. En este sentido, OCP3 es necesaria para la correcta edición del transcrito *NdhB*. Además, la regulación de la edición del RNA de las diferente subunidades del complejo NDH, se ven alteradas por la infección con *P. cucumerina*.
7. A través de la regulación de la subunidad NdhB, OCP3 juega un papel crucial en la actividad del complejo NDH implicado en el CEF. Dicho complejo participa de manera positiva en los procesos fotosintéticos, pero de manera negativa en la resistencia frente a patógenos. De tal forma que,

mutantes con nula actividad del complejo NDH presentan un incremento, tanto en la resistencia como en la deposición de calosa frente a *P. cucumerina*.

8. Dada la importancia del ABA en los mecanismos de defensa relacionados con la proteína OCP3, identificamos a PYR1 como el principal receptor implicado en la percepción de esta hormona. Siendo necesario para promover la activación de las defensas frente a *P. cucumerina*, y activando la señalización dependiente de las SnRK2s.
9. La expresión de *PYR1* se da de una manera restingida a la zona de infección por el patógeno y en la vasculatura próxima.
10. Tras observar la respuesta de la plantas mutantes *pyr1* a patógenos biotrofos y necrotrofos, podemos proponer que PYR1 juega un papel dual en la defensa. Por un lado, favorece la activación de resistencia frente a necrotrofos y por otro lado, reprime la resistencia frente a biotrofos.
11. El ABA percibido a través de PYR1 regula de manera negativa la vía dependiente de SA, mostrando el mutante *pyr1* en condiciones basales de-reprimidas las respuestas a esta hromona: (1) activación constitutiva de MPKs; (2) acumulación de SA e inducción de PR1; (3) represión del gen *NRPD2*; (4) Activación de la subtilasa SBT3.3; (5) modificaciones de cromatina a través de marcas de histonas de la región promotora de genes relacionados en la defensa a biotrofos.
12. Conclusión general:

    Con los resultado obtenidos en la presente Tesis Doctoral, podríamos proponer un nuevo nivel de complejidad de la regulación de la inmunidad vegetal. Esto implica: 1) La regulación de la edición del RNA cloroplástico, en el que OCP3 regula el transcrito *NdhB*; 2) el complejo NDH del cloroplasto y su función dual fotosíntesis vs inmunidad; 3) percepción del ABA a través del receptor PYR1 para la activación de los mecanismos de defensa como la deposición de calosa.

Bibliografía

1. **Acosta IF, Laparra H, Romero SP, Schmelz E, Hamberg M, Mottinger JP, Moreno MA, Dellaporta SL** (2009) Tasselseed1 is a lipoxygenase affecting jasmonic acid signaling in sex determination of maize. Science 323, 262-265.
2. **Adie, B.A., Perez-Perez, J., Perez-Perez, M.M., Godoy, M., Sanchez-Serrano, J.J., Schmelz, E.A. and Solano, R**. (2007) ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in Arabidopsis. Plant Cell, 19, 1665-1681.
3. **Agorio A, Vera P.** (2007) ARGONAUTE4 is required for resistance to Pseudomonas syringae in Arabidopsis. Plant Cell. 19 (11), 3778-90.
4. **Agrios, G. N.** (2005). Plant Pathology. Academic Press, London.
5. **Albertsson, P**. (2001) A quantitative model of the domain structure of the photosynthetic membrane. Trends Plant Sci 6, 349-358
6. **Anderson, J.M., and Andersson, B.** (1982) The architecture of photosynthetic membranes: lateral and transversal organization Trends Biochem. Sci. 7, 288-292
7. **Antoni R, Gonzalez-Guzman M, Rodriguez L, Peirats-Llobet M, Pizzio GA, Fernandez MA, De Winne N, De Jaeger G, Dietrich D, Bennett MJ, Rodriguez PL.** (2013) PYRABACTIN RESISTANCE1-LIKE8 plays an important role for the regulation of abscisic acid signaling in root. Plant Physiol. 161 (2), 931-41
8. **Antoni R, Gonzalez-Guzman M, Rodriguez L, Rodrigues A, Pizzio GA, Rodriguez PL.** (2012) Selective inhibition of clade A phosphatases type 2C by PYR/PYL/RCAR abscisic acid receptors. Plant Physiol 158, 970-980.
9. **Arabidopsis Interactome Mapping Consortium** (2011) Evidence for network evolution in an Arabidopsis interactome map. Science. 29; 333 (6042), 601-7
10. **Ariizumi T. and Toriyama K.** (2011) Genetic Regulation of Sporopollenin Synthesis and Pollen Exine Development. Annual Review of Plant Biology. 62, 437-460
11. **Asselbergh, B., De Vleesschauwer, D. and Hofte, M**. (2008) Global switches and fine-tuning-ABA modulates plant pathogen defense. Mol Plant Microbe Interact, 21, 709-719.
12. **Barber, J.** (1998) Photosystem two. Biochim. Biophys. Acta 1365, 269-277

13. **Bari R, Jones JD.** (2009) Role of plant hormones in plant defence responses. Plant Mol Biol. 69 (4), 473-88
14. **Baxter A, Mittler R, Suzuki N.** (2014). ROS as key players in plant stress signalling. Journal of Experimental Botany 65, 1229–1240
15. **Ben-Shem, A., Frolow, F., and Nelson, N**. (2003) Crystal structure of plant photosystem I. Nature 426, 630-635
16. **Bendall, D.S., and Manasse, R.S.** (1995) Cyclic photophosphorylation and electron transport. Biochim. Biophys. Acta 1229, 23–38
17. **Bentolila S, Heller WP, Sun T, Babina AM, Friso G, van Wijk KJ, Hanson MR.** (2012) RIP1, a member of an Arabidopsis protein family, interacts with the protein RARE1 and broadly affects RNA editing. Proc Natl Acad Sci U S A. 109 (22), E1453-61.
18. **Bentolila S, Oh J, Hanson MR, Bukowski R.** (2013) Comprehensive high-resolution analysis of the role of an Arabidopsis gene family in RNA editing. PLoS Genet. 9(6):e1003584
19. **Bessire M, Chassot C, Jacquat AC, Humphry M, Borel S, Petétot JM, Métraux JP, Nawrath C.** (2007) A permeable cuticle in Arabidopsis leads to a strong resistance to Botrytis cinerea. EMBO J. 26 (8), 2158-68.
20. **Bestwick, C.S., Brown, I.R., Bennett, M.H. and Mansfield, J.W.** (1997) Localization of hydrogen peroxide accumulation during the hypersensitive reaction of lettuce cells to Pseudomonas syringae pv phaseolicola. Plant Cell, 9, 209-221.
21. **Boekema, E.J., van Breemen, J.F., van Roon, H., and Dekker, J.P**. (2000) Arrangement of photosystem II supercomplexes in crystalline macrodomains within the thylakoid membrane of green plant chloroplasts. J. Mol. Biol. 301, 1123-1133
22. **Bolwell GP, Daudi A.** (2009). Reactive oxygen species in plant–pathogen interactions. In: del Río LA, Puppo A, eds. Reactive oxygen species in plant signaling . Berlin, Heidelberg: Springer-Verlag, 113–133.
23. **Boyer, P.D.** (1989) A perspective of the binding change mechanism for ATP synthesis. Faseb J. 3, 2164-217
24. **Browse J, Howe GA** (2008) New weapons and a rapid response against insect attack. Plant Physiol 146, 832-838.

25. **Bukhov N., Egorova E. and Carpentier R.** (2002) Electron flow to photosystem I from stromal reductants in vivo: the size of the pool of stromal reductants controls the rate of electron donation to both rapidly and slowly reducing photosystem I units. Planta 215: 812-820
26. **Burrows P.A., Sazanov L.A., Svab Z., Maliga P. and Nixon PJ.** (1998) Identification of a functional respiratory complex in chloroplasts through analysis of tobacco mutants containing disrupted plastid ndh genes. EMBO Journal 17, 868-876
27. **C. Chang, I. Damiani, A. Puppo, P. Frendo** (2009) Redox changes during the legume-Rhizobium symbiosis, Mol. Plant 2, 370-377.
28. **Cai W, Okuda K, Peng L, Shikanai T.** (2011) PROTON GRADIENT REGULATION 3 recognizes multiple targets with limited similarity and mediates translation and RNA stabilization in plastids. Plant J. 67 (2), 318-27
29. **Campbell W.J. and Ogren W.L.** (1992) Light activation of Rubisco by Rubisco activase and thylakoid membranes. Plant Cell Physiol 33, 751-756
30. **Canet JV, Dobón A, Roig A, Tornero P.** (2010) Structure-function analysis of npr1 alleles in Arabidopsis reveals a role for its paralogs in the perception of salicylic acid. Plant Cell Environ. 33 (11), 1911-22
31. **Cao H, Bowling SA, Gordon AS, Dong XN** (1994) Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell 6, 1583-1592.
32. **Cao H, Glazebrook J, Clarke JD, Volko S, Dong XN** (1997) The Arabidopsis NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell 88, 57-63.
33. **Carpita, N., and McCann, M.** (2000). The cell wall. In Biochemistry and Molecular Biology of Plants, B.B. Buchanan, G. Wilhelm, and R.L. Jones, eds (Rockville, IL: American Society of Plant Physiologists), pp. 52–108.
34. **Casano LM, Martín M, Sabater B** (2001) Hydrogen peroxide mediates the induction of chloroplastic Ndh complex under photooxidative stress in barley. Plant Physiol. 125 (3), 1450-8.
35. **Chassot C, Nawrath C, Métraux JP.** (2008) The cuticle: Not only a barrier for plant defence: A novel defence

syndrome in plants with cuticular defects. Plant Signal Behav. 3 (2), 142-4.
36. **Chateigner-Boutin AL, Small I.** (2007) A rapid high-throughput method for the detection and quantification of RNA editing based on high-resolution melting of amplicons. Nucleic Acids Res. 35 (17), e114.
37. **Cheng H, Song S, Xiao L, Soo HM, Cheng Z, Xie D, Peng J** (2009) Gibberellin acts through jasmonate to control the expression of MYB21, MYB24, and MYB57 to promote stamen filament growth in Arabidopsis. PLoS Genet 5, e1000440 doi: 10.1371/journal.pgen.1000440.
38. **Chico JM, Chini A, Fonseca S, Solano R** (2008) JAZ repressors set the rhythm in jasmonate signaling. Curr Opin Plant Biol 11, 486-494.
39. **Chini A, Fonseca S, Fernandez G, Adie B, Chico JM, Lorenzo O, Garcia-Casado G, Lopez-Vidriero I, Lozano FM, Ponce MR et al.** (2007) The JAZ family of repressors is the missing link in jasmonate signalling. Nature 448, 666-671.
40. **Coego A, Ramirez V, Ellul P, Mayda E, Vera P** (2005). The H2O2-regulated Ep5C gene encodes a peroxidase required for bacterial speck susceptibility in tomato. Plant J 42, 283-293.
41. **Coego A, Ramirez V, Gil MJ, Flors V, Mauch-Mani B, Vera P** (2005). An Arabidopsis homeodomain transcription factor, OVEREXPRESSOR OF CATIONIC PEROXIDASE 3, mediates resistance to infection by necrotrophic pathogens. Plant Cell 17, 2123-2137.
42. **Coll NS, Epple P, Dangl JL.** (2011) Programmed cell death in the plant immune system. Cell Death Differ. 18 (8), 1247-56.
43. **Covello PS, Gray MW.** (1989) RNA editing in plant mitochondria. Nature. 341 (6243), 662-6.
44. **Darvill A G and Albersheim P.** (1984) Phytoalexins and their elicitors—a defense against microbial infection in plants. Annu. Rev. Plant Physiol. 35, 243-75.
45. **Devoto A, Nieto-Rostro M, Xie D, Ellis C, Harmston R, Patrick E, Davis J, Sherratt L, Coleman M, Turner JG** (2002) COI1 links jasmonate signalling and fertility to the SCF ubiquitin–ligase complex in Arabidopsis. Plant J 32, 457-466.
46. **Eggert D., Naumann M., Reimer R., Voigt C.A**. (2014) Nanoscale glucan polymer network causes pathogen resistance. Sci Rep. 24 (4), 4159

47. **Endo T., Shikanai T., Takabayashi A., Asada K. and Sato F.** (1999) The role of chloroplastic NAD(P)H dehydrogenase in photoprotection. FEBS Letters 457, 5-8
48. **Estavillo GM, Crisp PA, Pornsiriwong W, Wirtz M, Collinge D, Carrie C, Giraud E, Whelan J, David P, Javot H, Brearley C, Hell R, Marin E, Pogson BJ.** (2011) Evidence for a SAL1-PAP chloroplast retrograde pathway that functions in drought and high light signaling in Arabidopsis. Plant Cell. 23 (11), 3992-4012.
49. **Fan, J., Hill, L., Crooks, C., Doerner, P. and Lamb, C.** (2009) Abscisic Acid Has a Key Role in Modulating Diverse Plant-Pathogen Interactions. Plant Physiol, 150, 1750-1761.
50. **Fernández AP, Strand A.** (2008) Retrograde signaling and plant stress: plastid signals initiate cellular stress responses. Curr Opin Plant Biol. 11 (5), 509-13.
51. **Fernández-Calvo P, Chini A, Fernández-Barbero G, Chico JM, Gimenez-Ibanez S, Geerinck J, Eeckhout D, Schweizer F, Godoy M, Franco-Zorrilla JM, Pauwels L, Witters E, Puga MI, Paz-Ares J, Goossens A, Reymond P, De Jaeger G, Solano R.** (2011) The Arabidopsis bHLH transcription factors MYC3 and MYC4 are targets of JAZ repressors and act additively with MYC2 in the activation of jasmonate responses. Plant Cell. 23 (2), 701-15.
52. **Flors, V., Ton, J., van Doorn, R., Jakab, G., Garcia-Agustin, P. and Mauch-Mani, B.** (2008) Interplay between JA, SA and ABA signalling during basal and induced resistance against Pseudomonas syringae and Alternaria brassicicola. Plant J, 54, 81-92.
53. **Foyer CH, Noctor G.** (2005). Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. Plant, Cell and Environment 28, 1056–1071.
54. **Francis SA, Dewey FM, Gurr SJ** (1996) The role of the cutinase in germling develment and infection by Erysiphe graminis f.sp. hordei. Physiological and Molecular Plant Pathology 49, 201-211
55. **Frenkel M, Külheim C, Jänkänpää HJ, Skogström O, Dall'Osto L, Agren J, Bassi R, Moritz T, Moen J, Jansson S.** (2009) Improper excess light energy dissipation in Arabidopsis results in a metabolic reprogramming. BMC Plant Biol. 26, 9-12.

56. **Friesen T.L., Faris J.D., Solomon P.S., Oliver R.P.** (2008) Host-specific toxins: effectors of necrotrophic pathogenicity. Cell Microbiol. 10 (7), 1421-8
57. **Fryer MJ, Ball L, Oxborough K, Karpinski S, Mullineaux PM, Baker NR.** (2003) Control of Ascorbate Peroxidase 2 expression by hydrogen peroxide and leaf water status during excess light stress reveals a functional organisation of Arabidopsis leaves. Plant J. 33 (4), 691-705.
58. **Fu ZQ, Yan SP, Saleh A, Wang W, Ruble J, Oka N, Mohan R, Spoel SH, Tada Y, Zheng N et al.** (2012) NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. Nature 486, 228-232.
59. **Fujii H, Chinnusamy V, Rodrigues A, Rubio S, Antoni R, Park SY, Cutler SR, Sheen J, Rodriguez PL, Zhu JK.** (2009) In vitro reconstitution of an abscisic acid signalling pathway. Nature 462, 660-664.
60. **Fujita M, Fujita Y, Noutoshi Y, Takahashi F, Narusaka Y, Yamaguchi-Shinozaki K, Shinozaki K** (2006) Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. Curr Opin Plant Biol 9, 436–442
61. **Furihata T, Maruyama K, Fujita Y, Umezawa T, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K.** (2006) Abscisic acid-dependent multisite phosphorylation regulates the activity of a transcription activator AREB1. Proc Natl Acad Sci USA 103, 1988-1993.
62. **García-Andrade J, Ramírez V, Flors V, Vera P** (2011) Arabidopsis ocp3 mutant reveals a mechanism linking ABA and JA to pathogen-induced callose deposition. Plant J 67, 783–794
63. **Gil MJ, Coego A, Mauch-Mani B, Jordá L, Vera P.** (2005) The Arabidopsis csb3 mutant reveals a regulatory link between salicylic acid-mediated disease resistance and the methyl-erythritol 4-phosphate pathway. Plant J. 44 (1), 155-66.
64. **Gilbert RD, Johnson AM, Dean RA.** (1996) Chemical signals response for appressorium formation in the rice blast fungus Magnaporthe grisea. Physiological and Molecular Plant Pathology 48, 335-346
65. **Glazebrook J.** (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu Rev Phytopathol. 43, 205-27

66. **Golding A.J. and Johnson G.N.** (2004) Down-regulation of linear and activation of cyclic electron transport during drought. Planta 218: 107-114 Erratum in: Planta. 218, 682
67. **Gonzalez-Guzman M, Pizzio GA, Antoni R, Vera-Sirera F, Merilo E, Bassel GW, Fernández MA, Holdsworth MJ, Perez-Amador MA, Kollist H, Rodriguez PL**. (2012) Arabidopsis PYR/PYL/RCAR receptors play a major role in quantitative regulation of stomatal aperture and transcriptional response to abscisic acid. Plant Cell 24, 2483-2496
68. **Grant, M.R. and Jones, J.D.** (2009) Hormone (dis)harmony moulds plant health and disease. Science 324, 750–752
69. **Gray MW, Covello PS**. (1993) RNA editing in plant mitochondria and chloroplasts. FASEB J. 7 (1), 64-71.
70. **Gualberto JM, Lamattina L, Bonnard G, Weil JH, Grienenberger JM.** (1989) RNA editing in wheat mitochondria results in the conservation of protein sequences. Nature. 341 (6243), 660-2.
71. **Halliwell B, Gutteridge JMC.** (2007). Free radicals in biology and medicine. Oxford University Press
72. **Halliwell B.** (2006). Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiology 141, 312–322.
73. **Hammani K, Okuda K, Tanz SK, Chateigner-Boutin AL, Shikanai T, Small I.** (2009) A study of new Arabidopsis chloroplast RNA editing mutants reveals general features of editing factors and their target sites. Plant Cell. 21 (11), 3686-99.
74. **Hashimoto M, Endo T, Peltier G, Tasaka M, Shikanai T.** (2003) A nucleus-encoded factor, CRR2, is essential for the expression of chloroplast ndhB in Arabidopsis. Plant J. 36 (4), 541-9.
75. **Heber U. and Walker D.** (1992) Concerning a dual function of coupled cyclic electron transport in leaves. Plant Physiol 100, 1621-1626
76. **Hématy, K., Cherk, C. and Somerville, S.** (2009) Host-pathogen warfare at the plant cell wall. Current Opinion in Plant Biology, 12, 406-413.
77. **Hertle AP, Blunder T, Wunder T, Pesaresi P, Pribil M, Armbruster U, Leister D**. (2013) PGRL1 is the elusive ferredoxin-plastoquinone reductase in photosynthetic cyclic electron flow. Mol Cell. 7; 49 (3), 511-23.

78. **Hiesel R, Wissinger B, Schuster W, Brennicke A.** (1989) RNA editing in plant mitochondria. Science. 246 (4937), 1632-4.
79. **Hobe, S., Forster, R., Klingler, J., and Paulsen, H.** (1995) N-proximal sequence motif in light-harvesting chlorophyll a/b-binding protein is essential for the trimerization of light-harvesting chlorophyll a/b complex. Biochemistry 34, 10224-10228
80. **Howard R.J., Valent B.** (1996) Breaking and entering: host penetration by the fungal rice blast pathogen Magnaporthe grisea. Annu Rev Microbiol 50, 491-512.
81. **Hückelhoven, R.** (2007) Cell wall-associated mechanisms of disease resistance and susceptibility. Annu Rev Phytopathol, 45, 101-127.
82. **Ifuku K, Endo T, Shikanai T, Aro EM.** (2011) Structure of the chloroplast NADH dehydrogenase-like complex: nomenclature for nuclear-encoded subunits. Plant Cell Physiol. 52 (9), 1560-8.
83. **Inzé A, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, Gollery M, Shulaev V, van Breusegem F.** (2012). A subcellular localization compendium of hydrogen peroxide-induced proteins. Plant, Cell and Environment 35, 308–320.
84. **Jacobs, A.K., Lipka, V., Burton, R.A., Panstruga, R., Strizhov, N., Schulze-Lefert, P. and Fincher, G.B.** (2003) An Arabidopsis Callose Synthase, GSL5, Is Required for Wound and Papillary Callose Formation. Plant Cell, 15, 2503-2513.
85. **Jensen MK, Hagedorn PH, de Torres-Zabala M, Grant MR, Rung JH, Collinge DB, Lyngkjaer MF.** (2008) Transcriptional regulation by an NAC (NAM-ATAF1,2-CUC2) transcription factor attenuates ABA signalling for efficient basal defence towards Blumeria graminis f. sp. hordei in Arabidopsis. Plant J. 56 (6), 867-80
86. **Johnson RR, Wagner RL, Verhey SD, Walker-Simmons MK.** (2002) The abscisic acid-responsive kinase PKABA1 interacts with a seed-specific abscisic acid response element-binding factor, TaABF, and phosphorylates TaABF peptide sequences. Plant Physiol 130, 837-846.
87. **Joliot P. and Joliot A.** (2002) Cyclic electron transfer in plant leaf. PNAS-USA 99: 10209-10214

88. **Jones JD, Dangl JL**. (2006) The plant immune system. Nature. 16; 444 (7117), 323-9
89. **Kaliff M, Staal J, Myrenås M, Dixelius C.** (2007) ABA is required for Leptosphaeria maculans resistance via ABI1- and ABI4-dependent signaling. Mol Plant Microbe Interact, 20, 335-345.
90. **Karpiński S, Szechyńska-Hebda M, Wituszyńska W, Burdiak P.** (2013) Light acclimation, retrograde signalling, cell death and immune defences in plants. Plant Cell Environ. 36 (4), 736-44.
91. **Kazan, K. and Manners, J.M.** (2011) The interplay between light and jasmonate signalling during defence and development. J. Exp. Bot. 62, 4087–4100
92. **Kieselbach, T., Hagman, Andersson, B., and Schroder, W.P.** (1998) The thylakoid lumen of chloroplasts. Isolation and characterization. J Biol Chem 273, 6710-6716
93. **Kim C, Meskauskiene R, Zhang S, Lee KP, Lakshmanan Ashok M, Blajecka K, Herrfurth C, Feussner I, Apel K.** (2012) Chloroplasts of Arabidopsis are the source and a primary target of a plant-specific programmed cell death signaling pathway. Plant Cell. 24 (7), 3026-39.
94. **Kofer W., Koop H.-U., Wanner G. and Steinmüller K.** (1998) Mutagenesis of the genes encoding subunits A, C, H, I, J and K of the plastid NAD(P)H-plastoquinone-oxidoreductase in tobacco by polyethylene glycol-mediated plastome transformation. Mol Gen Genet 258, 166-173
95. **Kotera E, Tasaka M, Shikanai T.** (2005) A pentatricopeptide repeat protein is essential for RNA editing in chloroplasts. Nature. 433 (7023), 326-30.
96. **Kunze G, Zipfel C, Robatzek S, Niehaus K, Boller T, Felix G.** (2004) The N terminus of bacterial elongation factor Tu elicits innate immunity in Arabidopsis plants. Plant Cell. 16(12), 3496-507
97. **Kuttkat, A., Hartmann, A., Hobe, S., and Paulsen, H.** (1996) The C-terminal domain of light-harvesting chlorophyll-a/b-binding protein is involved in the stabilisation of trimeric light-harvesting complex. Eur. J. Biochem. 242, 288-292
98. **L'Haridon F, Besson-Bard A, Binda M, Serrano M, Abou-Mansour E, Balet F, Schoonbeek HJ, Hess S, Mir R, Léon J, Lamotte O, Métraux JP**. (2011) A permeable cuticle is

associated with the release of reactive oxygen species and induction of innate immunity. PLoS Pathog. 7 (7), e1002148.
99. **Laisk A., Eichelmann H., Oja V. and Peterson R. B.** (2005) Control of cytochrome b6/f at low and high light intensity and cyclic electron transport in leaves. Biochim Biophys Acta 1708, 79-90
100. **Laluk, K., & Mengiste, T**. (2010). Necrotroph Attacks on Plants: Wanton Destruction or Covert Extortion? The Arabidopsis Book / American Society of Plant Biologists, 8, e0136.
101. **López, A., Ramírez, V., García-Andrade, J., Flors, V. and Vera, P.** (2011) The RNA silencing enzyme RNA Polymerase V is required for plant immunity. PLoS Genet. 7, e1002434.
102. **Luna E, Bruce TJ, Roberts MR, Flors V, Ton J.** (2012) Next-generation systemic acquired resistance. Plant Physiol. 158 (2), 844-53.
103. **Luna E, Pastor V, Robert J, Flors V, Mauch-Mani B, Ton J** (2011) Callose deposition: a multifaceted plant defense response. Mol Plant Microbe Interact 24, 183–193
104. **Luna E, Ton J.** (2012) The epigenetic machinery controlling transgenerational systemic acquired resistance. Plant Signal Behav. 7 (6), 615-8.
105. **Lurin C, Andrés C, Aubourg S, Bellaoui M, Bitton F, Bruyère C, Caboche M, Debast C, Gualberto J, Hoffmann B, Lecharny A, Le Ret M, Martin-Magniette ML, Mireau H, Peeters N, Renou JP, Szurek B, Taconnat L, Small I.** (2004) Genome-wide analysis of Arabidopsis pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. Plant Cell. 16 (8), 2089-103.
106. **Mandaokar A, Thines B, Shin B, Lange BM, Choi G, Koo YJ, Yoo YJ, Choi YD, Browse J** (2006) Transcriptional regulators of stamen development in Arabidopsis identified by transcriptional profiling. Plant J 46, 984-1008.
107. **Mateo A, Mühlenbock P, Rustérucci C, Chang C, Miszalski Z, Karpinska B, Parker JE, Mullineaux PM and Karpinski S** (2004) LESION SIMULATING DISEASE 1 is required for acclimation to conditions that promote excess excitation energy. Plant Physiol. 136, 2818–2830
108. **Mauch-Mani B, Mauch F.** (2005) The role of abscisic acid in plant-pathogen interactions. Curr Opin Plant Biol. 8 (4), 409-14

109. **Melcher K, Ng LM, Zhou XE, Soon FF, Xu Y, Suino-Powell KM, Park SY, Weiner JJ, Fujii H, Chinnusamy V, Kovach A, Li J, Wang Y, Li J, Peterson FC, Jensen DR, Yong EL, Volkman BF, Cutler SR, Zhu JK, Xu HE.** (2009) A gate-latch-lock mechanism for hormone signalling by abscisic acid receptors. Nature 462, 602-608.
110. **Melcher K, Xu Y, Ng LM, Zhou XE, Soon FF, Chinnusamy V, Suino-Powell KM, Kovach A, Tham FS, Cutler SR, Li J, Yong EL, Zhu JK, Xu HE.** (2010) Identification and mechanism of ABA receptor antagonism. Nat Struct Mol Biol 17, 1102-1108.
111. **Merchant S, Sawaya MR**. (2005) The light reactions: a guide to recent acquisitions for the picture gallery. Plant Cell. 17 (3), 648-63.
112. **Mi H., Endo T., Schreiber U. and Asada K.** (1992) Donation of electrons from cytosolic components to the intersystem chain in the cyanobacterium Synechococcus sp. 7002 as determined by the reduction of P700+. Plant Cell Physiol 33, 1099-1105
113. **Mittler R, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, Gollery M, Shulaev V, van Breusegem F.** (2011). ROS signaling: the new wave? Trends in Plant Science 16, 300–309.
114. **Miyake C., Horiguchi S., Makino A., Shinzaki Y., Yamamoto H. and Tomizawa K**. (2005) Effects of light intensity on cyclic electron flow around PSI and its relationship to nonphotochemical quenching of Chl fluorescence in tobacco leaves. Plant Cell Physiol 46: 1819-1830
115. **Miyazono K, Miyakawa T, Sawano Y, Kubota K, Kang HJ, Asano A, Miyauchi Y, Takahashi M, Zhi Y, Fujita Y, Yoshida T, Kodaira KS, Yamaguchi-Shinozaki K, Tanokura M.** (2009) Structural basis of abscisic acid signalling. Nature 462, 609-614.
116. **Mou Z, Fan WH, Dong XN** (2003) Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell 113, 935-944.
117. **Mühlenbock P, Plaszczyca M, Plaszczyca M, Mellerowicz E, Karpinski S**. (2007) Lysigenous aerenchyma formation in Arabidopsis is controlled by LESION SIMULATING DISEASE1. Plant Cell. 19 (11), 3819-30.

118. **Mühlenbock P, Szechyńska-Hebdaa M, Płaszczycaa M, Baudoa M, Mateoa A, Mullineauxb PM, Parkerc JE, Karpińskad B, and Karpiński S** (2008) Chloroplast signaling and LESION SIMULATING DISEASE1 regulate crosstalk between light acclimation and immunity in Arabidopsis. Plant Cell 20, 2339–2356
119. **Mullineaux PM, Baker NR.** (2010) Oxidative stress: antagonistic signaling for acclimation or cell death? Plant Physiol. 154 (2), 521-5.
120. **Munekage Y, Hashimoto M, Miyake C, Tomizawa K, Endo T, Tasaka M, Shikanai T.** (2004) Cyclic electron flow around photosystem I is essential for photosynthesis. Nature. 3; 429 (6991), 579-82.
121. **Munekage Y., Hojo M., Meurer J., Endo T., Tasaka M. and Shikanai T.** (2002) PGR5 is involved in cyclic electron flow around photosystem I and is essential for photoprotection in Arabidopsis. Cell 110: 361-371
122. **Mustilli AC, Merlot S, Vavasseur A, Fenzi F, Giraudat J.** (2002) Arabidopsis OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. Plant Cell 14, 3089-3099.
123. **Nandha B., Finazzi G., Joliot P., Haid S. and Johnson G.** (2007) The role of PGR5 in the redox poising of photosynthetic electron transport. Biochim Biophys Acta 1767:1252-1259
124. **Nawrath C.** (2006) Unraveling the complex network of cuticular structure and function. Curr Opin Plant Biol 9, 281-287
125. **Nishimura, M.T., Stein, M., Hou, B.H., Vogel, J.P., Edwards, H. and Somerville, S.C.** (2003) Loss of a callose synthase results in salicylic acid-dependent disease resistance. Science, 301, 969-972.
126. **O'Brien JA, Daudi A, Butt VS, Bolwell GP.** (2012) Reactive oxygen species and their role in plant defence and cell wall metabolism. Planta. 236 (3), 765-79.
127. **Oide S, Bejai S, Staal J, Guan N, Kaliff M, Dixelius C.** (2013) A novel role of PR2 in abscisic acid (ABA) mediated, pathogen-induced callose deposition in Arabidopsis thaliana. New Phytol. 200(4), 1187-99
128. **Okuda K, Chateigner-Boutin AL, Nakamura T, Delannoy E, Sugita M, Myouga F, Motohashi R, Shinozaki**

K, Small I, Shikanai T. (2009) Pentatricopeptide repeat proteins with the DYW motif have distinct molecular functions in RNA editing and RNA cleavage in Arabidopsis chloroplasts. Plant Cell. 21 (1), 146-56.

129. **Okuda K, Myouga F, Motohashi R, Shinozaki K, Shikanai T** (2007) Conserved domain structure of pentatricopeptide repeat proteins involved in chloroplast RNA editing. Proc Natl Acad Sci U S A. 104 (19), 8178-83.
130. **Orozco-Cárdenas ML1, Narváez-Vásquez J, Ryan CA**. (2001) Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. Plant Cell. 13 (1), 179-91.
131. **Osmond C.B.** (1981) Photorespiration and photoinhibition: Some implications for the energetics of photosynthesis. Biochim Biophys Acta 639, 77-98
132. **Ospina-Giraldo M.D., Mullins E., Kang S.** (2003) Loss of function of the Fusarium oxysporum SNF1 gene reduces virulence on cabbage and Arabidopsis. Curr Genet 44 (1), 49-57
133. **Park S-Y, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J, Rodrigues A, Chow TF, et al** (2009) Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. Science 324, 1068–1071
134. **Pauwels L1, Barbero GF, Geerinck J, Tilleman S, Grunewald W, Pérez AC, Chico JM, Bossche RV, Sewell J, Gil E, García-Casado G, Witters E, Inzé D, Long JA, De Jaeger G, Solano R, Goossens A**. (2010) NINJA connects the co-repressor TOPLESS to jasmonate signalling. Nature 464, 788–791
135. **Payankaulam, S. et al.** (2010) Transcriptional repression: conserved and evolved features. Curr. Biol. 20, R764–R771
136. **Peltier, J.B., Friso, G., Kalume, D.E., Roepstorff, P., Nilsson, F., Adamska, I., and van Wijk, K. J.** (2000) Proteomics of the chloroplast: systematic identification and targeting analysis of lumenal and peripheral thylakoid proteins. Plant Cell 12, 319-341
137. **Pfalz J, Bayraktar OA, Prikryl J, Barkan A.** (2009) Site-specific binding of a PPR protein defines and stabilizes 5' and 3' mRNA termini in chloroplasts. EMBO J. 28 (14), 2042-52.

138. **Pieterse CM, Leon-Reyes A, Van der Ent S, Van Wees SC.** (2009) Networking by small-molecule hormones in plant immunity. Nat Chem Biol. 5(5), 308-16
139. **Qi T., Song S., Ren Q., Wu D., Huang H., Chen Y., Fan M., Peng W., Ren C., Xie D.** (2011) The Jasmonate-ZIM-domain proteins interact with the WD-Repeat/bHLH/MYB complexes to regulate jasmonatemediated anthocyanin accumulation and trichome initiation in Arabidopsis thaliana. Plant Cell 23, 1795–1814
140. **Ramel F, Birtic S, Ginies C, Soubigou-Taconnat L, Triantaphylidès C, Havaux M.** (2012) Carotenoid oxidation products are stress signals that mediate gene responses to singlet oxygen in plants. Proc Natl Acad Sci U S A. 3; 109 (14), 5535-40.
141. **Ramírez R, Van der Ent S, García-Andrade J, Coego A, Pieterse CMJ, Vera P** (2010). OCP3 is an important modulator of NPR1-mediated jasmonic acid-dependent induced defenses in Arabidopsis. BMC Plant Biol 10, 199.
142. **Ramírez V, Coego A, López A, Agorio A, Flors V, Vera P.** (2009) Drought tolerance in Arabidopsis is controlled by the OCP3 disease resistance regulator. Plant J. 58 (4), 578-91.
143. **Ramos B., González-Melendi P., Sánchez-Vallet A., Sánchez-Rodríguez C., López G., Molina A.** (2013) Functional genomics tools to decipher the pathogenicity mechanisms of the necrotrophic fungus Plectosphaerella cucumerina in Arabidopsis thaliana. Mol Plant Pathol. 14 (1), 44-57
144. **Robert A. Cramer, Christopher B. Lawrence,** (2004) Identification of Alternaria brassicicola genes expressed in planta during pathogenesis of Arabidopsis thaliana. Fungal Genetics and Biology, 41 (2), 115-128
145. **Robert-Seilaniantz A1, Grant M, Jones JD** (2011) Hormone crosstalk in plant disease and defense: More than just JASMONATE-SALICYLATE antagonism. Annu. Rev. Phytopathol. 49, 317–343
146. **Roberts, K.** (1990) Structures at the plant cell surface. Curr Opin Cell Biol, 2, 920-928.
147. **Rossel JB, Wilson PB, Hussain D, Woo NS, Gordon MJ, Mewett OP, Howell KA, Whelan J, Kazan K, Pogson BJ.** (2007) Systemic and intracellular responses to photooxidative stress in Arabidopsis. Plant Cell. 19 (12), 4091-110.

148. **Ruwe H, Castandet B, Schmitz-Linneweber C, Stern DB.** (2013) Arabidopsis chloroplast quantitative editotype. FEBS Lett. 2013 May 2;587(9):1429-33.
149. **Sánchez-Vallet A, López G, Ramos B, Delgado-Cerezo M, Riviere MP, Llorente F, Fernández PV, Miedes E, Estevez JM, Grant M, Molina A.** (2012) Disruption of abscisic acid signaling constitutively activates Arabidopsis resistance to the necrotrophic fungus Plectosphaerella cucumerina. Plant Physiol. 160 (4), 2109-24
150. **Santiago J, Dupeux F, Round A, Antoni R, Park SY, Jamin M, Cutler SR, Rodriguez PL, Márquez JA.** (2009) The abscisic acid receptor receptor PYR1 in complex with abscisic acid. Nature 462, 665-668.
151. **Scheller, H.V., Jensen, P.E., Haldrup, A., Lunde, C., and Knoetzel, J.** (2001) Role of subunits in eukaryotic Photosystem I. Biochim. Biophys. Acta 1507, 41-60
152. **Schmitz-Linneweber C, Small I.** (2008) Pentatricopeptide repeat proteins: a socket set for organelle gene expression. Trends Plant Sci. 13 (12), 663-70.
153. **Schreiber U., Schliwa U. and Bilger W.** (1986) Continuous recording of photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. Photosynth Res 10, 51-62
154. **Schröder A, Fink P.** (2011) Functional somatic syndromes and somatoform disorders in special psychosomatic units: organizational aspects and evidence-based treatment. Psychiatr Clin North Am. 34 (3), 673-87.
155. **Schubert, M., Petersson, U.A., Haas, B.J., Funk, C., Schroder, W.P., and Kieselbach, T.** (2002) Proteome map of the chloroplast lumen of Arabidopsis thaliana. J. Biol. Chem. 277, 8354-8365
156. **Sharma, J., Panico, M., Barber, J., and Morris, H.R.** (1997) Primary structure characterization of the photosystem II D1 and D2 subunits. J. Biol. Chem. 272, 3935-3943
157. **Shikanai T.** (2007) Cyclic electron transport around Photosystem I: genetic approaches. Annu Rev Plant Biol 58: 199-217
158. **Shikanai T., Endo T., Hashimoto T., Yamada Y., Asada K. and Yokota A.** (1998) Directed disruption of the tobacco ndhB gene impairs cyclic electron flow around photosystem I. PNASUSA 95: 9705-9709

159. **Simpson C., Thomas C., Findlay K., Bayer E. and Maule J.A.** (2009) An Arabidopsis GPI-Anchor Plasmodesmal Neck Protein with Callose Binding Activity and Potential to Regulate Cell-to-Cell Trafficking. The Plant Cell. 21, 581–594
160. **Sugiura M., (**1992) The chloroplast genome. Plant Mol Biol. 19 (1), 149-68.
161. **Sun T, Germain A, Giloteaux L, Hammani K, Barkan A, Hanson MR, Bentolila S.** (2013) An RNA recognition motif-containing protein is required for plastid RNA editing in Arabidopsis and maize. Proc Natl Acad Sci U S A. 110 (12), E1169-78.
162. **Suorsa M, Sirpiö S, Aro EM**. (2009) Towards characterization of the chloroplast NAD(P)H dehydrogenase complex. Mol Plant. 2 (6), 1127-40.
163. **Suzuki N, Miller G, Morales J, Shulaev V, Torres MA, Mittler R.** (2011). Respiratory burst oxidases: the engines of ROS signaling. Current Opinion in Plant Biology 14, 691–699.
164. **Svensson, B., Etchebest, C., Tuffery, P., van Kan, P., Smith, J., and Styring, S.** (1996) A model for the photosystem II reaction center core including the structure of the primary donor P680. Biochemistry 35, 14486-14502
165. **Szostkiewicz I, Richter K, Kepka M, Demmel S, Ma Y, Korte A, Assaad FF, Christmann A, Grill E.** (2010) Closely related receptor complexes differ in their ABA selectivity and sensitivity.Plant J. 61 (1), 25-35.
166. **Takenaka M, Zehrmann A, Verbitskiy D, Kugelmann M, Härtel B, Brennicke A.** (2012) Multiple organellar RNA editing factor (MORF) family proteins are required for RNA editing in mitochondria and plastids of plants. Proc Natl Acad Sci U S A. 109 (13), 5104-9.
167. **Tang D, Simonich MT, Innes RW.** (2007) Mutations in LACS2, a Long-Chain Acyl-Coenzyme A Synthetase, Enhance Susceptibility to Avirulent Pseudomonas syringae But Confer Resistance to Botrytis cinerea in Arabidopsis. Plant Physiology, 144, 1093–110
168. **Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, Browse J** (2007) JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. Nature 448, 661-665.

169. **Thordal-Christensen, H., Zhang, Z., Wei, Y., and Collinge, D.B.** (1997) Subcellular localization of H2O2 in plants. H2O2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. Plant Journal, 11, 1187–1194
170. **Ton J, Flors V, Mauch-Mani B** (2009) The multifaceted role of ABA in disease resistance. Trends Plant Sci 14, 310–317
171. **Traverso JA, Pulido A, Rodríguez-García MI, Alché JD.** (2013) Thiol-based redox regulation in sexual plant reproduction: new insights and perspectives. Front Plant Sci. 14; 4, 4
172. **Triantaphylidès C, Havaux M.** (2009). Singlet oxygen in plants: production, detoxification and signalling. Trends in Plant Science 14, 219–228.
173. **Triantaphylidès C, Krischke M, Hoeberichts FA, Ksas B, Gresser G, Havaux M, Van Breusegem F, Mueller MJ.** (2008) Singlet oxygen is the major reactive oxygen species involved in photooxidative damage to plants. Plant Physiol. 148 (2), 960-8.
174. **Umezawa T, Sugiyama N, Mizoguchi M, Hayashi S, Myouga F, Yamaguchi-Shinozaki K, Ishihama Y, Hirayama T, Shinozaki K.** (2009) Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in Arabidopsis. Proc Natl Acad Sci USA 106, 17588-17593.
175. **van Kan, J.A.** (2006) Licensed to kill: the lifestyle of a necrotrophic plant pathogen. Trends Plant Sci. 11, 247–253.
176. **Varner, J.E. and Lin, L.S.,** (1988) Plant cell wall architecture. Cell, 56 231
177. Vorwerk, S., Somerville, S. and Somerville, C. (2004) The role of plant cell wall polysaccharide composition in disease resistance. Trends Plant Sci, 9, 203-209.
178. **Wang D, Amornsiripanitch N, Dong XN** (206) A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. PLoS Pathog 2, 1042-1050.
179. **Wasilewska A, Vlad F, Sirichandra C, Redko Y, Jammes F, Valon C, Frei dit Frey N, Leung J** (2008) An update on abscisic acid signaling in plants and more.... Mol Plant 1, 198–217

180. **Wasternack C** (2007) Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. Ann Bot (Lond) 100, 681-697.
181. **Williamson B., Tudzynski B., Tudzynski P., van Kan J.A.** (2007) Botrytis cinerea: the cause of grey mould disease. Mol Plant Pathol 8 (5), 561-80.
182. **Xiao F, Goodwin SM, Xiao Y, Sun Z, Baker D, Tang X, Jenks MA, Zhou JM.** (2004) Arabidopsis CYP86A2 represses Pseudomonas syringae type III genes and is required for cuticle development. EMBO J. 23, 2903–291
183. **Xu L, Liu F, Lechner E, Genschik P, Crosby WL, Ma H, Peng W, Huang D, Xie D** (2002) The SCF(COI1) ubiquitin–ligase complexes are required for jasmonate response in Arabidopsis. Plant Cell 14, 1919-1935.
184. **Yan S. and Dong X.** (2014) Perception of the plant immune signal salicylic acid. Curr Opin Plant Biol. 20, 64-8Yang J, Tian L, Sun MX, Huang XY, Zhu J, Guan YF, Jia QS, Yang ZN (2013) AUXIN RESPONSE FACTOR17 is essential for pollen wall pattern formation in Arabidopsis. Plant Physiol. 162(2), 720-31
185. **Yang J, Tian L, Sun MX, Huang XY, Zhu J, Guan YF, Jia QS, Yang ZN** (2013) AUXIN RESPONSE FACTOR17 is essential for pollen wall pattern formation in Arabidopsis. Plant Physiol. 162(2), 720-31
186. **Yin P, Fan H, Hao Q, Yuan X, Wu D, Pang Y, Yan C, Li W, Wang J, Yan N**. (2009) Structural insights into the mechanism of abscisic acid signaling by PYL proteins. Nat Struct Mol Biol 16, 1230-1236.
187. **Zavaliev R, Ueki S, Epel BL, Citovsky V.** (2011) Biology of callose (β-1,3-glucan) turnover at plasmodesmata. Protoplasma. 248 (1), 117-30
188. **Zhang Y, Fan W, Kinkema M, Li X, Dong X** (1999) Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR-1 gene. Proc Natl Acad Sci USA 96, 6523-6528.
189. **Zhou JM, Trifa Y, Silva H, Pontier D, Lam E, Shah J, Klessig DF** (2000) NPR1 differentially interacts with members of the TGA/OBF family of transcription factors that bind an element of the PR-1 gene required for induction by salicylic acid. Mol Plant Microbe Interact 13, 191-202.

190. **Zhu Q, Dugardeyn J, Zhang C, Takenaka M, Kühn K, Craddock C, Smalle J, Karampelias M, Denecke J, Peters J, Gerats T, Brennicke A, Eastmond P, Meyer EH, Van Der Straeten D.** (2012) SLO2, a mitochondrial pentatricopeptide repeat protein affecting several RNA editing sites, is required for energy metabolism. Plant J. 71 (5), 836-49.