UNIVERSIDAD POLITECNICA DE VALENCIA

Departamento de Biotecnología

Ruta de transducción de señal del ácido abscísico: Regulación por HAB1 y dianas de interacción. La inactivación combinada de PP2Cs como herramienta biotecnológica para incrementar la tolerancia a sequía en plantas.

Memoria presentada por:

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DOCTOR

Director

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A José María y Adrián.
A mis padres y a mi hermana.

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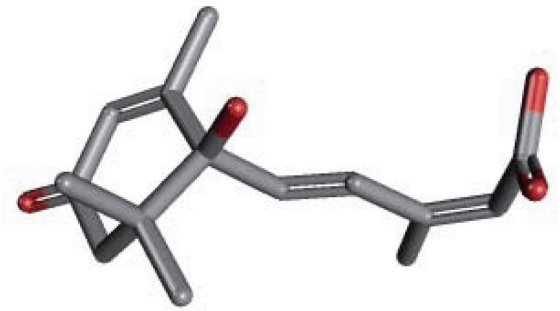

Resumen,

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Abstract

El ácido abscísico (ABA) es una hormonal vegetal que tiene un papel crucial en las respuestas adaptativas de las plantas al estrés por sequía, salinidad o frío, además de regular importantes procesos del desarrollo de las plantas como el desarrollo del embrión, dormición, germinación, crecimiento vegetativo, organogénesis y floración. En el campo de la señalización por ABA existen múltiples evidencias de que las proteínas fosfatasas 2C (PP2Cs) son claves para comprender los procesos en los que media esta hormona. Entre estas PP2Cs se encuentran las fosfatasas de la especie modelo *Arabidopsis thaliana* ABI1 y ABI2 (ABA-INSENSITIVE) que actúan como reguladores negativos de la ruta de transducción de señal de ABA. Nuestro trabajo se centra en la PP2C HAB1 (HYPERSENSITIVE TO ABA) cuyo secuencia genómica fue clonada por homología con *ABI1 y ABI2.*

Para el estudio y caracterización de esta nueva PP2C, comprobamos que HAB1 se expresa de forma ubicua en sitios importantes de la acción del ABA como células oclusivas o semillas y que su expresión es inducible por ABA. Realizamos un abordaje de genética reversa, novedoso en el campo de la señalización por ABA, con el aislamiento y caracterización de un alelo de pérdida de función *hab1-1* y, con la generación y el estudio de líneas que sobre expresan HAB1. La hipersensibilidad del mutante *hab1-1* y la insensibilidad de las plantas *35S:HAB1* proporcionan una nueva evidencia genética del papel de la PP2C HAB1 como regulador negativo de la señalización por ABA.

Con la intención de conocer y profundizar más en los detalles moleculares de la función de HAB1 en la ruta de señalización por ABA, realizamos una búsqueda por doble híbrido de las posibles dianas de interacción de HAB1 en una librería de cDNA de Arabidopsis. HAB1 interacciona con la proteína SWI3B, un homólogo de la subunidad SWI3B del complejo remodelador de cromatina SWI/SNF de levaduras. La interacción mapea en la mitad del dominio N-terminal de AtSWI3B, es específica para HAB1 y requiere un dominio catalítico intacto de la fosfatasa.

Confirmamos la interacción de HAB1 y SWI3B en núcleo mediante ensayos de interacción proteína-proteína por complementación bimolecular de la fluorescencia (BiFC) en *Nicotiana benthamiana* y, en ensayos de coinmunoprecipitación. Mutantes *swi3b* muestran una reducida sensibilidad a ABA en ensayos de inhibición de germinación y crecimiento, asi como una

reducida expresión de los genes de respuesta a ABA *RAB18* y *RD29B*. Experimentos de inmunoprecipitación de cromatina (ChIP) demuestran que la presencia de HAB1 en los promotores de los genes marcadores *RAB18* y *RD29B* es suprimida por la presencia de ABA, lo que involucra directamente a HAB1 en la regulación de la transcripción en respuesta a ABA. Adicionalmente, estos resultados muestran el papel de SWI3B como un nuevo regulador positivo de la señalización por ABA, modulando HAB1 la respuesta a ABA a través de un supuesto complejo remodelador de cromatina del tipo SWI/SNF.

Debido a que la familia de las PP2Cs del grupo A, HAB1, ABI1, ABI2 y PP2CA actúan como reguladores negativos claves en la ruta de señalización por ABA, aislamos y caracterizamos mutantes sencillos para generar posteriormente diferentes combinaciones de mutantes dobles y triples de pérdida de función en esas PP2Cs. Estudios previos a esta tesis no habían analizado mutantes de pérdida de función sencillos, dobles y triples en PP2Cs de plantas. El objetivo es determinar su contribución a la ruta de señalización por ABA y desentrañar posibles interacciones génicas y una posible redundancia funcional entre ellas. La comparación de las respuestas a ABA en diferente mutantes en *pp2c*s muestra un incremento progresivo de la sensibilidad a ABA obtenido a través de la inactivación combinada de esas PP2Cs. Estos resultados indican que la respuesta a ABA está regulada sutilmente por la acción integrada de estos genes, la cuál se requiere para prevenir una respuesta constitutiva al ABA endógeno que podría tener efectos deletéreos en el crecimiento y desarrollo en ausencia de estrés ambiental. El ácido abscísico tiene un papel esencial en la respuesta a la sequía. A pesar de los numerosos mutantes hipersensibles a ABA descritos, pocos de ellos muestran tolerancia a sequía.

En esta tesis hemos generado mutantes hipersensibles a ABA tolerantes a la sequía por inactivación combinada de las PP2Cs HAB1 y ABI1. Los dobles mutantes *hab1-1abi1-2* y *hab1-1abi1-3* presentan un reforzamiento de la respuesta a ABA tanto en semilla como en tejido vegetativo, son especialmente sensibles a la inhibición de la germinación de la semilla mediada por ABA y, muestran hipersensibilidad en ensayos de crecimiento, cierre estomatal e inducción de genes de respuesta a ABA en comparación con los mutantes sencillos. En experimentos de pérdida de agua por transpiración en

condiciones de sequía *hab1-1abi1-2* y *hab1-1abi1-3* muestran una reducción notable en la pérdida de agua respecto a los mutantes parentales sencillos. Estos resultados muestran que la inactivación combinada de PP2Cs específicas involucradas en la señalización por ABA puede ser una herramienta biotecnológica en la mejora de cultivos tolerantes a la sequía.

L'àcid abscísic (ABA) és una hormonal vegetal que té un paper crucial en les respostes adaptatives de les plantes a l'estrès per sequera, salinitat o fred, a més de regular importants processos del desenvolupament de les plantes com el desenvolupament de l'embrió, dormició, germinació, creixement vegetatiu, organogènesi i floració. En el camp de la senyalització per ABA existeixen múltiples evidències que les proteïnes fosfatases 2C (PP2Cs) són claus per comprendre els processos en els que hi ha aquesta hormona. Entre aquestes PP2Cs es troben les fosfatases de l'espècie model *Arabidopsis thaliana* ABI1 i ABI2 (ABA-insensitive) que actuen com a reguladors negatius de la ruta de transducció de senyal de ABA. El nostre treball es centra en la PP2C HAB1 (HYPERSENSITIVE TO ABA) la seqüència genòmica va ser clonada per homologia amb *ABI1* i *ABI2.* Per a l'estudi i caracterització d'aquesta nova PP2C, comprovem que HAB1 s'expressa de manera ubiqua en llocs importants de l'acció del ABA com cèl·lules oclusives o llavors i que la seva expressió és induïble per ABA. Realitzem un abordatge de genètica inversa, nou en el camp de la senyalització per ABA, amb l'aïllament i caracterització un al·lel de pèrdua de funció *hab1-1* i, amb la generació i l'estudi de línies que sobre expressen HAB1. La hipersensibilitat del mutant *hab1-1* i la insensibilitat de les plantes *35S: HAB1* proporcionen una nova evidència genètica del paper de la PP2C HAB1 com a regulador negatiu de la senyalització per ABA. Amb la intenció d'aprofundir més en els detalls moleculars desconeguts de la funció de HAB1 en la ruta de senyalització per ABA, fem una recerca per doble híbrid de les possibles dianes d'interacció d'HAB1 en una llibreria de cDNA d'Arabidopsis. HAB1 interacciona amb la proteïna SWI3B, un homòleg de la subunitat SWI3B del complex remodelat de cromatina SWI / SNF de llevats. La interacció mapeja en la meitat del domini N-terminal de AtSWI3B, és específica per HAB1 i requereix un domini catalític intacte de la fosfatasa.

Confirmem la interacció de HAB1 i SWI3B en nucli mitjançant assajos d'interacció proteïna-proteïna per complementació bimolecular de la fluorescència (BiFC) a *Nicotiana benthamiana* i, en assaigs de coinmunoprecipitación. Mutants swi3b mostren una reduïda sensibilitat a ABA en assaigs d'inhibició de germinació i creixement, així com una reduïda

expressió dels gens de resposta a ABA *RAB18* i *RD29B*. Experiments de immunoprecipitació de cromatina (ChIP) demostren que la presència de HAB1 en els promotors dels gens marcadors *RAB18* i *RD29B* és suprimida per la presència de ABA, el que involucra directament a HAB1 en la regulació de la transcripció en resposta a ABA. Addicionalment, aquests resultats mostren el paper de SWI3B com un nou regulador positiu de la senyalització per ABA, modulant HAB1 la resposta a ABA a través d'un supòsit complex remodelat de cromatina del tipus SWI / SNF.

Com que la família de les PP2Cs del grup A, HAB1, ABI1, ABI2 i PP2CA actuen com a reguladors negatius claus en la ruta de senyalització per ABA, aïllem i caracteritzem mutants senzills per generar posteriorment diferents combinacions de mutants dobles i triples de pèrdua de funció en aquestes PP2Cs. Estudis previs a aquesta tesi no havien analitzat mutants de pèrdua de funció senzills, dobles i triples en PP2Cs de plantes. L'objectiu és determinar la seva contribució a la ruta de senyalització per ABA i desentranyar possibles interaccions gèniques i una possible redundància funcional entre elles. La comparació de les respostes a ABA a diferent mutants en *pp2cs* mostra un increment progressiu de la sensibilitat a ABA obtingut a través de la inactivació combinada d'aquestes PP2Cs. Aquests resultats indiquen que la resposta a ABA està regulada subtilment per l'acció integrada d'aquests gens, la qual es requereix per prevenir una resposta constitutiva a ABA endogen que podria tenir efectes deleteris en el creixement i desenvolupament en absència d'estrès ambiental.

L'àcid abscísic té un paper essencial en la resposta a la sequera. Malgrat els nombrosos mutants hipersensibles a ABA descrits, pocs d'ells mostren tolerància a sequera. En aquesta tesi hem generat mutants hipersensibles a ABA tolerants a la sequera per inactivació combinada de les PP2Cs HAB1 i ABI1. Els dobles mutants *hab1-1abi1-2* i *hab1-1abi1-3* presenten un reforçament de la resposta a ABA tant en llavor com en teixit vegetatiu, són especialment sensibles a la inhibició de la germinació de la llavor mediada per ABA i, mostren hipersensibilitat a assaigs de creixement, tancament estomatal i inducció de gens de resposta a ABA en comparació amb els mutants senzills. En experiments de pèrdua d'aigua per transpiració en

condicions de sequera *hab1-1abi1-2* i *hab1-1abi1-3* mostren una reducció notable en la pèrdua d'aigua pel que fa als mutants parentals senzills. Aquests resultats mostren que la inactivació combinada de PP2Cs específiques involucrades en la senyalització per ABA pot ser una eina biotecnològica en la millora de cultius tolerants a la sequera.

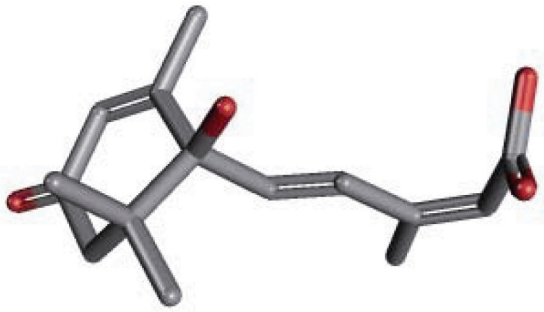
Abscisic acid (ABA) is a plant hormone that plays a crucial role in adaptive responses of plants to drought stress, salinity or cold, in addition to regulating important processes of plant development such as embryo development, dormancy, germination, vegetative growth, organogenesis and flowering. In the field of ABA signaling, there are many evidences showing that protein phosphatase 2C (PP2Cs) are key players in the processes that mediates ABA. Among PP2C phosphatases from *Arabidopsis thaliana*, the protein ABI1 and ABI2 (ABA-INSENSITIVE) act as negative regulators of signal transduction pathway of ABA. Our work focuses on the PP2C HAB1 (HYPERSENSITIVE TO ABA), whose genome sequence was cloned by homology to *ABI1* and *ABI2*. To study and characterize this new PP2C, we found that HAB1 is ubiquitously expressed at important sites of action of ABA as guard cells or seeds and its expression is inducible by ABA.

We performed a reverse genetic approach, new in the field of ABA signalling, with the isolation and characterization of the loss of function allele *hab1-1* and, with the generation and study of lines that overexpress HAB1. The hypersensitivity showed by *hab1-1* mutant and the insensitivity of plants *35S: HAB1* provide a new genetic evidence of the role of PP2C HAB1 as negative regulator of ABA signaling. In order to improve the knoledge and to go deeper into the molecular details of HAB1 function in ABA-signaling pathway, we performed a yeast two-hybrid screening for potential interaction targets of HAB1 using a cDNA library of Arabidopsis. In this screening we detected that HAB1 interacts with the protein SWI3B, a homolog of the SWI3B subunit of the chromatin remodeling complex SWI/SNF in yeast. The interaction mapped to the middle of the N-terminal domain of AtSWI3B, is HAB1 specific and requires an intact catalytic domain of the phosphatase. We confirmed the nuclear interaction between SWI3B and HAB1 by assays of protein-protein interaction of bimolecular fluorescent complementation (BIFC) in *Nicotiana benthamiana* and in coimmunoprecipitation experiments. *Swi3b* mutants show reduced sensitivity to ABA in inhibition of germination and growth assays, as well as a reduced expression of ABA-response genes *RAB18* and *RD29B.* Chromatin immunoprecipitation experiments (ChIP) demonstrate that the presence of HAB1 in the promoters of the marker genes *RAB18* and *RD29B* is suppressed by the presence of ABA, which directly involves HAB1 in the regulation of

transcription in response to ABA. Additionally, these results show the role of SWI3B as a new positive regulator of ABA signaling, being HAB1 a modulator of the response to ABA through a putative chromatin remodeling complex type SWI / SNF.

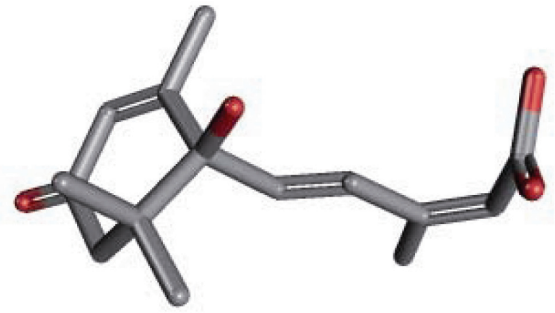
Because clade A PP2Cs family of proteins constituted by HAB1, ABI1, ABI2 and PP2CA act as key negative regulators of ABA signaling pathway, we isolated and characterized single mutants subsequently to generate different combinations of double and triple mutant null for these PP2Cs. Previous studies to this thesis have not analyzed single, double and triple knockout mutants in plant PP2Cs. The objective was to determine its contribution to the ABA signaling pathway and unravel possible interactions between genes and possible functional redundancy between PP2Cs. The comparison of responses to ABA of different *pp2cs* mutants showed a progressive increase in ABA sensitivity obtained through the combined inactivation of these PP2Cs. These results indicate that the response to ABA is finely regulated by the integrated action of these genes, which is required to prevent constitutive response to endogenous ABA which could have deleterious effects on growth and development in the absence of environmental stress. Abscisic acid has an essential role in the response to drought. Despite of the fact that it has been described many mutants that are hypersensitive to ABA, few of them show tolerance to drought.

In this thesis we have generated hypersensitive to ABA mutants that are drought tolerant by the combined inactivation of the PP2Cs HAB1 and ABI1. The double mutants *hab1-1abi1-2* and *hab1-1abi1-3* show enhanced responses to ABA in both seed and vegetative tissue, they are also particularly sensitive to inhibition of seed germination mediated by ABA showing hypersensitivity to ABA in growth stomatal closure and genes induction of ABA-response assays, compared with single mutants. In experiments of water loss through transpiration under drought *hab1-1abi1-2* and *hab1-1abi1-3* showed a significant reduction in the loss of water compared to single parental mutants. These results show that combined inactivation of specific PP2Cs involved in ABA signaling may be a biotechnological tool in the improvement of drought-tolerant crops.



Índice

	Página
Resumen	I
Resum	V
Abstract	IX
1. Introducción	1
1.1. Descubrimiento del ácido abscísico	3
1.2. Localización y movimiento del ácido abscísico	4
1.3. Transportadores del ácido abscísico	4
1.4. Características químicas de la molécula del ácido abscísico	8
1.5. Biosíntesis del ácido abscísico	10
1.6. Funciones fisiológicas del ácido abscísico	15
1.7. Cascadas de señalización del ácido abscísico	17
1.7.1. Proteínas quinasas involucradas en la señalización por ácido abscísico	18
1.7.2. Receptores del ácido abscísico	23
1.7.2.1. Primeras proteínas identificadas como receptores de ácido abscísico	23
1.7.2.2. Receptores del ácido abscísico de tipo PYR/PYL/RCAR	29
1.7.3. Papel de las proteínas fofatasas en la señalización por ácido abscísico	34
1.7.3.1. Papel de las fosfatasas de tipo 2C en la señalización por ácido abscísico	36
1.8. Expresión génica regulada por ácido abscísico	45
1.9. Elementos *cis* y *trans* de la señalización por ABA	46
2. Objetivos	49
3. Capítulo I	55
4. Capítulo II	57
5. Capítulo III	59
6. Caplítulo IV	61
7. Discusión	63
8. Conclusiones	95
9. Abreviaturas	99
10. Bibliografía	105



Introducción

1.1. Descubrimiento del ácido abscísico

El ácido abscísico (ABA) es un ácido débil de 15 carbonos, ópticamente activo que fue identificado en la década de los años 60 como un inhibidor del crecimiento que se acumulaba en el fruto del algodón en proceso de abscisión (recibiendo el nombre de abscisina II) y, en hojas de sicomoro inducidas por fotoperiodo que permanecían durmientes (denominado como dormina) [49]. El ABA se caracterizó por laboratorios que estaban interesados en el aislamiento de reguladores de crecimiento endógenos. Concretamente, el grupo de Addicott buscaba un compuesto del algodón que promoviese la abscisión de la hoja a partir de cotiledones en proceso de abscisión [177]. El compuesto obtenido, denominado abscisina II, tenía un efecto inhibidor del crecimiento del coleóptilo de avena aunque el efecto del ABA como promotor de la abscisión era un efecto indirecto producido por la inducción de la biosíntesis de etileno [28]. Por otro lado, otro grupo se interesó en la búsqueda de compuestos que promovieran la dormancia de las yemas como potenciales inhibidores del crecimiento de acción generalizada. En estos estudios aislaron un compuesto que denominaron dormina, que era un inhibidor de la germinación del embrión en maíz y que se encontraba en extractos de hoja del sicomoro. Análisis químicos posteriores determinaron que la dormina y la abscisina II eran el mismo compuesto [25]. Desde ese momento este compuesto recibió el nombre de ácido abscísico. Experimentos posteriores permitieron encontrar un inhibidor del crecimiento en tubérculos de *Aegopodium* que fue denominado inhibidor β y que posteriormente se comprobó que también era ABA [156]. El ABA fue inmediatamente conocido como regulador del crecimiento en plantas además

de estar presente como compuesto endógeno en algunos hongos y animales [168, 260]

1.2. Localización y movimiento del ácido abscisico

El ABA se encuentra de forma ubicua tanto en plantas superiores como inferiores, también se produce en algunos hongos fitopatógenos [104] y se ha encontrado en tejido cerebral de mamíferos [125]. Debido a que es un sesquiterpenoide, durante mucho tiempo se creyó que se sintetizaba directamente del farnesil pirofosfato como ocurre en hongos, sin embargo en actualidad se sabe que se sintetiza a partir de carotenoides [49]. Al ser un ácido débil (pKa=4.8) el ABA no se encuentra cargado en el apoplasto que es relativamente acídico por lo que puede atravesar con cierta facilidad la membrana plasmática y entrar en la célula. La distribución del ABA en los compartimentos celulares se rige por el concepto de “trampa de aniones” que consiste en que la forma disociada del ácido débil (anión), se acumula en los compartimentos alcalinos (ej. en los cloroplastos iluminados) y se distribuye de acuerdo con el gradiente de pH a través de las membranas. Además de este reparto dependiente del pH relativo de cada compartimento, existen transportadores específicos que contribuyen a mantener una baja concentración de ABA en el apoplasto de plantas no estresadas. A pesar de la facilidad con la que el ABA puede entrar en las células, existen evidencias tanto de la percepción intracelular como extracelular de ABA, revisado en [132, 202].

1.3. Transportadores de ABA

El ABA se produce de forma predominante en tejidos vasculares y ejerce su respuesta hormonal en distintos tipos celulares, incluyendo las células

oclusivas. El ABA se mueve dentro de la planta y los transportadores de ABA podrían ser de especial relevancia en condiciones de estrés, ya que en estas situaciones se incrementa el pH celular [261]. El incremento de este pH causa que el ABA en su forma protonada no cargada (ABAH) se disocie a su forma aniónica cargada (ABA^-), la cuál no puede difundir pasivamente a través de la bicapa lipídica, en contradicción con la necesidad del reparto rápido de la hormona del estrés en el interior de la célula para obtener una respuesta en el tiempo oportuno.

Recientemente se han identificado dos transportadores de ABA pertenecientes a la gran familia de los transportadores ABC (ATP- binding cassette) PDR12 (Pleiotropic drug resistant transporter) y AtABCG25 [100, 117]. Esta familia de transportadores presenta miembros en todos los filos [252]. La mayoría de estas proteínas son proteínas integrales de membrana que actúan como transportadores impulsados por ATP para una gran variedad de sustratos como lípidos, metales pesados y auxinas [197].

El transportador ABC de *Arabidopsis thaliana* PDR12 (AtPDR12)/ABCG40 es una proteína de membrana que funciona como transportador para la toma de ABA. Ha sido identificado en una búsqueda de potenciales transportadores de ABA partiendo de la base de que el ABA es un sesquiterpeno y existe una subfamilia denominada PDR/ABCG de transportadores de terpenoides dentro de los transportadores ABC [21, 197] que podrían ser posibles candidatos a transportadores de ABA, estos transportadores están implicados en respuesta a patógenos [233] y a distintos tipos de estrés como salinidad, frío y metales pesados [197] [161] [126]. Para encontrar al posible candidato, realizan ensayos de germinación y de apertura

y cierre de estomas en 13 mutantes de pérdida de función para la subfamilia de transportadores ABC PDR (*atabcg*29 a *atabcg41*) en Arabidopsis. La toma de ABA se vió incrementada en experimentos realizados en levaduras y en células BY2 (Bright Yellow 2) que expresaban AtABCG40, en contra de lo que ocurría en protoplastos de plantas *atabcg40*. En las plantas *atabcg40* la inducción de genes de respuesta a ABA tras la aplicación de ABA exógeno está fuertemente retrasada, indicando que ABCG40 es necesario para las repuestas a ABA en un tiempo oportuno. En las plantas de pérdida de función *atabcg40* los estomas se cierran más lentamente en respuesta a ABA, lo que resulta en una reducida tolerancia a la sequía [100].

El transportador ABC AtABCG25 de *Arabidopsis thaliana* se ha identificado en una búsqueda de mutantes sensibles a ABA en germinación y establecimiento de plántula. La búsqueda se realizó en una colección de mutantes de inserción marcados con el transposon *Ds* [118]. *AtABCG25* se expresa fundamentalmente en tejidos vasculares. La fusión de GFP a AtABCG25 se localiza en la membrana plasmática de células vegetales. AtABCG25 muestra un transporte de ABA dependiente de ATP en membranas devesículas derivadas de la expresión de *AtABCG25* en células de insecto. Plantas que sobre expresan *AtABCG25* muestran una elevación de la temperatura en hojas como consecuencia de la influencia de este transportador en la regulación de estomas. Estos resultados señalan a AtABCG25 como un transportador de ABA involucrado en la señalización por ABA intracelular [117].

Estos resultados podrían promover la producción de plantas con mayor tolerancia a la sequía por ejemplo a través de la expresión específica de transportadores de ABA en células oclusivas. Además la manipulación de un

paso específico para la toma de ABA localizado al comienzo de la ruta de señalización por ABA, podría permitir el desarrollo de plantas cebadas permanentemente para responder de forma rápida ante una situación de estrés (Figura 1).

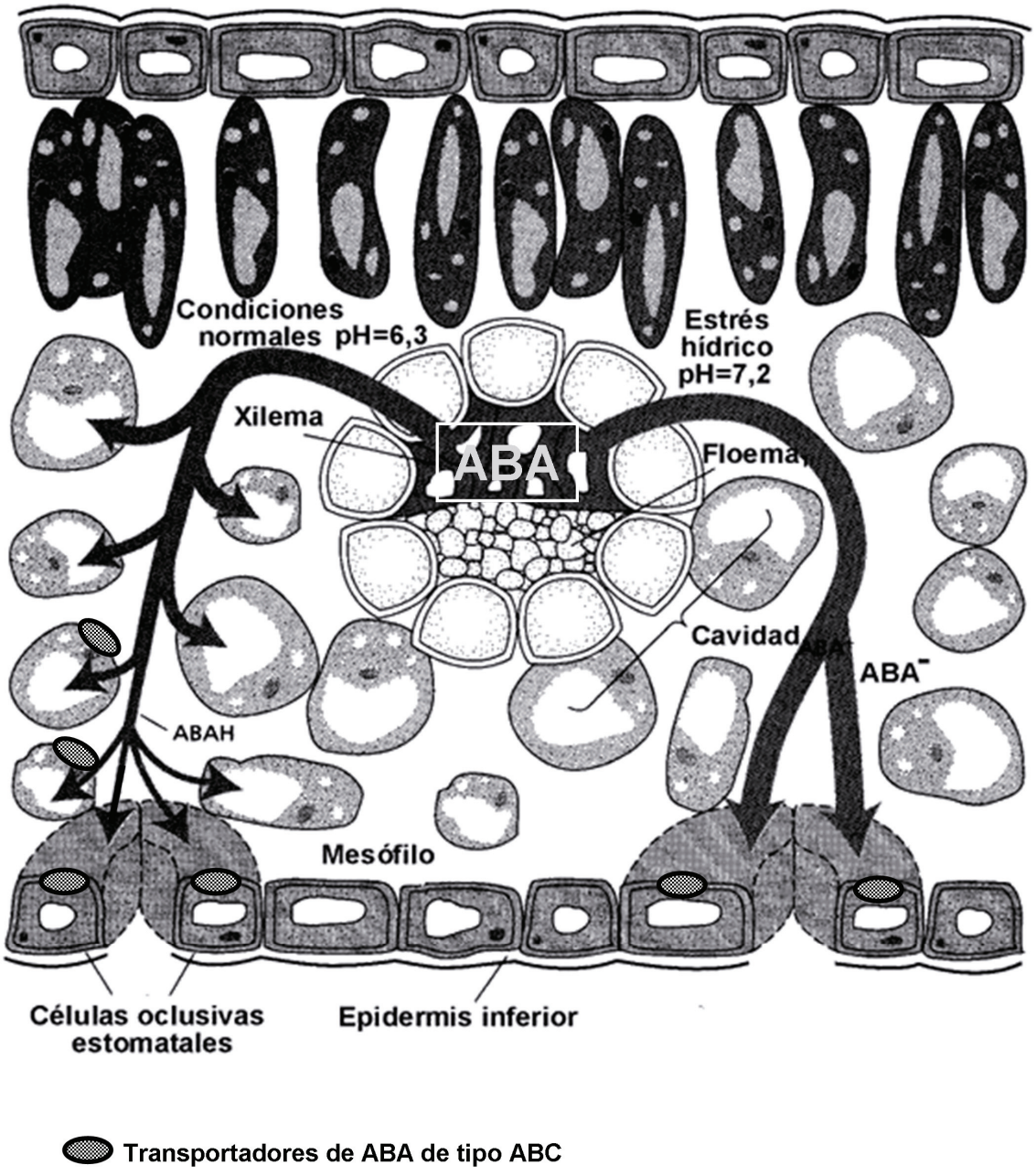


Figura 1. Redistribución del Ácido Abscísico. Adaptado de: Taiz y Zeiger. 1998. Plant physiology. Second edition. Sinauer Associates Inc., Sunderland. 792 p.

1.4. Características químicas de la molécula del ácido abscísico

La estructura molecular del ABA fue deducida por métodos de espectroscopia poco después de su descubrimiento confirmándose posteriormente mediante la síntesis química de la molécula [26, 176]. La estructura molecular del ABA posee una serie de características químicas que son importantes para su actividad biológica en plantas.

El ABA es un sesquiterpeno de 15 carbonos ($C_{15}H_{20}O_4$) derivado del caroteno con un carbono asimétrico ópticamente activo C-1'. El ABA posee en la cadena lateral de su molécula dos enlaces dobles conjugados en el ácido carboxílico, (Figura 1), la configuración del doble enlace adyacente al anillo es en trans y la del doble enlace proximal al grupo ácido es en cis. El ABA tiene por tanto dos isómeros, cis o (+) ABA y trans o (-) ABA, interconventibles entre ellos en la planta y dos enantiómeros R y S que no son interconvertibles. La exposición a la luz ultravioleta provoca que la forma biológicamente activa del ABA que es 2-*cis*, 4-*trans* ABA se isomerize a su forma inactiva 2-*trans*, 4-*trans*. En la naturaleza se encuentra la forma S(+)ABA (Figura 2); donde la cadena lateral de cinco carbonos del ABA es por definición 2-cis,-4-trans. La forma trans, trans-ABA es inactiva biológicamente, y la forma R(-)ABA no se encuentra en la naturaleza pero se encuentra en un 50% del ABA no radiactivo que está disponible comercialmente teniendo además cierta actividad biológica.

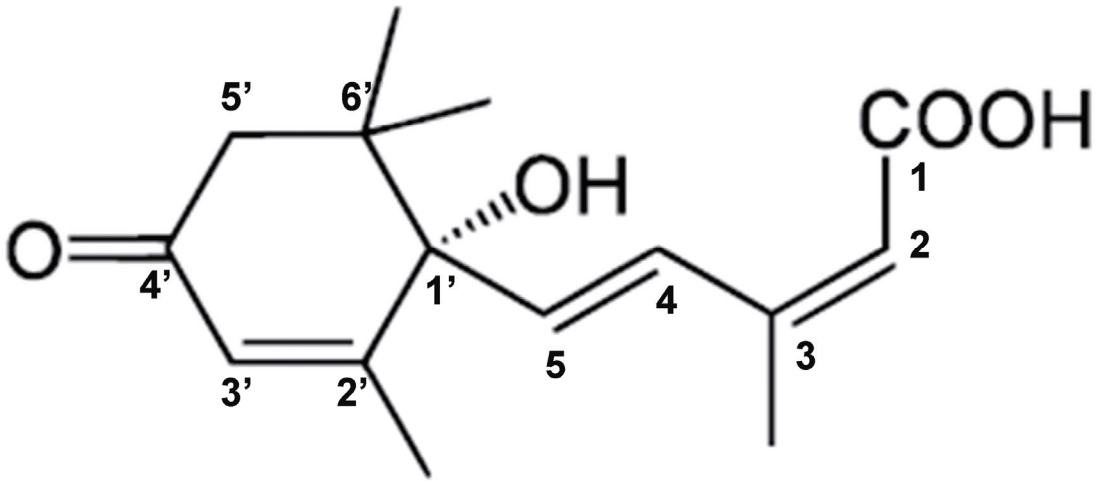


Figura 2. Ácido abscísico. (S)-5-(1-hidroxi-2,6,6-trimetil-4-oxo-1-ciclohexil)-3-metil-cis, trans-penta-2,4-dienoico, S(+)ABA. Forma activa en la naturaleza.

La regulación de los procesos fisiológicos controlados por ABA se produce principalmente a partir del ABA que se sintetiza de *novo* y de su recambio. Esto conlleva una mayor cantidad de ABA procedente de la síntesis de *novo* de enzimas relevantes que de la redistribución del ABA ya existente [74, 157, 278, 279].

En condiciones de sequía, las plantas pueden reducir el consumo de agua limitando al mínimo la transpiración a través de los estomas en las hojas. El ABA es la fitohormona que causa el cierre de estomas, y es la señal designada para la comunicación del déficit de agua desde la raíz al ápice. La respuesta del ápice a la escasez de agua en el suelo no se ve afectada por la capacidad de generación de ABA en la raíz, si no que esta respuesta requiere de la biosíntesis de ABA y de la señalización en el ápice. El estrés hídrico del suelo provoca una respuesta hidráulica en el ápice, que precede a la señalización por ABA y al cierre de estomas [41] (Figura 1).

1.5. Biosíntesis del ácido abscísico.

El ABA ($C_{15}H_{20}O_4$) es un sesquiterpeno que se sintetiza vía escisión oxidativa de los epoxicarotenoides neoxantina y violaxantina. Su biosíntesis ha sido caracterizada mediante el estudio de mutantes, cuyo fenotipo a sido revertido mediante la adición de ABA exógeno.

Síntesis de los epoxi-carotenoides: La zeaxantina se produce como un isómero trans después de la ciclación e hidroxilación del todo-trans-licopeno a través de β-caroteno (Figura 3). Los pasos siguientes consisten en la síntesis de isómeros cis de violaxantina y neoxantina que serán cortados para formar un precursor de quince carbonos de ABA.

La conversión de zeaxantina a violaxantina está catalizada por la enzima zeaxantina epoxidasa (ZEP) a través del intermediario anteraxantina. El gen ZEP, que fue clonado por primera vez en *Nicotiana plumbaginifolia* mediante mutagénesis por inserción, codifica una proteína similar a las monooxigenasas que unen FAD y requieren ferredoxina [14, 143]. Se han aislado mutantes en la enzima ZEP en varias especies, incluyendo Arabidopsis [151, 174, 270], *N. plumbaginifolia* [143], y arroz [3]. Estos mutantes acumulan zeaxantina y muestran una reducción severa en el contenido en ABA, que produce un fenotipo marchito y semillas que tienen alterada la dormición. En Arabidopsis, las mutaciones producidas por sustituciones de ácido aminos en el dominio monooxigenasa alteran la función de la enzima, indicando que este dominio podría ser importante para su actividad [151, 269].

A partir de violaxantina se produce la síntesis de neoxantina por la neoxantina sintasa (NSY) identificada en tomate y patata por homología de

secuencia con la enzima, licopeno ciclasa β (LCYB) y capsantina capsorubina sintasa de pimienta [4, 13] y Arabidopsis [175]. Además, las mutaciones en el putativo gen NSY de tomate afectan a la síntesis de β-caroteno a partir de licopeno, demostrando que este gen codifica una isoforma LCYB [205]. También se han identificado mutantes que carecen de isómeros de neoxantina en Arabidopsis [175] y tomate [58].

Corte de la xantofila: La 9-cis-epoxicarotenoide dioxigenasa (NCED) corta los isómeros de violaxantina y neoxanthin formando un producto de 15 carbonos, xantoxina, y un metabolito de 25 carbonos. En maiz se clonó el primer gen de NCED (VP14) por mutagénesis por inserción [219, 242]. La enzima VP14 recombinante es capaz de cortar 9-cis-violaxantina y 9'-cis-neoxantina pero no isómeros de trans-xantofila [219]. Además requiere hierro y oxígeno para formar isómero cis de xantoxina [219]. En todas las especies vegetales analizadas, los genes NCED pertenecen a una familia multigénica. Como ocurre con los mutantes Vp14 de maiz y notabilis de tomate, los mutantes nced, presentan un fenotipo leve de deficiencia a ABA a debido a la redundancia genética [19] [242]. En Arabidopsis, se han identificado nueve secuencias relacionadas con NCED de las que cinco de ellas están probablemente involucrados en la biosíntesis de ABA como indican experimentos de análisis funcional (*AtNCED2, 3, 5, 6* y *9*) [90, 218].

Como es el caso de otras enzimas de la biosíntesis de carotenoides, la proteínas NCED de distintas especies están localizadas en los cloroplastos [91, 190, 240, 241]. Debido a que las siguientes reacciones enzimáticas tienen lugar en el citosol [35], tiene que haber un mecanismo de trasporte de xantosina entre el plastidio y el citosol.

Vía citosólica de los C15. La forma biológicamente activa del ABA se produce a partir cis-xantoxina en dos pasos enzimáticos pasando por el intermediario abscísico aldehído En Arabidopsis, la conversión de xantoxina a abscísico aldehído está catalizada por la enzima AtABA2 perteneciente a la familia SDR. Este gen fue identificado por clonación basada en mapeo [35, 67] después del aislamiento de numerosos alelos mutantes de Arabidopsis en varias búsquedas genéticas [67, 129, 151, 167, 192, 206]. La proteína AtABA2 está codificada por un único gen en el genoma de Arabidopsis, por lo que la pérdida de función de este gen conduce a una deficiencia severa en ABA. Las mutaciones han sido identificadas en supuestos dominios funcionales (dominio de unión a NAD, centro catalítico, hélice de interacción con subunidades, y el sitio de unión a sustrato) afectando a la producción de ABA, por lo que se deduce que estos dominios son de gran importancia en la actividad de la enzima [67].

Por otra parte, la complementación entre los alelos mutantes intragénicos sugiere que AtABA2 podría tener una estructura multimérica, de manera similar a la estructura dimérica o tetramérica de la mayoría de las proteínas de la familia SDR de varios organismos [99, 151, 206].

La oxidación del abscísico aldehído a ácido carboxílico es el paso final de la biosíntesis de ABA, este paso está catalizado por una abscísico aldehído oxidasa (AAOS). Se han encontrado 4 enzimas AAOs en Arabidosis siendo AAO3 la que procesa el aldehído abscísico [224]. El mutante *aao3-1*, que contiene una mutación en un sitio de empalme de intrones, muestra un fenotipo de marchitamiento pero con una pequeña reducción en la dormición de semillas, en comparación con otros mutantes de ABA en Arabidopsis afectados

en genes únicos. Este fenotipo leve en las semillas se ha atribuido a la redundancia de genes postulándose que otros genes de *AAOs* podrían estar implicados en la biosíntesis de ABA [222]. Aunque la identificación de alelos nulos *aao3* que exhiben fenotipos deficientes en ABA importantes en semillas indican que probablemente *AAO3* es único gen de las *AAOs* que participa en la síntesis de ABA [66, 221].

La enzima aldehído oxidasa requiere un cofactor de molibdeno (MoCo) para su actividad catalítica. Por lo que las mutaciones en los genes de la biosíntesis del MoCo producen deficiencia en ABA. Consistente con esto, las mutaciones en *FLACCA* en tomate [211] y en *AtABA3* en Arabidopsis [10, 45] que codifican una MoCo sulfurasa producen los esperados fenotipos deficientes en ABA [168].

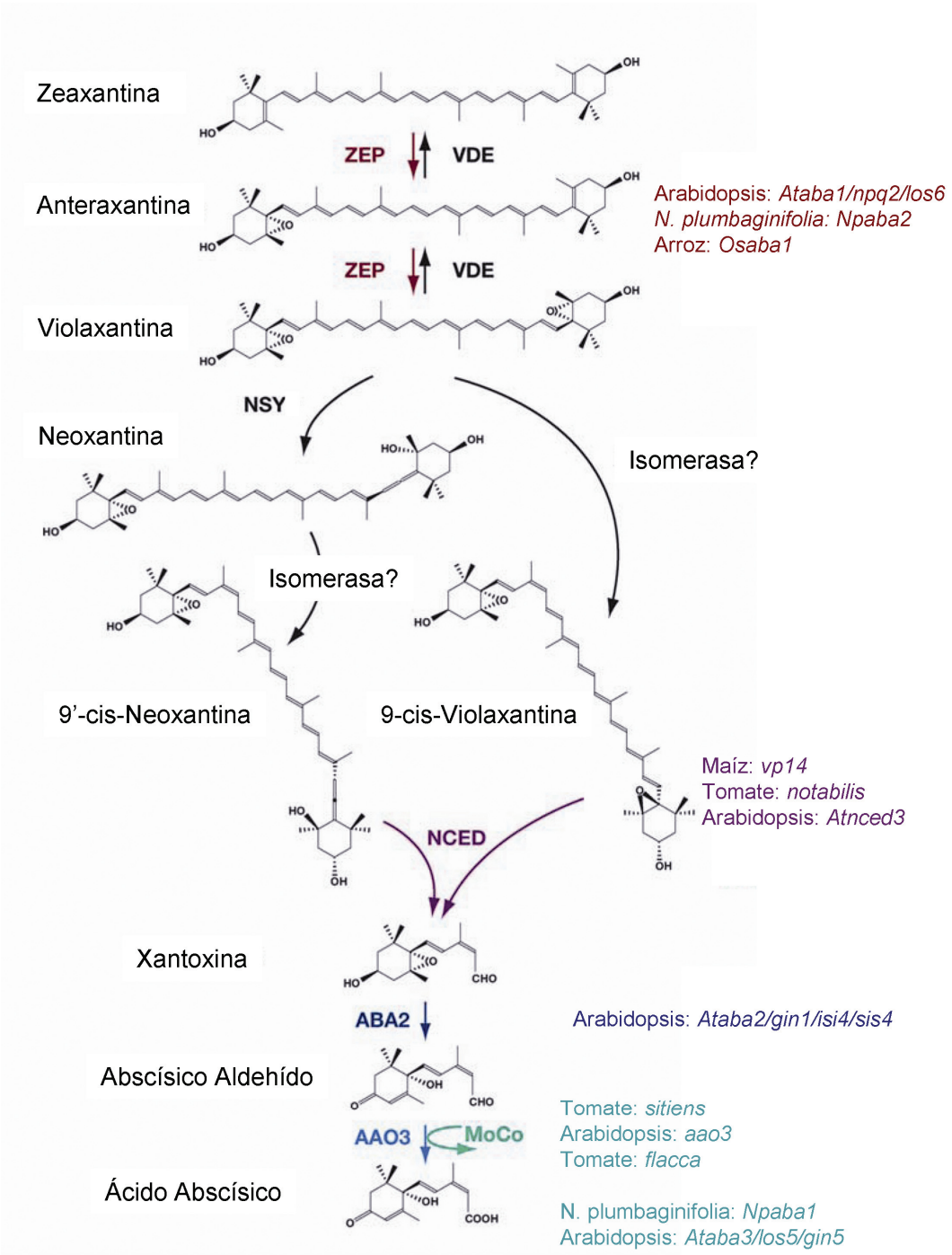


Figura 3. Biosíntesis del ácido abscísico. La síntesis de la violaxantina está catalizada por la zeaxantina epoxidasa (ZEP). En condiciones de alta luminosidad en los cloroplastos la violaxantina de-epoxidasa (VDE) cataliza la reacción inversa. La formación de los isómeros *cis* de la violaxantina y neoxantina es realizada por dos enzimas, una neoxantina sintasa (NSY) y una isomerasa. Una familia de 9-*cis*-epoxicarotenoide dioxigenasa (NCED) se encarga de catalizar el corte de las *cis*-xantofilas. La xantoxina es convertida por una alcohol deshidrogenasa de cadena corta (ABA2) en abscísico aldehído que es oxidado para formar ABA por una abscísico aldehído oxidasa (AAO3). AAO3 contiene como cofactor molibdeno que es activado por una sulfatasa MoCo. En el lado derecho se muestra una lista de mutantes defectivos en cada paso enzimático. Adaptado de Nambara y Marion-Poll. Annu. Rev. Plant Biol.2005. 56:165–85

1.6. Funciones fisiológicas del ácido abscísico.

Al ABA se le ha atribuido un papel regulador de muchos aspectos del crecimiento y del desarrollo vegetal, entre los que se incluyen maduración del embrión, dormición de la semilla, germinación, división celular y elongación, respuestas al estrés ambiental (sequía, salinidad, frío, ataque por patógenos y radiación UV) y regulación de la expresión génica [132, 202]. Pese a que su nombre puede indicar lo contrario, no participa directamente en el control de la abscisión sino más bien en los procesos previos a ésta, actuando como promotor de senescencia y/o en respuestas frente al estrés. Aunque históricamente se le ha considerado un inhibidor del crecimiento, existen niveles elevados de ABA en tejidos jóvenes, además los mutantes deficientes en ABA tienen impedido su desarrollo debido a que tienen alteradas la capacidad de reducir la transpiración y mantener la turgencia, estos efectos se revierten con el tratamiento con ABA exógeno que restauran la expansión celular y el crecimiento en estos mutantes [49].

Las funciones del ABA se han conseguido dilucidar en muchos casos mediante estudios genéticos en Arabidopsis como resultado del aislamiento de mutantes deficientes en ABA que subrayan la importancia y la directa participación del ABA en el metabolismo y en el desarrollo de la planta y proporcionan información para determinar la ruta de biosíntesis y señalización por ABA. No solo lo experimentos con Arabidopsis han sido esenciales para establecer los componentes de la ruta de señalización y biosíntesis de ABA sino que algunos pasos de la ruta han sido descritos gracias a la caracterización de mutantes en maíz, tomate y, *Nicotiana plumbaginifolia*, siendo sus ortólogos identificados en Arabidopsis. Además, algunos de los

factores implicados en la ruta del ABA observados en Arabidopsis no han sido encontrados en otras plantas a pesar de evidencias que muestran que la ruta de biosíntesis de ABA está conservada en todas las plantas (revisado en [30, 111, 137, 157, 223, 280]).

Entre las funciones fisiológicas del ABA se encuentra su capacidad para actuar como antagonista de algunos de los efectos provocados por giberelinas (GA), como son el promover el crecimiento de la plántula y la síntesis de α-amilasa [244]. Otra de las principales funciones del ABA está relacionada con el control hídrico de la planta, ejerciendo un papel en las células oclusivas. Ejemplo de ello es el mutante de tomate *flacca,* deficiente en ABA, que presenta un fenotipo de marchitamiento. Este fenotipo puede ser rescatado con la aplicación exógena de ABA [88, 101] que además produce el cierre de los estomas. La oclusión estomatal por la aplicación de ABA exógeno ha sido además observado en *Xanthium* [98]. Corroborando el papel fundamental del ABA en la regulación de la células oclusivas se ha observado la elevación de los niveles de ABA endógenos en plantas tras un periodo de sequía. Además del cierre estomatal, el mantenimiento del crecimiento de la raíz es clave en la respuesta adaptativa para garantizar el abastecimiento de agua a toda la planta en situaciones de déficit hídrico. En este proceso participa el ABA junto con la acción coordinada de otras rutas de señalización hormonal [225]. Es interesante resaltar que en algunos casos donde la respuesta adaptativa primaria no equilibra la pérdida y ganancia de agua, se ponen en marcha diferentes mecanismos que impiden y/o toleran la deshidratación y que involucran la regulación de genes de respuesta a estrés a través de la acción del ABA entre otras rutas de señalización [285]. En concreto se produce la

acumulación de osmolitos compatibles y la síntesis regulada de dehidrinas y proteínas implicadas en la tolerancia a desecación de tipo LEA (late embryogenesis abundant), desempeñando funciones de retención de agua y protección de proteínas y membranas en condiciones de estrés [89].

Recientemente, se ha descubierto que el ABA está además implicado en la respuesta a patógenos impidiendo la entrada de patógenos a través del estoma. Además del papel del ABA en la respuesta al estrés biótico y abiótico, el ABA regula importantes aspectos del desarrollo y crecimiento de la planta, entre ellos el desarrollo del embrión y de la semilla, promueve la tolerancia a la desecación y dormición, promueve la germinación, el establecimiento de la plántula, el desarrollo vegetativo incluyendo la heterofilia (hojas de una misma planta que presentan formas diferentes) así como el crecimiento en general y la reproducción. Por este motivo, mutantes deficientes en ABA afectados de forma severa y los insensibles a ABA muestran un fenotipo raquítico incluso bajo condiciones de buen riego acompañado de una producción de semillas alterada [5, 35, 56, 166].

1.7. Cascadas de señalización del ácido abscísico.

El ABA regula multitud de procesos del desarrollo y de la respuesta al estrés en plantas. Se han identificado muchos de los componentes de la ruta de señalización y se ha estudiado la interconexión existente entre ellos para elucidar la estructura de la cascada de señalización del ABA. Estudios de genética molecular, bioquímica y farmacología han llevado a la identificación de más de 100 loci y numerosos mensajeros secundarios que participan en la ruta de señalización por ABA. Entre ellos se encuentran el Ca^{2+}, especies reactivas

de oxigeno (ROS), nucleótidos y fosfolípidos así como diferentes enzimas. Recientemente se han identificado y caracterizado los receptores de ABA llegándose incluso a publicar su estructura tridimensional. También se ha establecido que la regulación por ABA de la actividad fosfatasa y quinasa en la célula es de crucial importancia en la planta. Actualmente se ha propuesto un modelo que describe la acción del ABA, en el cuál los receptores solubles PYR/PYL/RCAR se encuentran en la cima de una ruta controlando directamente a los reguladores negativos que son las proteínas fosfatasas de tipo 2C (PP2C) y también a las proteínas quinasas SnRK2 (ABA activated SNF-1 related protein kinase 2) que actúan en sentido contrario. Este modelo unifica criterios previos acerca de los componentes de la señalización y pone de manifiesto la importancia que en un futuro tendrá la caracterización de las dianas de interacción tanto de las PP2Cs como de las SnRK2, los mecanismos de interacción entre diferentes hormonas y, la posibilidad de establecer conexiones entre esta nueva ruta de regulación negativa y otros factores implicados en la señalización por ABA.

1.7.1. Proteínas quinasas involucradas en la señalización por ácido abscísico.

La fosforilación reversible de proteínas tiene un papel fundamental en la regulación de las rutas de transducción de señal en la mayoría de los organismos. Estas modificaciones post-traduccionales son realizadas por las quinasas, proteínas que tienen mucha relevancia en la ruta de señalización por ABA. Como ejemplo, el ABA controla la expresión de diferentes quinasas [64, 81, 86, 128, 155, 188] y/o la activación de quinasas que se expresan

constitutivamente [20, 134], estos efectos tienen lugar en procesos regulados por ABA como son el control de los estomas y la expresión génica involucrada en la señalización por ABA. Estas observaciones se han obtenido empleando inhibidores farmacológicos y alelos dominantes negativos que muestran la implicación de quinasas en procesos controlados por ABA [135, 226].

Entre las quinasas que actúan en la ruta de señalización por ABA se encuentran tanto quinasas independientes de calcio SnRK2s (SNF1-RELATED PROTEIN KINASE 2) como quinasas reguladas por calcio SnRK3s/CIPKs (SNF1-RELATED PROTEIN KINASE3/CBL-INTERACTING PROTEIN KINASE) y CDPKs/CPKs (CALCIUM DEPENDENT PROTEIN KINASE).

Cronológicamente, la primera quinasa independiente de calcio descrita en plantas fue PKABA1. Esta quinasa, que fue descrita en maíz, es inducida por ABA, fosfoforila al factor de transcripción TaABF1 (un miembro de la familia de los factores de transcripción ABF/AREB que reconocen secuencias de elementos de respuesta a ABA ó cajas ABRE) y media la supresión de la expresión de genes inducidos por GA en granos de cereales [64]. Otro ejemplo es la Ser/Thr quinasa codificada por el gen *AAPK* (ABA ACTIVATED SERIN-THREONINE PROTEIN KINASE) de *Vicia faba* específica de células oclusivas e implicada en la regulación del cierre del estoma inducido por ABA [135]. La expresión de una versión de AAPK alterada en el cambio de un aminoácido (Lys_{43} por Ala_{43}) produce insensibilidad al cierre de estomas inducido por ABA, por eliminación de la activación por ABA de los canales de aniones de membrana plasmática [135].

El genoma de *Arabidopsis thaliana* contiene 10 SnRK2s, entre ellas SnRK2.2 (también conocida como SRK2D), SnRK2.3 (SRK2I) y

SnRK2.6/OST1 (OPEN STOMATA1)/SRK2E son activadas por ABA. Se ha descrito que SnRK2.2 y SnRK2.3 regulan las respuestas mediadas por ABA en germinación, crecimiento y regulación de la expresión génica, mientras que SnRK2.6/OST1, el supuesto ortólogo de AAPK en Arabidopsis, que se identificó en una búsqueda de plantas con defectos en transpiración utilizando termografía por infrarrojos, regula la apertura estomatal [55, 135, 164, 274]. Además, SnRK2.6/OST1 interacciona con ABI1 [275] y el mutante dominante de ABI1 *abi1-1D* muestra una inactivación constitutiva de SnRK2.6/OST1 en respuesta a ABA de mayor índole que el genotipo silvestre [164, 275]. AAPK y OST1/SnRK2.6 participan de forma específica en la ruta de señalización por ABA ya que alteraciones de la función de AAPK u OST1 no afectan a la regulación del estoma por luz o por CO_2. El doble mutante *snrk2.2snrk2.3* muestra una leve alteración en el control de los estomas por ABA a pesar de que las quinasas correspondientes activadas por ABA SnRK2.2 y SnRK2.3 se encuentran relacionadas con OST1 [55]. Sin embargo, el doble mutante *snrk2.2snrk2.3* muestra una fuerte insensibilidad a ABA en ensayos de germinación de semilla e inhibición del crecimiento de raíz junto con una menor expresión de genes inducibles por ABA [55].

Finalmente, en el mutante defectivo en las tres quinasas SnRK2.2, SnRK2.3 y OST1/SnRK2.6 es capaz de germinar y crecer en 50μM ABA, condiciones un orden de magnitud superiores a las que es capaz de germinar y crecer el mutante insensible a ABA *abi1-1D* [56] por lo que de este fenotipo severo se puede deducir que estas tres quinasas actúan como reguladores positivos en la ruta de señalización por ABA [56, 165] .

Debido a la práctica eliminación de las respuestas a ABA que presenta el mutante *snrk2.2/2.3/2.6*, será de gran importancia en un futuro para el completo conocimiento de la ruta de señalización por ABA el llegar a elucidar los posibles sustratos de estas quinasas. Con este fin se están llevando a cabo búsquedas para identificar los sustratos de estás quinasas [255]. Por otro lado se sabe que SnRK2s fosforila factores de transcripción de tipo bZIP como son ABF/AREB/ABI5 [55, 57, 96, 109]. Además se ha descrito que las SnRKs están relacionadas con proteínas de unión a ADN involucradas en la activación en respuesta a ABA.

No se conoce con seguridad si las SnRKs requieren de quinasas para su activación *in vivo* o si la autofosforilación es suficiente para la activación. En experimentos *in vitro* con inmunoprecipitados de SnRKs se ha observado su sensibilidad a la estaurosporina un inhibidor de quinasas de amplio espectro [12]. Sin embargo en tratamientos de protoplastos la utilización de este compuesto no bloquea la activación de la quinasa SnRK2 por ABA, lo que hace suponer que existe una quinasa localizada previamente en la ruta de señalización que debe activar a SnRK2 [12].

En Arabidopsis se han encontrado hasta el momento 25 quinasas reguladas por calcio del tipo SnRK3 (SnRK3s/CIPKs) y 34 quinasas del tipo CDPKs/CPKs [34, 43] que se caracterizan por tener un dominio de activación por Ca2+ con 4 sitios de unión de calcio (EF hand Ca2+-binding sites) [73, 82]. Las SnRK3s/CIPKs interaccionan con proteínas de unión a calcio como SOS3/CaBPs/CBL (SALT OVERLY SENSITIVE/ CALCINEURIN B-LIKE PROTEIN) y, algunos miembros regulan la señalización por ABA dependiente de Ca^{2+}. Un ejemplo de ello es la interacción entre la proteína de unión a calcio

SCaBP5/CBL1 (CALCINEURIN B-LIKE PROTEIN 1) y PKS3/CIPK15 (CBL-INTERACTING PROTEIN KINASE) que funcionan como reguladores negativos en germinación y en respuesta de estomas a ABA [71]. El ABA induce un incremento transitorio de calcio citosólico que es percibido tanto por sensores de calcio, que son reguladores positivos de la señalización por ABA (CDPKs/CPKs), como por SCaBP/PSK3 que suprime la represión de PKS3 en la señalización por ABA. CIPK3/PKS12 está también involucrada en la regulación negativa de la señalización por ABA en germinación de semilla, a pesar de que el cierre de los estomas inducido por ABA no está afectado en el mutante de pérdida de función *cipk3* [103]. Además CIPK3 participa de forma general en la modulación de la expresión génica en respuesta al frío y salinidad. El mutante de pérdida de función *cipk23* pierde menos agua por transpiración y presenta una respuesta reforzada en el cierre de los estomas inducido por ABA junto con mayor inhibición de la apertura de los estomas sin tener afectadas las respuesta a ABA en germinación [36]. Resumiendo, PKS37/CIPK15, CIPK3/PSK12 y CIPK23/PKS17 actúan como reguladores negativos de la señalización por ABA.

En cambio, la familia de quinasas dependientes de Ca^{2+} CDPKs/CPPS funcionan como reguladores positivos de la señalización por ABA. Poseen un dominio regulador de tipo calmodulina en su extremo C-terminal. Además poseen el dominio sensor de calcio y el dominio donde reside su actividad quinasa en un solo polipéptido La expresión constitutiva de las proteínas CPK10/CDPK1 y CPK30/CDPK en protoplastos de maíz origina la activación de un promotor inducible por ABA siendo esta la primera evidencia que involucraba a las CDPK en la señalización por ABA [227]. Más tarde se

observó que la sobre expresión constitutiva de CPK32 resulta en hipersensibilidad a ABA en inhibición de la germinación [39]. La utilización de mutaciones de pérdida de función en CPK3 y CPK6 sirven para identificar estas proteínas como reguladores positivos de la apertura de estomas regulada por ABA, a pesar de que estos mutantes no muestran fenotipo de respuesta a ABA en germinación o en establecimiento de plántula [162]. Por último, los mutantes *cpk4* y *cpk11* muestran un fenotipo de insensibilidad a ABA pleiotrópico en respuestas tempranas así como en apertura y cierre de estomas, lo que indica que son reguladores positivos en la ruta de señalización por ABA mediada por proteínas CDPK dependientes de calcio.

También se han descrito otras familias de quinasas que participan en la ruta de señalización por ABA pero se desconocen los factores a los que regulan. Un ejemplo es el receptor RPK1 (RECEPTOR LIKE KINASE1) que es un regulador positivo de la señalización por ABA si atendemos a los fenotipos de insensibilidad a ABA del mutante de pérdida de función y de las plantas transgénicas antisentido RPK1 [179]. Por otro lado, el ABA induce la activación de MAPK en protoplastos de aleurona de cebada aunque no existe una evidencia genética en líneas de pérdida de función [107].

1.7.2. Receptores del ácido abscísico.

1.7.2.1. Primeras proteínas identificadas como receptores de ácido abscísico.

La primera proteína de unión a ABA que se aisló fue a partir de su habilidad para unir un anticuerpo anti-idiotípico de ABA, un anticuerpo contra otro anticuerpo de ABA en aleurona de cebada, esta proteína fue denominada

ABAP1 (ARMADILLO BTB ARABIDOPSIS PROTEIN 1) [196]. ABAP1 es una proteína de 472 aminoácidos, inducida por ABA en las capas de aleurona de cebada, que contiene un dominio de interacción con proteínas denominado WW. ABAP1 se ha detectado en monocotiledonias y dicotiledoneas como maíz, tabaco, alfalfa, guisante de jardín entre otras plantas [196], ABAP1 es capaz de unir ABA *in vitro* de forma reversible [196]. Por otro lado también se publicó que proteínas que eran capaces de unir ARN o unir a proteínas encargadas del procesamiento del ARN podrían unir ABA. Este es el caso de la proteína FCA (putative cytosine deaminase), una proteína que une RNA en el proceso de floración [195]. FCA y ABAP1 presentan homología de secuencia pero a pesar de ello, FCA y ABAP1 muestran diferencias fundamentales, entre ellas que FCA1 es una proteína nuclear con dos dominios de unión a ARN y ABAP1 está asociada a membrana y no presenta los dominios de unión a ARN. Posteriormente, ensayos con radioligandos para intentar reproducir la capacidad de unión a ABA de FCA mostraron que FCA no unía ABA y el articulo de FCA como proteína de unión a ABA fue retirado [200].

Otra proteína publicada como receptor de ABA es la proteína ChlH/ABAR (Mg Chelatase subunit/ABA-RESPONSIVE PROTEIN). ChlH fue aislada a partir de preparaciones de proteínas de epidermis de hojas de judía (*Vicia faba*) utilizando una matriz de afinidad construida por unión del carboxilo de ABA a una resina con grupos amino [282]. La proteína obtenida tenía un peso molecular de 42 KDa, un punto isoeléctrico de 4.86 y una Kd de 32nM por lo que se la denominó ABAR (receptor de ABA). Esta proteína es un componente de una Mg-quelatasa, un complejo plastídico compuesto por muchas subunidades que funciona insertando Mg^{2+} en el interior de la

protoporfirina IX (mg-proto), que es a su vez un precursor de la clorofila [144]. Se ha logrado modular la acción de la proteína ChlH/ABAR a través de la utilización de alelos de inserción de RNAi o de T-DNA observando fenotipos consistentes con un papel de ChlH/ABAR en la respuestas a ABA [228].

La forma Mg-proto funciona como una señal que coordina la expresión génica tanto en núcleo como en cloroplasto en Arabidopsis [234] y en *Clamydomonas* [114]. El factor de transcripción ABI4 es un componente de esta ruta de señalización que se encuentra más abajo en la ruta de señalización [113]. Aunque no están muy claro que ABI4 pudiera actuar de forma retrógrada en la ruta de señalización por ABA, si que hay indicios de que existe comunicación entre los factores de señalización mediados por ABA y el cloroplasto. Sin embargo ChlH no parece participar en la señalización retrógrada de ABA [228].

Otros experimentos mostraban que ChlH podría unir ABA, usando una columna de afinidad [264]. Sin embargo, estos resultados no se han corroborado con XanF, que se corresponde con ChlH en cebada, ya que XanF no puede unir ABA y los mutantes de pérdida de función *xan F* presentan una respuesta normal a ABA, sugiriendo que en esta planta XanF no funciona como receptor de ABA [163] o bien que ChlH se comporta de manera diferencial en monocotiledóneas y en dicotiledóneas difiriendo en cuanto a su capacidad de unión y señalización por ABA.

Otro candidato a unirse a ABA son las proteínas acopladas a proteínas G, GPCRs, ya que estudios farmacológicos han mostrado que estas proteínas participan en la ruta de transducción de señal de ABA en plantas [47]. Alelos de pérdida de función del gen G-alfa (*GPA1*) de Arabidopsis muestran alteraciones

en su respuesta a ABA, concretamente se ha observado hipersensibilidad a ABA en germinación y sensibilidad reducida a la inhibición por ABA de la apertura de las células oclusivas, sin alteraciones en el cierre estomatal inducido por ABA [259]. Estos experimentos sugieren que GPCR debe participar en la ruta de transducción de la señal de ABA. En este sentido la sobre expresión de GCR1 en Arabidopsis reduce la dormición de la semilla [24]. Sin embargo los estudios realizados en alelos de pérdida de función no implican a GCR1 de forma directa en la percepción de ABA. El mutante de pérdida de función *gcr1* muestra hipersensibilidad a ABA, pero existen datos que confirman que GCR1 se encuentra en otras rutas de señalización por lo que tiene efectos pleiotrópicos [33].

Otro supuesto receptor de ABA aislado, GCR2, se propuso también como un receptor acoplado a proteínas G. Actualmente existe cierta controversia acerca tanto sobre su papel en la señalización por ABA [59, 70] como en su inclusión en el grupo de receptores acoplados a proteínas G [87, 97]. Por un lado esta proteína muestra cierta similitud con una enzima soluble de bacterias de la superfamilia LanC (lantibiotic synthetase component C) [97]. Por otro lado se han realizado medidas posteriores de ABA unido GCR2 sin éxito [199] .

También se han realizado abordajes bioinformáticos en búsqueda de posibles receptores de ABA acoplados a proteínas G en el genoma de Arabidopsis [183] obteniendo como resultado las proteínas GTG1 y GTG2 (GPCR-TYPE G PROTEIN1 y 2) basándose en su similitud a GPCRs [183]. Las proteínas de Arabidopsis GTGs contienen un dominio de unión a nucleótidos y un dominio con actividad GTPasa que hacen de ellas unos GPCRs diferentes.

Por otro lado en humanos existe una proteína denominada GPR98/GPHR (G protein-coupled receptor 89B) homologa a las proteínas GTGs de Arabidopsis que fue identificada en una búsqueda genética como un factor necesario para el transporte de proteínas a través del retículo endoplásmico, además de ser un transportador de iones involucrado en la acidificación del Golgi [140]. La proteína de humanos carece de los dominios de de unión a nucleótidos y de actividad GTPasa. GTG se une a ABA como muestran experimentos de unión usando proteína GTG reconstituida en presencia de fosfatidilcolina donde la especificidad de unión a (+) ABA (el receptor unido a (+) ABA no era desplazado por un exceso de > 1000 veces de (-) ABA). Sin embargo hay que señalar que sólo un 1% de la proteína recombinante unía ABA [40, 46]. Pandey y colaboradores atribuyen la estequiometría de la unión a las grandes diferencias de plegamiento existentes en las proteínas de membranas con su forma funcionalmente activa [183] . Es interesante mencionar que la unión de ABA a las proteínas GTGs es estimulada por GDP, sugiriendo que la unión de GDP al receptor hace que esté en un estado de alta afinidad de unión. En ensayos de fusión de GTGs a la proteína fluorescente verde (GFP) se ha observado la localización de estas proteínas en la periferia de los protoplastos y en el sedimento de la fracción microsomal [183]. Estos resultados parecen indicar que las GTGs se encuentran localizadas en la membrana plasmática funcionando con GPCRs y controlando la señalización por ABA. Consistente con la regulación de GTG a través de la señalización por proteínas G, se han estudiado interacciones entre GPA1 (GTP-BINDING ALPHA SUBUNIT GCR1) y GTGs viéndose que GPA1 funciona inhibiendo la actividad GTPasa intrínseca a las GTGs, pero sin modificar las propiedades de unión a ABA. Además, la

actividad GTPasa de GTGs es regulada negativamente por la unión de GTP a GPA1, que ha sido sugerida como la forma mayoritaria de GPA1 *in vivo* [97]. Cuando GPA1 une GDP, es la forma inactiva de GPA1, ésta se une a PLDα1 inhibiéndola. La señalización por ABA activa GPA1, permitiendo la liberación de PLD y la producción de ácido fosfatídico, el cuál promueve el cierre de los estomas inducido por ABA y la expresión génica al mismo tiempo que promueve otras respuestas frente al estrés, para ello se dan múltiples mecanismos de retroalimentación. Entre estos mecanismos están involucrados la unión a una gran variedad de dianas, incluyendo proteínas fosfatasa, proteínas quinasa y enzimas del metabolismo (revisado en [136]) .

El doble mutante de Arabidopsis *gtg1/gtg2* muestra una sensibilidad a ABA reducida en germinación de semilla, crecimiento de raíz, respuesta de estomas y expresión génica como se ha observado en ensayos de respuesta a ABA [183]. De forma contraria al fenotipo que muestra el mutante *gpa1*, el doble mutante *gtg1/gtg2* tiene un fenotipo silvestre en respuesta a la inhibición por ABA de la apertura de estomas. Los mutantes sencillos del loci *GTG* no muestran fenotipos obvios lo que sugiere que las proteínas GTGs son redundantes funcionalmente en la señalización por ABA. Desde que se supo que el mutante de pérdida de función *gpa1* llevaba a un fenotipo tanto de incremento como de disminución de la respuesta a ABA dependido del tipo de tejido en el que se diera, la observación de que GPA1 podría regular la unión de GTGs a ABA insinúa que el fenotipo del mutante *gpa1* en relación con el ABA podría ser en parte debido a la acción a través de las GTGs. Los datos de GTG implican que la subunidad Gα de GPA1 no está implicada en la transducción de señal corriente debajo de los receptores GTG, el cuál hace que

el sistema de señalización de GPCRs no tenga precedentes. Un objetivo interesante para el futuro podría ser identificar las dianas de GTGs corriente abajo y relacionar su posible acción con otros factores involucrados en la señalización por ABA.

1.7.2.2. Receptores del ácido abscísico de tipo PYR/PYL/RCAR

Los receptores proteicos PYR/PYL/RCAR fueron aislados por diferentes grupos y métodos. En el caso de PYR, se observó que un agonista sintético de ABA denominado pirabactina inhibía el crecimiento a través de una proteína de tipo START denominada PYRABACTIN RESISTANCE 1 (PYR1) que pertenecía a la familia PYR/PYLs y, que era necesaria tanto para la función de pirabactina como para la señalización por ABA in vivo [184]. En más detalle, los análisis genéticos llevados a cabo en este trabajo mostraron que PYR1 es necesario para la acción de la pirabactina *in vivo*, a pesar de que alelos de pérdida de función carecían de fenotipos relacionados con el ABA ya que existía redundancia génica. Para confirmar la hipótesis de que PYR1 podría llevar a cabo su función por unión a una proteína efectora en respuesta a pirabactina, se realizó una búsqueda de proteínas de unión a PYR1 con un ensayo de doble híbrido con pirabactina en el medio de crecimiento. En este ensayo como en ensayos posteriores se observa que tanto en presencia de pirabactina como en presencia de ABA, la proteína PYR1 se une e inhibe al grupo A de proteínas fosfatasas de tipo 2C (PP2Cs) insensibles a ABA (ABI1 y ABI2) y a la proteína hipersensible a ABA 1 (HAB1). Esta familia de proteínas relacionadas con la ruta de transducción de señal de ABA es denominada PYR/PYL, incluyéndose en ella a PYR1 y a 13 miembros más que forman el

grupo de proteínas PYL (PYRABACTIN RESISTANCE 1-LIKE). Con el mismo tipo de ensayos pero con un abordaje a la inversa, la utilización de proteínas fosfatasa de tipo 2C como ABI2 (ABA-INSENSITIVE 2) como cebo en una búsqueda por doble híbrido para encontrar proteínas que pudieran unirse a ABI2 , se identificaron PYL9 (Regulatory Component of ABA Receptor1, RCAR1) y PYL8 [236] que se unen de forma constitutiva a ABI2, es decir, en ausencia de ABA en un ensayo de doble híbrido. De manera independiente con experimentos parecidos identificamos a PYL5 por su interacción constitutiva con HAB1 en un ensayo de doble híbrido [213]. Otro método utilizado para la identificación de proteínas PYR/PYL fue la copurificación constitutiva con ABI1 a partir de extractos de plantas. No se conoce la base molecular de la diferente interacción entre PP2Cs y PYL1 ya que en el caso controlada por ABA en el caso de la proteína PYL1 y las proteínas PYLs 1 a 4 y, la interacción constitutiva, es decir, en ausencia de ABA de las proteínas PYLs 5,8 y 9.

Como se ha comentado previamente, las proteínas PYR/PYL/RCAR son miembros de una superfamilia de proteínas de unión a ligandos solubles denominada superfamilia de proteínas con dominio START [93] y, más recientemente nombrada como superfamilia Bet v 1 en reconocimiento a un dominio conservado que se identificó en abedul (*Betula verrucosa*) [194]. Las proteínas que poseen un dominio START se caracterizan por tener un plegamiento que forma un bolsillo central hidrofóbico al que se une el ligando [93]. En animales y plantas, otras proteínas con dominio START se han propuesto como hipotéticos receptores para la señalización de moléculas [147, 189, 217] sin embargo, las proteínas PYR/PYL son las únicas proteínas START descritas en la actualidad que funcionan directamente como receptores

involucrados en la transducción de señal y las únicas que funcionan como reguladoras de la actividad enzimática de PP2Cs.

PYL9 une (+)ABA con una Kd de 660nM, pero su afinidad por esta molécula se incrementa en un factor de 10 veces (Kd de 64nM) cuando se añade la PP2C ABI2 en el ensayo [139]. Esta interacción de tipo cooperativa también se ha visto con las proteínas PYL5 y HAB1 [213] lo que sugiere que es una característica común de las proteínas fosfatasas de tipo 2C mejorar la afinidad de unión por el ligando de las PYR/PYL. Incidiendo en este hecho, estudios estructurales de las proteínas PYR1, PYL1 y PYL2 solas y en complejos con ABA y PP2Cs han mostrado esta cooperatividad [149, 159, 171, 212, 273] Concretamente, estos estudios han explicado cómo el ABA se une a una cavidad central hidrofóbica de las proteínas PYR1, PYL1 y PYL2 induciendo un cambio conformacional en los dos bucles que flanquean el bolsillo hidrofóbico donde se aloja el ABA. Estos dos bucles denominados puerta y pestillo se cierran en respuesta a la unión de ABA lo que crea una superficie de interacción que favorece la unión de la PP2C. La PP2C a través de un residuo de triptófano (Trp_{385} de HAB1 y el Trp_{300} de ABI1) establece un puente de hidrógeno con una molécula de H_2O, la cuál a su vez forma un puente de hidrógeno con el grupo cetona del ABA [149, 159, 273].

En general las proteínas PYL unen de forma preferente la del estereoisómero (+)ABA natural, aunque algunas pueden unir (-)ABA con menor afinidad. Esto explica que se hubiese detectado bioactividad de la forma (-)ABA en ensayos anteriores (Figura 4).

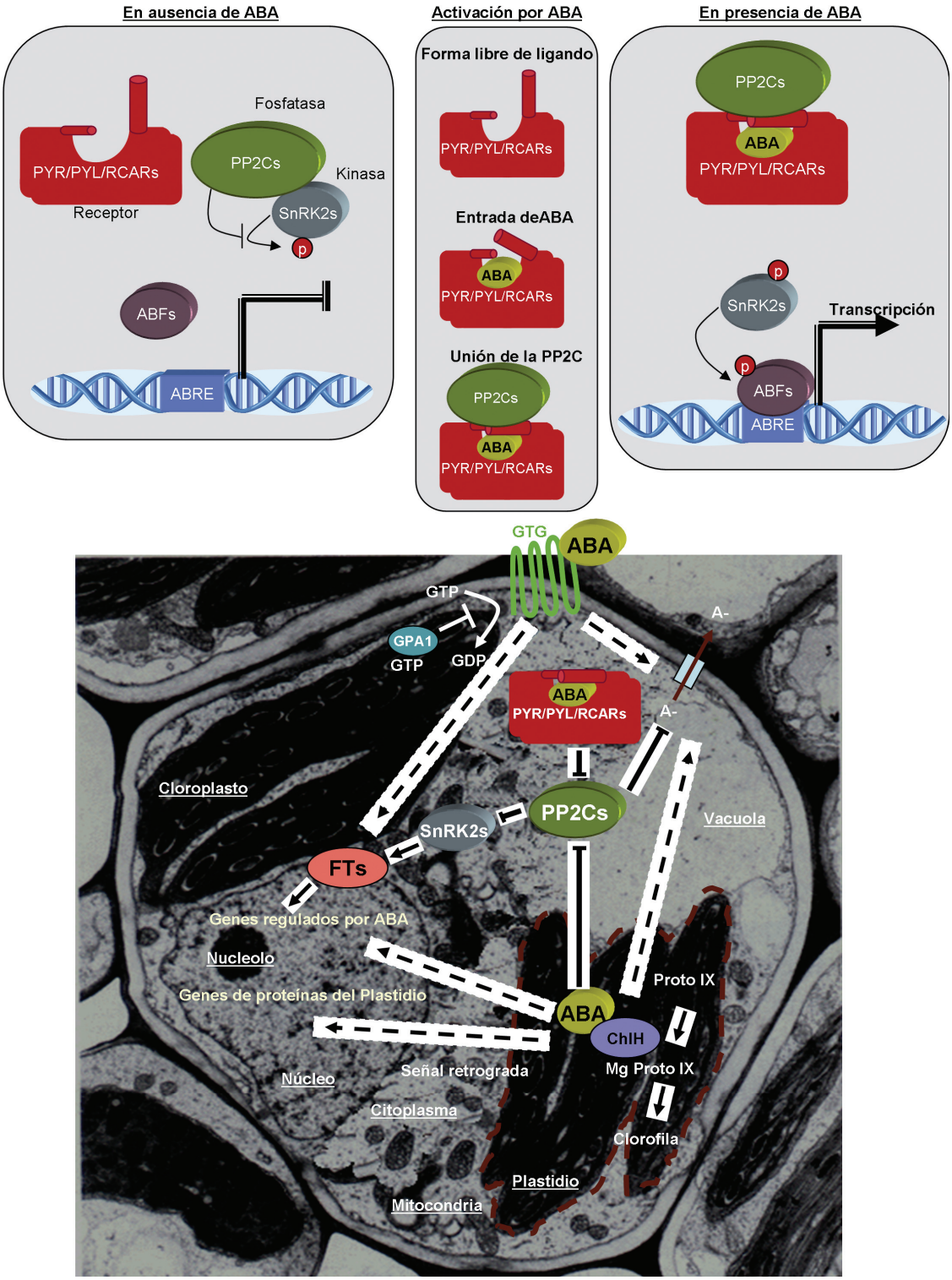


Figura 4. Esquema de la señalización por ABA. Arriba. Señalización por la familia del receptor de ABA PYR/PYL/RCAR (Pyrabactin resistance 1/Pyrabactin resistance 1-like/Regulatory Component of the ABA Receptor). Izquierda: En ausencia de ABA, el receptor PYR/PYL/RCARs no se encuentra unido a las fosfatasas PP2Cs (protein phosphatase 2C) por lo que la actividad PP2C es alta impidiendo la autofosforilación y activación de las quinasas de la familia SnRK2 (Snf1-related protein kinase). Centro: Mecanismo estructural de la acción del ABA. En la forma libre de ligando el receptor de ABA PYR/PYL/RCAR presenta una cavidad abierta y accesible con dos dominios proteicos que controlan la entrada a la cavidad, El ABA

produce cambios alostéricos del receptor que secuestran el ABA en el interior del receptor. La fosfatasa PP2C se une al receptor lo que impide que se una a sus sustratos. Derecha: En presencia de ABA, el receptor PYR/PYL/RCAR se une e inhibe las PP2Cs lo que permite la acumulación de SnRK2s fosforilado y la consiguiente fosforilación del los factores de unión a los elementos de respuesta a ABA (ABRE). Abajo. Modelo de las interacciones del ABA con varias clases de receptores. Los receptores de ABA identificados incluyen ChIH (plastid-localized magnesium cheletase), GTGs localizados en la membrana plasmática (GPCR type G-proteins) y nucleocitoplasmatico PYR/PYL/RCARs. Todas estas proteínas median efectos en la expresión de genes y el la electrofisiología de las células oclusivas (representado como canal A- para simplificar) No se conoce las interconexiones entre algunos de los factores. Líneas continuas indican interacciones directas y líneas discontinuas indican interacciones no conocidas. Las interacciones positivas se representan como flechas, las interacciones negativas como barras. Adaptado de Cutler y cols. Annu. Rev. Plant Biol 61:26.1-26.29 2010 y Sheard y Zeng 2009: Nature (462) 575-576 2009. Imagen de microscopía electrónica de una célula de una hoja de maíz modificada. http://library.thinkquest.org/3564/gallery.html

1.7.3. Papel de las proteínas fosfatasas en la señalización por ácido abscísico.

Las proteínas fosfatasas (PPs) se clasifican según su especificidad de sustrato en Serina-Treonina fosfatasas (Ser-Thr fosfatasas), Tirosina fosfatasas (Tyr fosfatasas) y fosfatasas de especificidad dual [138]. Las PPs se clasifican en 4 grupos principales: proteínas fosfatasa de tipo 1 (PP1), de tipo 2A (PP2A), de tipo 2B (PP2B) y de tipo 2C (PP2C). A diferencia de la mayoría de las enzimas, las proteínas fosfatasa de tipo Ser/Thr presentan una especificidad de sustrato muy amplia y solapante.

Las PP1 defosforilan la subunidad β de la fosforilasa quinasa (PHK) y son inhibidas por concentraciones nanomolares del inhibidor de proteínas termoestable 1 y 2. Las proteínas fosfatasa de tipo 2 defosforilan la subunidad α de la PHK y no son afectadas por los inhibidores mencionados anteriormente.

Las fosfatasas de tipo 2 están compuestas por 3 enzimas, PP2A, PP2B, y PP2C que se pueden distinguir por sus requerimientos catiónicos, PP2A, al igual que PP1, no necesita la presencia de cationes bivalentes mientras que PP2B y PP2C son Ca2+/Calmodulina y Mg2+ dependientes respectivamente [23]. Por otro lado el ácido okadaico, una toxina producida por dinoflagelados, inhibe la PP2A completamente a una concentración de 1nM. Concentraciones 10-15 nm inhiben la PP1 sin prácticamente inhibir a PP2B y sin afectar en absoluto a PP2C [23]. Las PP2A son holoenzimas compuestas por tres subunidades, una subunidad catalítica C que forma un complejo con la subunidad A cuya función es de andamiaje y que forman el núcleo de la enzima. A este complejo AC se le une la subunidad reguladora B.

El mutante roots curl in npa 1 (*rcn1*) esta afectado en la subunidad de andamiaje A, muestra niveles de actividad reducidos en un principio se aisló como un mutante en transporte de auxinas [60]. De hecho, las fosfatasas PP2A regulan el flujo de auxinas en la raíz a través de las proteínas PIN [154]. Además, la interrupción el gen RCN1 conlleva una mejora en la sensibilidad a etileno y reduce la sensibilidad a ABA en Arabidopsis, en concreto el mutante *rcn1* muestra insensibilidad a ABA en la respuesta en estomas debido a la ausencia de la activación de canales de aniones, sugiriendo que RCN1 funciona como un transductor positivo de la señalización por ABA [119, 124]. Volviendo a las características de las PP2As encontramos que en Arabidopsis existen 5 tipos de subunidad catalítica (PP2Ac-5), la mutación recesiva de PP2Ac-2 lleva a una mayor sensibilidad a ABA en distintos procesos [186] , lo cuál contrasta con el aparente papel de regulador positivo de RCN1. A pesar de que PP2Ac-2 es una subunidad catalítica con una aparente especificidad en la señalización por ABA, RCN1 muestra efectos pleiotrópicos en distintas rutas hormonales, lo que podría explicar su aparente paradoja. Por otro lado *pp2ac-2* suprime parcialmente el fenotipo de insensibilidad de *abi1-1D*, sugiriendo que ambas fosfatasas podrían actuar en la misma ruta de señalización por ABA [186]. El ácido okadaico es un inhibidor farmacológico de las fosfatasas PP1/PP2A que dependiendo de la especie inhibe o estimula la respuesta a ABA [69, 116, 185, 265], por ejemplo en Arabidopsis, el ácido okadaico inhibe parcialmente la activación por ABA de canales de aniones de tipo S y el cierre estomatal [185], induce el cierre estomatal en haba [216] y activa promotores de respuesta a ABA en hipocotilo de tomate [265]

No solo las PP2A están relacionadas con la ruta de señalización por ABA sino que las PP2Cs tienen un papel clave. Numerosas evidencias genéticas sugieren que las PP2Cs son reguladores negativos de la ruta de señalización de ABA y que esta función se encuentra conservada desde el musgo hasta Arabidopsis [110]

1.7.3.1. Papel de las fosfatasas de tipo 2C en la señalización por ácido abscísico.

La familia de proteínas fosfatasa PP2C esta integrada en Arabidopsis por 76 miembros [220] (Figura 5). Estas fosfatasas tienen un núcleo catalítico similar en tamaño y aminoácidos, con 11 motivos conservados [220]. El fragmento N-terminal de las PPs presenta baja similitud de secuencia y un tamaño variable, hecho que sugiere que esta zona de la proteína medie posiblemente la interacción con sustratos, proteínas reguladoras o segundos mensajeros [148, 203]. Apoyando esta posibilidad se ha observado que la región de unión del ácido fosfatídico a la proteína ABI1 está localizado en la zona N-terminal como indica que la mutación puntual R73A en ABI1 impida la unión de ABI1 con este ácido además de bloquear el efecto inhibitorio del ácido fosfatídico en la función fosfatasa ABI1 [158, 283]. Por otro lado el fragmento de ABI1 que comprende la región aminoacídica del 1 al 93 es suficiente para unir la proteína quinasa MAPK6 *in vitro* y en ensayos de doble híbrido [133]. A diferencia de otras fosfatasas, las PP2C son enzimas monoméricas que no están reguladas por proteínas inhibidoras o subunidades reguladoras, por lo tanto se piensa que estas proteínas son reguladas a diferentes niveles por interacción con diferentes sustratos u otras proteínas de señalización, por

expresión de la proteína, por compartimentalización, secuestro, proteolisis o por segundos mensajeros [220]. No obstante, este concepto debe cambiarse tras el descubrimiento de los receptores de ABA, ya que éstos regulan la actividad de las PP2Cs dependiente de ABA. Las PP2Cs generalmente se testan midiendo su capacidad de desfosforilar fosfo-caseína, un sustrato artificial usado en reacciones *in vitro*. A diferencia de otras PPs, las PP2C requirieren de Mn^{2+} o Mg^{2+} para su actividad, además de ser insensibles a inhibidores y toxinas como el ácido okadaico, que como se ha comentado antes es un inhibidor de PP1 y PP2A y otras fosfatasas [23]. Pese a requerir grandes concentraciones de Mn^{2+} o Mg^{2+} no se piensa que estas moléculas regulen la actividad de las PP2Cs puesto que las concentraciones de estos cationes a penas varía in vivo [220]. Otros posibles reguladores de estas proteínas son el balance redox que podría alterar residuos de cisteína [148] Ca^{2+}/calmodulina [239] o ácidos grasos como el ácido fosfatídico que se une directamente a ABI1, dando como resultado la disminución de la actividad fosfatasa y secuestro en la membrana plasmática que hace que se limite el acceso a los factores nucleares [106, 136], aunque es necesario un mayor número de experimentos para comprobar estas opciones.

Las PP2Cs están localizadas tanto en el núcleo como en el citosol, por lo que sería esperable que su función se ejerza en ambos compartimentos. Se podría establecer cierta jerarquía en cuanto a su función de acuerdo a los niveles de expresión génica, según sus patrones de expresión en distintos tejidos y en cuanto a los ensayos de respuesta a ABA llevados a cabo en líneas de pérdida de función en *pp2c* [207]

Dentro de las PP2Cs, la mayoría de las proteínas asociadas a la señalización por ABA pertenecen al grupo A [68, 115, 130, 150, 173, 207, 208, 276] mientras que los miembros del grupo B se caracterizan por tener homología con MPC2, una PP2C de alfalfa que regula la señalización por MAPK, y los componentes el grupo C incluyen fosfatasas del tipo POL (POLTERGEIST) que están involucradas en el desarrollo de las flores. El grupo A de PP2Cs esta dividido a su vez en 2 subgrupos, las ramas ABI1 y PP2CA.

Un característica a destacar en las PP2Cs que son represores en la ruta del ABA, es que tienen un funcionamiento análogo al de otras proteínas que intervienen como represores en otras rutas de señalización por hormonas como son las proteínas DELLA, Aux-/IAAs, JAZ o CTR que regulan respectivamente giberelinas, auxinas, ácido jasmónico, o señalización por etileno. Esas rutas de señalización tienen una característica común y es un mecanismo de represión de la señalización, o bien por regulación de la actividad o por degradación proteolítica. En el caso de la señalización por ABA, la inhibición de la función de las PP2Cs está dirigido por las proteínas PYL/PYR/RCAR que son los receptores que conectan la percepción de ABA con la liberación de las PP2C como sistema fisiológico de freno [184, 213]

Experimentos con los mutantes *abi1-1D* y *abi2-1D*, la reversión de estos mutantes, estudios de expresión transitoria y el análisis novedoso en el campo de la señalización por ABA que se presenta en esta tesis de plantas transgénicas con pérdida de función en PP2C mediante mutagénesis insercional de T-DNA, junto con la ganancia de función para la PP2C HAB1, han mostrado que las PP2Cs actúan como reguladores negativos de la señalización por ABA [68, 150, 203, 208, 227]. Además experimentos de

expresión transitoria atribuyen específicamente este papel a las PP2Cs similares a ABI pero no las PP2C similares a KAPP o a otras fosfatasas como PP1, PP2A o PP2B [227]. En más detalle, las primeras evidencias que mostraban que las fosfatasas de tipo PP2C estaban implicadas en la regulación de la señalización por ABA se obtuvieron por la identificación de los mutantes *abi1-1D* y *abi2-1D*, que eran insensibles a ABA, y la clonación de su correspondiente loci [112, 131, 152, 203]. Estas mutaciones son dominantes y llevan a una gran cantidad de alteraciones fenotípicas incluyendo resistencia a ABA en germinación de la semilla y crecimiento de la plántula junto con dormición de la semilla reducida, regulación estomatal anormal y defectos en respuesta a distintos tipos de estrés [203]. El aislamiento y análisis de los mutantes revertientes de *abi1-1D* y *abi2-1D* [68] permitieron corroborar que ABI1 y ABI2 regulaban negativamente la señalización por ABA. La caracterización de los alelos de pérdida de función mostraron que ABI1 y ABI2 eran inhibidores de la señalización por ABA con funciones solapantes. Mutaciones en la parte catalítica de ABI1 y ABI2 producen una perdida de función de la proteína casi total confiriendo una heredabilidad recesiva causante de un fenotipo hipersensible a ABA. ABI1 y ABI2 contribuyen en un 50% de la actividad PP2C inducida por ABA poniendo de relieve que otras PP2Cs están implicadas en la señalización por ABA [150]. Contando a ABI1 y ABI2 se ha demostrado que hay al menos seis PP2Cs en Arabidopsis thaliana, incluyendo AHG1 (ABA-HYPERSENSITIVE GERMINATION1), PP2CA/AHG3, HAB1 (HYPERSENSITIVE TO ABA1) y HAB2 siendo todos reguladores negativos de la señalización por ABA [65, 68, 115, 130, 150, 173, 208, 209, 238, 276]. AHG1 y PP2CA/AHG3 (ABA-HYPERSENSITIVE AT

GERMINATION) parecen tener un papel relevante en la señalización por ABA que controla germinación y crecimiento postgerminación ([115, 173, 276]. Además, PP2CA/AHG3 tiene un papel importante en la señalización por ABA en semilla y tejido vegetativo como muestran la hipersensibilidad a ABA en germinación, crecimiento y cierre estomatal del mutante *pp2ca*. Por el contrario, la sobre expresión de PP2CA bajo el control del promotor 35S produce insensibilidad a ABA en germinación de la semilla y cierre estomatal inducido por ABA [115], confirmando resultados previos que mostraban que la sobre expresión de PP2CA podía bloquear la inducción de genes por ABA en protoplastos [227]. La reducción de expresión de ABI1, ABI2 y HAB1 o la pérdida de función alélica de estas fosfatasas tiene efectos fenotípicos a diferentes niveles en la señalización por ABA como muestra el análisis de dobles mutantes que se presenta en esta tesis, poniendo de manifiesto la redundancia funcional entre alguna de ellas [150, 209]. Un ejemplo de esta redundancia funcional se observa por la doble inactivación de HAB1 y de ABI1 que produce una respuesta a ABA de índole mayor que la observada en los mutantes monogénicos de *hab1-1* o *abi1-2*. Un resultado similar se obtuvo con la combinación de los alelos revertientes *abi1-1R4* y *abi2-1R1* [150]. Por lo tanto la inactivación combinada de miembros cercanos de una familia génica es necesaria para desenmarañar la redundancia funcional genética y para establecer la jerarquía entre los diferentes miembros. Corrobora esta posibilidad los experimentos realizados en vías de señalización hormonal [2, 75, 83, 92, 120]. De forma alternativa el empleo de aproximaciones donde se realice la ganancia de función de una proteína pueden circunvalar redundancia genética como se deduce del fenotipo de insensibilidad a ABA global que se ha

encontrado en los dominantes negativos *abi1-1D* y *abi2-1D* así como en las líneas transgénicas *35S:HAB1*, *35S: PP2CA* y *35S:hab1Gly-246Asp* [112, 115, 201, 208].

El estudio de triple mutantes en PP2Cs, *hab1-1abi1-2abi2-2* y *hab1-1abi1-2pp2ca-1* realizado en esta memoria permite confirmar la redundancia funcional de estas proteínas y la precisa respuesta que estas proteínas realizan en la señalización por ABA ya que estos mutantes muestran una respuesta extrema a ABA exógeno, un crecimiento alterado y una respuesta constitutiva a ABA endógeno [207].

Pese a la importancia de las PP2C en la regulación de la señalización por ABA solo recientemente se han identificado los sustratos de estas proteínas. En esta línea, recientemente Umezawa y cols. han mostrado que un triple mutante SnRK2.2, SnRK2.3 y OST1/SnRK2.6 (sk2dei) presenta insensibilidad a ABA, resultado también obtenido previamente [56, 165], además de una reducción muy significativa de la actividad quinasa. Este trabajo describe la interacción entre OST1/SnRK2.6 y otras PPC2, especialmente del grupo A, como ABI2 y At5g59200 [248, 255]. Este estudio muestra que las PP2Cs del grupo A que interaccionan con OST1/SnRK2.6 producen la inactivación de las SnRK2 de subclase III por la defosforilación directa de estas proteínas. Finalmente estos autores proponen el siguiente modelo que combina los resultados de la regulación negativa de la señalización por ABA de las PP2Cs de clase A con sus hallazgos del efecto de las PP2C en estas quinasas de tipo SNRK2. En primer lugar las PP2Cs interaccionan con el dominio II en la zona C terminal de SnRK2 [275], de forma constante e independiente de ABA. En ausencia de ABA, las PP2Cs reprimen la

señalización por ABA mediante la desfosforilación e inactivación de las SnRK2s. Después de la inducción de ABA por estímulos ambientales o de desarrollo, RCAR/PYR se une a PP2C [139, 184] liberando y activando a SnRK2. Este hecho permite que SnRK2 fosforile a sustratos más abajo en la señalización incluyendo los factores de transcripción bZIP (AREB/ABFs) [57] entre otros para activar la expresión de genes de respuesta a ABA u otras respuestas. En sentido inverso, las mutaciones dominantes en PP2Cs como *abi1-1D* permite a la proteína evitar la unión a RCAR/PYR [139, 184] produciendo la inactivación de las SnRK2s incluso en presencia de ABA [248].

ABI1 y AB12 interaccionan con GPX3 (Glutathione Peroxidase 3) una proteína relacionada con la regulación de la transpiración en plantas [153] y con la quinasa PKS3/CIPK15/SnRK3.1 (CBL-INTERACTING PROTEIN KINASE) [71]. ABI1 por otro lado también interacciona con OST1 (OPEN STOMATA 1)/SnRK2.6 quinasa crucial en el control de los estomas, desfosforilando la Ser175 de OST1/SnRK2.6 [275]. ABI1 también interacciona con ATHB6 [76] y ABI2 con SALT OVERLY SENSITIVE (SOS2/SnRK3.11), una quinasa de la familia SnKR3 que juega un papel primordial en la tolerancia a la salinidad en Arabidopsis [178] a través de la regulación de un antiportador Na^+/H^+ que codifica *SOS1,* así como SnRK3.15/PKS24 [178] y Fibillina, una proteína de unión a lípidos que se induce en condiciones de estrés abiótico y que podría ser la clave para establecer un mecanismo de control mediado por ABA del estrés por luz guiado por la fotoinhibición regulada por PP2Cs [272], a pesar que estas dos proteínas se localizan en distintos compartimentos celulares lo que complica el modelo de regulación [272]. Finalmente, PP2CA interacciona con el canal de potasio AKT2, interacción que podría conectar el

ABA con el transporte de K^+ y la polarización de la membrana en situaciones de estrés [37] y at1g0743c, otra PP2C de la misma rama, interacciona a su vez con el canal de potasio AKT1 [127] (Figura 4).

Por otra parte se ha de mencionar que el inositol polifosfato 1'fosfatasa (SAL1/FIERY1),que funciona en el catabolismo del inositol 1,4,5-trifosfato (IP3) y que no es una fosfatasa, también actúa como regulador negativo de ABA y de la señalización por estrés en Arabidopsis [268]. El mutante *fry1/sal1* es capaz de acumular más cantidad de IP3 en respuesta a ABA, esto evidencia de forma genética la conexión entre el recambio de fosfoinositoles y la señalización por ABA. Recientemente se ha caracterizado el alelo *sal1*, (nombrado como *alx8*) así como la conexión de *SAL1* con una ruta de respuesta al estrés independiente de ABA [262].

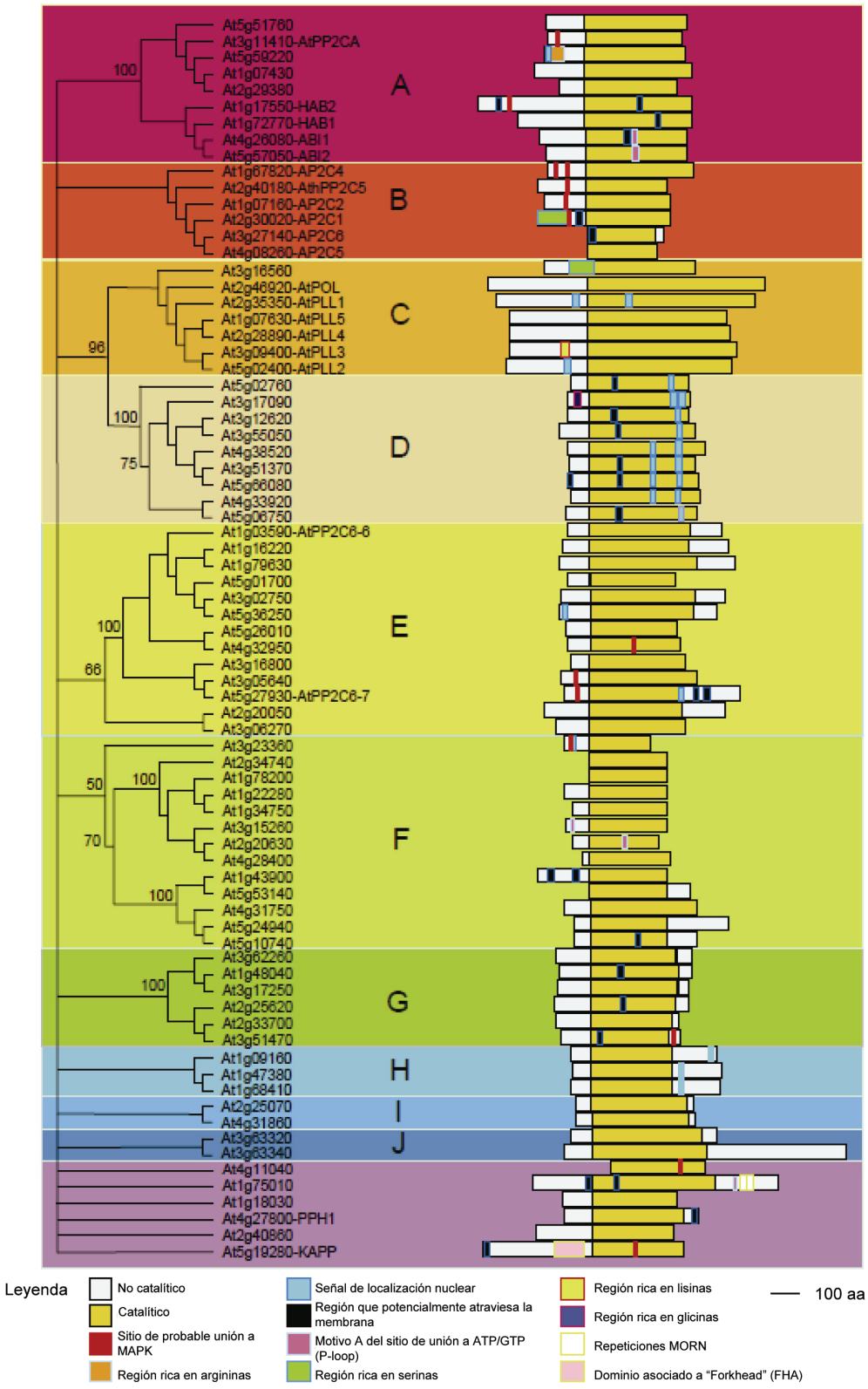


Figura 5. Cladograma topográfico y alineamiento de los dominios estructurales de las PP2Cs de Arabidopsis basándose en la secuencia aminoacídica usando el programa de análisis T-Cofee. Se agruparon 76 genes de Arabidopsis en 10 grupos (A-J), y seis genes no se agruparon. Adaptado de Schweighofer y cols. TRENDS in Plant Science. (9);5: 236-243 2004.

1.8. Expresión génica regulada por ácido abscísico.

La activación de las rutas de señalización controladas por ABA está asociada a cambios en la expresión génica. Estos cambios en la expresión génica contribuyen a tolerar condiciones de sequía tanto inducidas por el desarrollo como es el caso de la maduración del polen o de la semilla o por condiciones ambientales adversas como sequía, salinidad y bajas temperaturas. Varios estudios realizados en arroz y en Arabidopsis en condiciones de exposición a ABA y a varios tipos de estrés abiótico han mostrado que se produce un cambio en el 5 al 10% de los genes siendo la mitad de ellos comunes a condiciones de sequía, salinidad y tratamiento con ABA [166, 229]. En plántulas de Arabidopsis se ha observado que en alrededor del 10% del genoma hay genes regulados por ABA, entre genes inducidos y reprimidos, lo que supone entre 2 y 6 veces los genes regulados por la mayoría de otras hormonas en plantas [169]. Teniendo en cuenta datos de transcriptómica procedentes de semillas en imbibición, menos de dos terceras partes de los genes caracterizados como genes inducidos por ABA en plántulas, se corresponden con el mismo criterio en semillas, pero si coinciden los grupos funcionales a los que estos genes se encuentran inducidas tanto en plántulas como en semillas. Entre los genes inducidos destacan los que codifican a proteínas involucradas en la tolerancia al estrés como por ejemplo dehidrinas y enzimas de detoxificación de especies reactivas de oxígeno, enzimas pertenecientes al metabolismo de solutos compatibles, una variedad de transportadores, proteínas reguladoras como los factores de transcripción, proteínas quinasas y fosfatasas y, enzimas involucradas en señalización por fosfolípidos. Atendiendo a los genes que se encuentran reprimidos por ABA se

han observado proteínas asociadas al crecimiento, incluyendo proteínas de pared celular, proteínas del ribosoma, de membrana plasmática y de cloroplastos.

También se han realizado estudios de metabolómica para saber cómo afecta la expresión génica al metabolismo, en ellos se ha comprobado que el ABA regula cambios globales en condiciones de deshidratación que afectan a la síntesis de glucosa, al metabolismo de aminoácidos alifáticos como la valina, leucina e isoleucina, un precursor de lisina, prolina y poliaminas [250].

Por otro lado se han estudiado con técnicas de transcriptómica los efectos de la regulación por ABA en genes involucrados con condiciones de estrés utilizando arrays observándose que hay más de 8000 unidades transcripcionales sin anotar presentes en regiones intergénicas. De un 5% al 10% de esas unidades transcripcionales están reguladas por ABA [145, 281]

1.9. Elementos *cis* y *trans* de la señalización por ABA

La regulación génica la llevan a cabo una serie de factores de transcripción que son regulados por la señalización por ABA [31, 50, 77, 166]. Las principales secuencias cis que confieren inducibilidad por ABA son los elementos de respuesta a ABA ABRE (T/CACGTGGC), que son reconocidos por la familia de factores de transcripción AREB/ABF [31, 38].

Entre los reguladores que activan la transcripción se encuentran las proteínas con elementos de respuesta a ABA en promotores (ABA-responsive element binding proteins) (ABI5/ABF/AREB/Bzip family) [8, 31, 38, 51, 249], ABI3 de *Arabidopsis thaliana*, el factor de transcripción VP1 (VIVIPAROUS1) de la familia de factores de transcripción con dominios B3 en maíz (Zea mays)

[63, 146], el factor de transcripción ABI4 de la familia de factores de transcripción con dominios APETALA2 [52] y los factores de transcripción ATMYC2 y ATMYB2 [1]. Además existen otros factores de transcripción que reprimen la actividad transcripcional [77, 181, 231]. A pesar del gran número de genes reprimidos por ABA, el mecanismo de represión no está tan bien caracterizado como para los genes inducidos por ABA [31]. Algunos factores de transcripción del tipo VP1 y ABI4 actúan tanto como activadores que como represores sobre diferentes genes diana [11, 80].

El estrés osmótico activa otros factores de transcripción que incluyen las proteínas ZFHD (ZINC FINGER HOMEODOMAIN) y las proteínas NAC (NAM TAF CUC2). ZFHD1 se une al motivo CACTAAATTGTCAC, denominado ZFHDR en la región del promotor de *ERD1* (*EARLY RESPONSE TO DEHYDRATION* 1). Las proteínas NAC reconocen secuencias diana del tipo MYC y activan *ERD1* [79, 245, 246] (Figura 6)

La unión del factor de transcripción requiere una cromatina accesible o abierta y como se ha comentado anteriormente algunas de las fosfatasa relacionadas con la ruta de señalización por ABA interaccionan directamente con un homólogo de SWI3, un componente del complejo remodelador de cromatina SWI/SNF y su interacción bloquea completamente la inducción de un subgrupo de genes regulados por ABA [210]. Al menos un miembro de la familia de PP2Cs, la fosfatasa HAB1, el ABA libera esta represión por inhibición de la actividad fosfatasa que se requiere para la interacción con SWI3B.

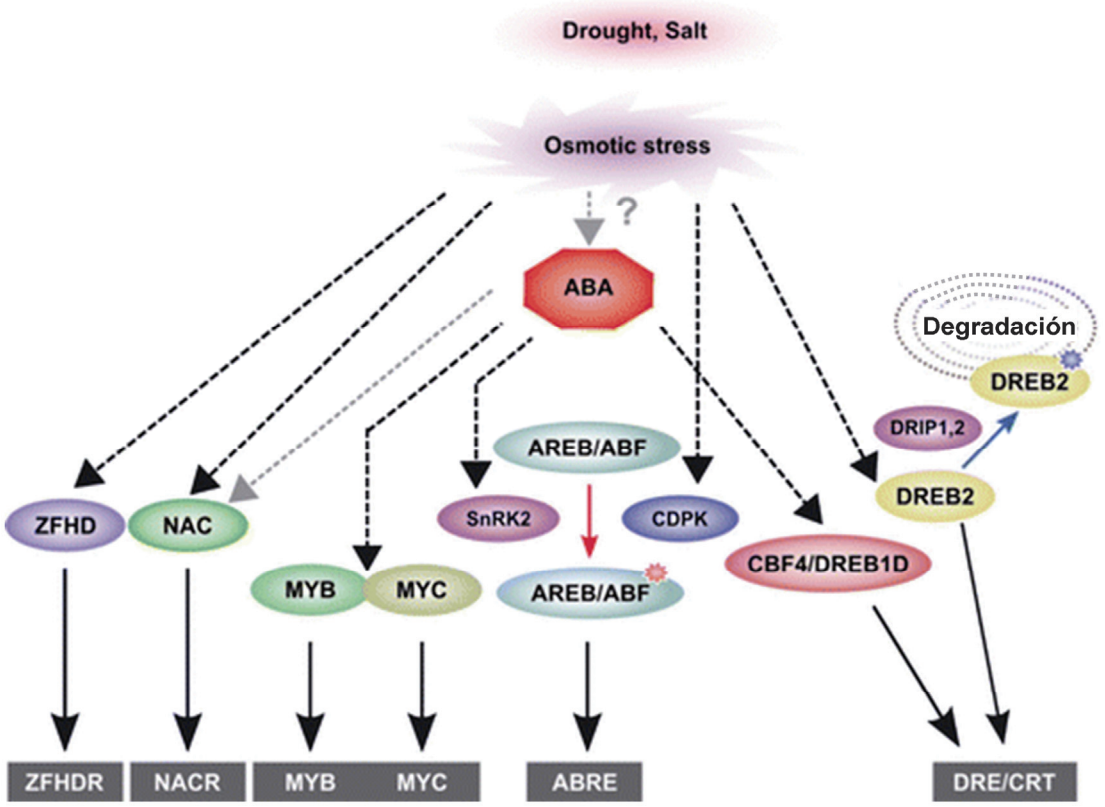
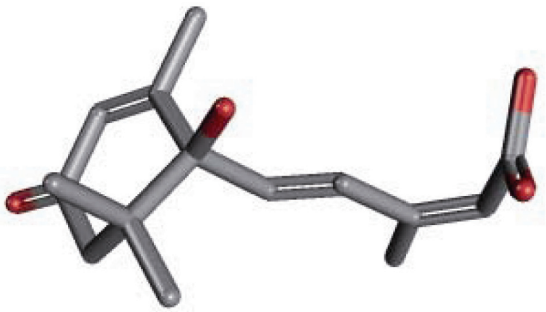


Figura 6. Red de regulación transcripcional en respuesta a estrés provocado por sequía y salinidad. Los objetos elípticos y las cajas grises indican proteínas funcionales y elementos *cis*, respectivamente. Líneas continuas y líneas discontinuas representan uniones directas e indirectas, respectivamente. Líneas coloreadas indican modificaciones. Líneas punteadas grises indican conexiones no conocidas. Adaptado de Hirayama y Shinozaki, The Plant Journal 61, 1041-1052, 2010



Objetivos

1. Caracterización del papel de la PP2C del grupo A HAB1 en la ruta del transducción de señal del ácido abscísico.

Estudio del promotor de *HAB1* mediante la generación y caracterización de líneas transgénicas $Pro_{HAB1:}$GFP.

Análisis de expresión del RNAm de *HAB1* y de otras PP2Cs en tratamientos con ABA exógeno.

Estudios de genética reversa con la identificación y posterior caracterización de líneas de pérdida de función *hab1-1* y con la generación y el análisis de líneas de ganancia de función *35S::HAB1*.

Complementación del mutante de pérdida de función HAB1 con la generación de las plantas *hab1-1::HAB1 cDNA*.

Análisis de la expresión de genes de respuesta a ABA en plantas *35S::HAB1*.

Ensayos de germinación y establecimiento de plántula en presencia de ABA exógeno.

Ensayos de germinación dosis-respuesta en presencia de NaCl, manitol y paclobutrazol.

Ensayos de crecimiento de raíz.

Ensayos de pérdida de agua por transpiración.

2. Búsqueda de las posibles dianas de interacción de la PP2C del grupo A HAB1.

Rastreo de doble híbrido en levaduras utilizando la región catalítica de *HAB1* como cebo frente a una librería de cDNA de Arabidopsis como presa.

Identificación de AtSWI3B como diana prevalente de interacción con HAB1.

Mapeo del dominio de interacción. Localización subcelular de HAB1 y SWI3B. Interacción de HAB1 y SWI3B *in planta* mediante complementación bimolecular de la fluorescencia (BiFC).

Confirmación de la interacción por coinmunoprecipitación de HAB1 y SWI3B en extractos de *Nicotiana benthamiana* procedentes de ensayos de BIFC.

Inmunoprecipitación de cromatina en líneas transgénicas generadas con un doble epítopo de hemaglutinina *proHAB1::HAB1dHA*.

Ensayos de germinación y crecimiento de mutantes *swi3b-1+/-* y *swi3b-2+/-* y en mutantes de TILLING *swi3b3-3* y *swi3b-4*.

3. Contribución de HAB1, ABI1, ABI2 y PP2CA a la señalización por ABA. Determinación de las funciones aditivas o redundantes de las PP2Cs HAB1 y ABI1, ABI2 y PP2CA en la ruta de señalización por ABA. Estudio de la interacción genética entre ABI1/HAB1 y PP2CA.

Generación de una colección de mutantes de pérdida de función en PP2Cs.

Aislamiento y caracterización de alelos pérdida de función de *abi1-2* y *abi2-2.*

Caracterización de mutantes sencillos, dobles y triples de pérdida de función en los genes de las *PP2Cs HAB1, ABI1, ABI2 y PP2CA*

Generación y análisis de los dobles mutantes de pérdida de función *hab1-1abi1-2, hab1-1abi1-3*, *hab1-1pp2ca* y *abi1-2pp2ca-1.*

Generación y análisis de los triples mutantes *hab1-1abi1-2pp2ca-1* y *hab1-1abi1-2abi2-2*

Análisis de la expresión génica de *HAB1*, *PP2CA*, *ABI1* y *ABI2* mediante qRT-PCR en los mutantes sencillos, dobles y triples.

Expresión de genes de respuesta a ABA en mutantes sencillos, dobles y triples

Ensayos de germinación y establecimiento de plántula en presencia de ABA exógeno. Ensayos de germinación dosis-respuesta en presencia de NaCl, manitol.

Cuantificación de peso fresco, superficie foliar y crecimiento radicular en distintas condiciones.

Medidas de apertura en estomas.

Medidas de conductancia e intercambio de gases a través de las hojas.

Estudio de la expresión génica con microarrays.

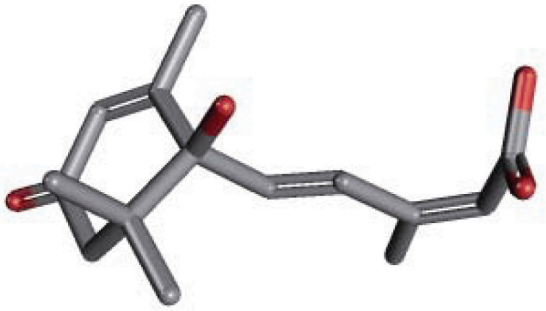
4. La inactivación combinada de PP2Cs del grupo A como herramienta biotecnológica para incrementar la tolerancia a sequía en plantas.

Generación y análisis de los dobles mutantes de pérdida de función *hab1-1abi1-2* y *hab1-1abi1-3*.

Medidas de apertura de estomas inducida por ABA.

Ensayos de pérdida de agua en hojas cortadas.

Ensayos de pérdida de agua por transpiración en condiciones de sequía.



Capítulo I

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Gain-of-function and loss-of-function phenotypes of the protein phosphatase 2C *HAB1* reveal its role as a negative regulator of abscisic acid signalling

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Summary

***HAB1* was originally cloned on the basis of sequence homology to *ABI1* and *ABI2*, and indeed, a multiple sequence alignment of 32 *Arabidopsis* protein phosphatases type-2C (PP2Cs) reveals a cluster composed by the four closely related proteins, ABI1, ABI2, HAB1 and At1g17550 (here named HAB2). Characterisation of transgenic plants harbouring a transcriptional fusion Pro_{HAB1}: green fluorescent protein (*GFP*) indicates that *HAB1* is broadly expressed within the plant, including key target sites of abscisic acid (ABA) action as guard cells or seeds. The expression of the *HAB1* mRNA in vegetative tissues is strongly upregulated in response to exogenous ABA. In this work, we show that constitutive expression of *HAB1* in *Arabidopsis* under a cauliflower mosaic virus (CaMV) 35S promoter led to reduced ABA sensitivity both in seeds and vegetative tissues, compared to wild-type plants. Thus, in the field of ABA signalling, this work represents an example of a stable phenotype *in planta* after sustained overexpression of a *PP2C* genes. Additionally, a recessive T-DNA insertion mutant of *HAB1* was analysed in this work, whereas previous studies of recessive alleles of *PP2C* genes were carried out with intragenic revertants of the *abi1-1* and *abi2-1* mutants that carry missense mutations in conserved regions of the PP2C domain. In the presence of exogenous ABA, *hab1-1* mutant shows ABA-hypersensitive inhibition of seed germination; however, its transpiration rate was similar to that of wild-type plants. The ABA-hypersensitive phenotype of *hab1-1* seeds together with the reduced ABA sensitivity of *35S:HAB1* plants are consistent with a role of *HAB1* as a negative regulator of ABA signalling. Finally, these results provide new genetic evidence on the function of a PP2C in ABA signalling.**

Keywords: ABA, PP2C, HAB1, negative regulation, *Arabidopsis*.

Introduction

Abscisic acid (ABA) plays a crucial role in seed development, as well as in the regulation of plant responses to adverse environmental conditions (Koornneef and Karssen, 1994; Leung and Giraudat, 1998; Rock and Quatrano, 1995; Zeevaart and Creelman, 1988). During seed development, ABA regulates the synthesis of seed storage proteins and lipids, desiccation tolerance, as well as the promotion of seed dormancy. The role of ABA as a mediator of physiological responses to low-water potential situations is further evidenced genetically by ABA-insensitive and ABA-deficient mutants, which are hypersensitive to drought and salinity (Leung and Giraudat, 1998). Water stress results in the increase of up to 40-fold in ABA levels, leading to a variety of adaptive responses, such as stomatal closure and differential gene expression (Zeevaart, 1999). Stomatal closure occurs through rapid changes of ion fluxes in guard cells and osmoregulation (reviewed by Schroeder *et al.*, 2001). A variety of second messengers contribute to the transmission of the ABA signal. These include cyclic ADP-ribose, reactive oxygen species (ROS), phosphoinositides, phosphatidic acid, sphingosine 1-phosphate, and Ca^{2+} (Allen *et al.*, 2001; Gilroy *et al.*, 1990; Jacob

et al., 1999; Leckie *et al.*, 1998; Lemtiri-Chlieh *et al.*, 2000; Ng *et al.*, 2001; Pei *et al.*, 2000; Wu *et al.*, 1997). Downstream signalling involves a complex network of both positive and negative regulators, particularly protein kinases/phosphatases, and a plethora of transcriptional regulators (reviewed by Finkelstein *et al.*, 2002). Mutations in the RNA-binding proteins hyponastic leaves 1 (HYL1), ABA-hypersensitive 1 (ABH1) or supersensitive to ABA and drought 1 (SAD1) also affect ABA signalling (Hugouvieux *et al.*, 2001; Lu and Fedoroff, 2000; Xiong *et al.*, 2001).

Protein phosphorylation/de-phosphorylation events in ABA signalling involve several known protein kinases and phosphatases (Finkelstein *et al.*, 2002; Leung and Giraudat, 1998). For instance, the guard-cell-specific protein kinase ABA-activated protein kinase (AAPK) from *Vicia faba* regulates ABA-induced stomatal closure (Li *et al.*, 2000). Expression of a dominant negative *AAPK* allele renders stomata insensitive to ABA-induced closure through inhibition of plasma membrane anion channels (Li *et al.*, 2000). Recently, the *Arabidopsis* open stromata 1 (OST1) protein kinase, which is related to AAPK, was shown to mediate the regulation of stomatal aperture by ABA (Mustilli *et al.*, 2002). ABA-induced ROS production was disrupted in *ost1* guard cells, indicating that OST1 acts upstream of ROS production (Mustilli *et al.*, 2002). Another protein kinase involved in ABA signalling is ABA-induced protein kinase 1 (PKABA1), which is induced by ABA and which suppresses gibberellin (GA)-inducible gene expression in barley aleurone layers (Gomez-Cadenas *et al.*, 1999). In addition to calcium-independent protein kinases, the calcium-dependent protein kinases (CDPK)1 and CDPK1a also mediate ABA signalling, as expression of constitutively active CDPK1 and CDPK1a leads to activation of the ABA-inducible *Hordeum vulgare* ABA-responsive 1 (*HVA1*) promoter (Sheen, 1996).

Conversely, ABA signalling is also regulated by several protein serine/threonine phosphatases (PP). PP are classified into four major classes, PP1, PP2A, PP2B and protein phosphatases type-2C (PP2C), on the basis of their substrate specificities, divalent cation requirements and sensitivity to inhibitors (Cohen, 1989). The use of okadaic acid (an inhibitor of PP1 and PP2A) enhances ABA-induced stomatal closing in fava bean (Schmidt *et al.*, 1995) and activates ABA-responsive promoters in tomato hypocotyls (Wu *et al.*, 1997). However, the same drug reduces ABA-induced stomatal closure in *Arabidopsis* (Pei *et al.*, 1997). Interestingly, it has been recently found that disruption of the PP2A regulatory subunit RCN1 confers ABA insensitivity in *Arabidopsis*, which suggests that RCN1 functions as a positive transducer of ABA signalling (Kwak *et al.*, 2002). Finally, inactivation of Ca^{2+}-sensitive inward K^+ channels in fava bean guard cells was prevented by inhibitors of PP2B (Luan *et al.*, 1993), and ABA responses in pea epidermal peels were reduced by an inhibitor of PP2B (Hey *et al.*, 1997). However, these results have to be interpreted with caution as no catalytic PP2B subunit is found in the *Arabidopsis* genome (Kerk *et al.*, 2002).

In case of PP2C, where no pharmacological inhibitor is available, genetic evidence has implicated three *Arabidopsis* PP2Cs (ABI1, ABI2 and PP2CA) as negative regulators of the ABA signal pathway (Gosti *et al.*, 1999; Merlot *et al.*, 2001; Tahtiharju and Palva, 2001). Whereas ABI1 and ABI2 are central regulators of ABA signalling both in seeds and vegetative tissues (Leung *et al.*, 1994, 1997; Meyer *et al.*, 1994; Rodriguez *et al.*, 1998a), PP2CA does not appear to regulate ABA signalling in dehydration responses (Tahtiharju and Palva, 2001). Additionally, PP2CA regulates the activity of the K^+ channel AKT2 (Cherel *et al.*, 2002). Other plant PP2Cs are involved in different signal transduction processes. Thus, the kinase-associated protein phosphatase (KAPP) protein phosphatase functions as a negative regulator of at least two receptor-like kinase (RLK) systems (Stone *et al.*, 1994; Williams *et al.*, 1997), and *in vitro* studies suggest that it could be a common component of more (Becraft, 2002). The *Medicago* protein phosphatase type-2C (MP2C) acts as a negative regulator of a stress-activated mitogen-activated protein kinase (MAPK) pathway (Meskiene *et al.*, 1998, 2003).

In this work, we present evidence for a novel *PP2C* gene, named *HAB1,* functioning as a negative regulator of ABA signalling. *HAB1* was originally cloned on the basis of sequence homology to *ABI1* and *ABI2*, and it was tentatively named *AtP2C-HA* for homology to *ABI1*/*ABI2* (Rodriguez *et al.*, 1998b). Following standards for nomenclature (Meinke and Koornneef, 1997), we have re-named the gene as *AtP2C-HAB1* and abbreviated it as *HAB1*. *HAB1* encodes a functional PP2C (Meskiene *et al.*, 2003), whose expression was previously detected in root, leaf, stem, flower and silique (Rodriguez *et al.*, 1998b). In this work, a detailed analysis of transgenic plants harbouring a transcriptional fusion between the green fluorescent protein (GFP) reporter gene and the *HAB1* promoter was performed. As a result, we could confirm that *HAB1* is expressed ubiquitously in the plant, including key target sites of ABA action, such as guard cells and seeds. *HAB1* expression in vegetative tissues was found to be low in the absence of ABA and was strongly upregulated by ABA (Rodriguez *et al.*, 1998b); however, genetic evidence involving this gene in ABA signalling was lacking. In this work, we show that transgenic *Arabidopsis* plants, expressing a *35S:HAB1* transgene, show a remarkable ABA insensitivity. Blockage of ABA signalling by other PP2Cs (ABI1 and PP2CA) in transient overexpression experiments was previously reported by Sheen (1998). Our work on *HAB1* and the one recently published by Gonzalez-Garcia *et al.* (2003) on *FsPP2C1* further show reduced ABA signalling *in planta* upon sustained overexpression of *PP2C* genes. Conversely, a T-DNA disrupted allele of *HAB1* (here named *hab1-1*)

displays enhanced response to ABA in seeds. Both phenotypes support the role of *HAB1* as a negative regulator of ABA signalling.

Results

HAB1 *in the context of the complex family of plant PP2Cs*

At least 69 PP2C candidate gene products can be found in the genome of *Arabidopsis* (Kerk *et al*., 2002) and additional PP2Cs have been described in other plant species (Lorenzo *et al*., 2001, 2002; Meskiene *et al*., 1998; Miyazaki *et al*., 1999). A full alignment including 169 PP2Cs has recently been reported by Kerk *et al*. (2002). This complex alignment, in addition to plant PP2Cs, includes bacterial, fungal and animal PP2Cs. Therefore, in order to provide a comprehensive overview on the sequence similarity among HAB1 and a representative subset of plant PP2Cs, we compiled a partial alignment including 32 *Arabidopsis* PP2Cs, as well as 7 PP2Cs from other plant species (Figure 1).

The alignment of the catalytic cores of these 39 PP2Cs defines six distinct clusters. Physiological characterisation of PP2Cs from cluster #1 has not yet been reported. Cluster #2 includes the recently characterised POLTERGEIST PP2C (Yu *et al*., 2003). In the case of cluster #3, the single distinctive feature shared by the members of the group is the presence of two bipartite nuclear targeting sequences next to the C-terminal region of the gene product (Lorenzo *et al*., 2002). However, experimental evidence on the subcellular localisation of this group of PP2Cs is lacking. Cluster #4 includes the *Arabidopsis* KAPP and KAPP-like proteins from rice and maize. Cluster #6 includes, as a representative member, the well-characterised alfalfa MP2C enzyme (Meskiene *et al*., 1998, 2003).

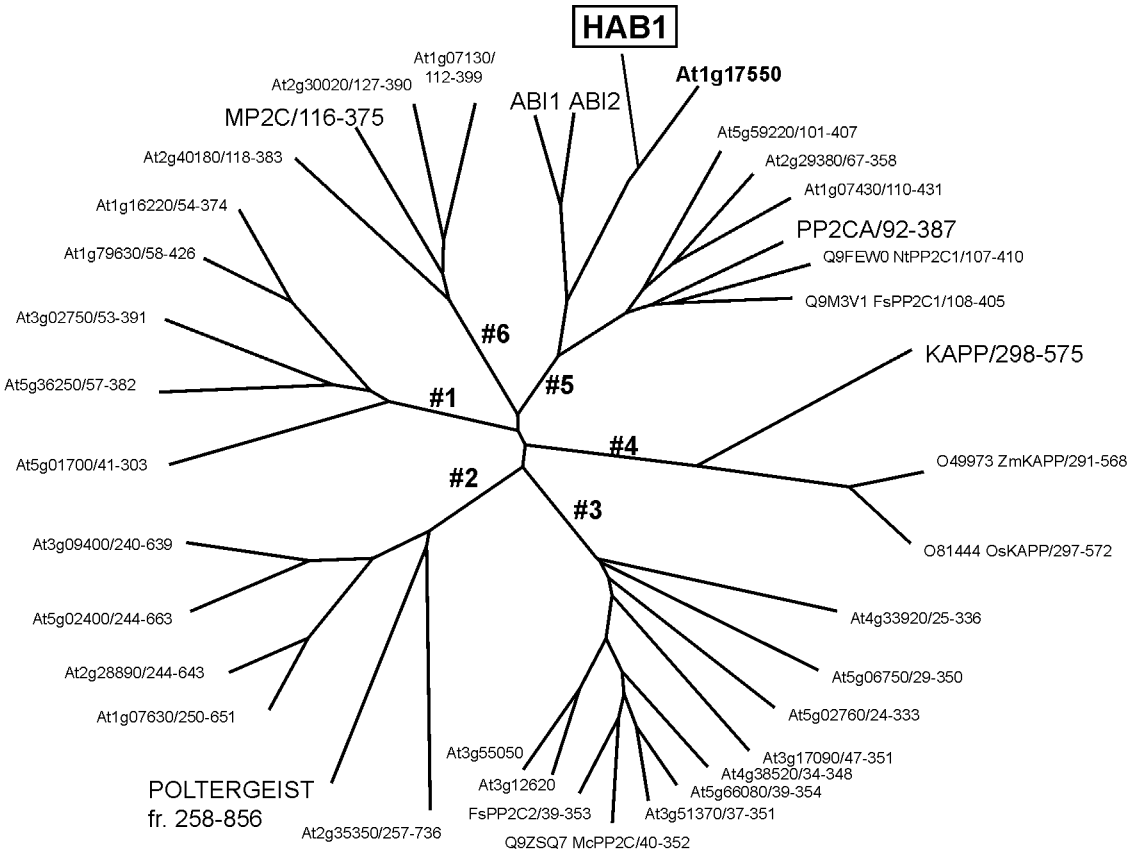

Figure 1. HAB1 in the context of plant PP2Cs.
Radial phylogenetic tree resulting from the alignment of the catalytic cores of 32 *Arabidopsis* PP2Cs: *Medicago sativa*, MP2C (O24078); *Fagus sylvatica*, FsPP2C1 (Q9M3V0) and FsPP2C2 (Q9M3V1); *Mesembryanthemum crystallinum*, McPP2C (Q9ZSQ7); *Zea mays*, ZmKAPP (O49973); *Oryza sativa*, OsKAPP (O81444); and *Nicotiana tabacum*, NtPP2C1 (Q9FEW0). A PSI-BLAST search for sequence similarity in TAIR and NCBI databases was performed using the amino acid sequence of HAB1 as a query. Representative members of the plant PP2C family were gathered and aligned with CLUSTALX 1.81 using the amino acid range indicated after the identifier. In the case of HAB1, At1g17550, ABI1 and ABI2, the amino acid range used was 180–511, 179–511, 118–434 and 103–423, respectively. Finally, a radial tree was generated and displayed with TREEVIEW 3.2. The AGI identifiers for *Arabidopsis* PP2Cs and SWISS-PROT TrEMBL (SPTREMBL) protein entries for PP2Cs from other plant species are indicated. *Arabidopsis* Genome Initiative (AGI) identifiers for ABI1, ABI2, HAB1, PP2CA, KAPP and POLTERGEIST are At4g26080, At5g57050, At1g72770, At3g11410, At5g19280 and At2g46920, respectively.

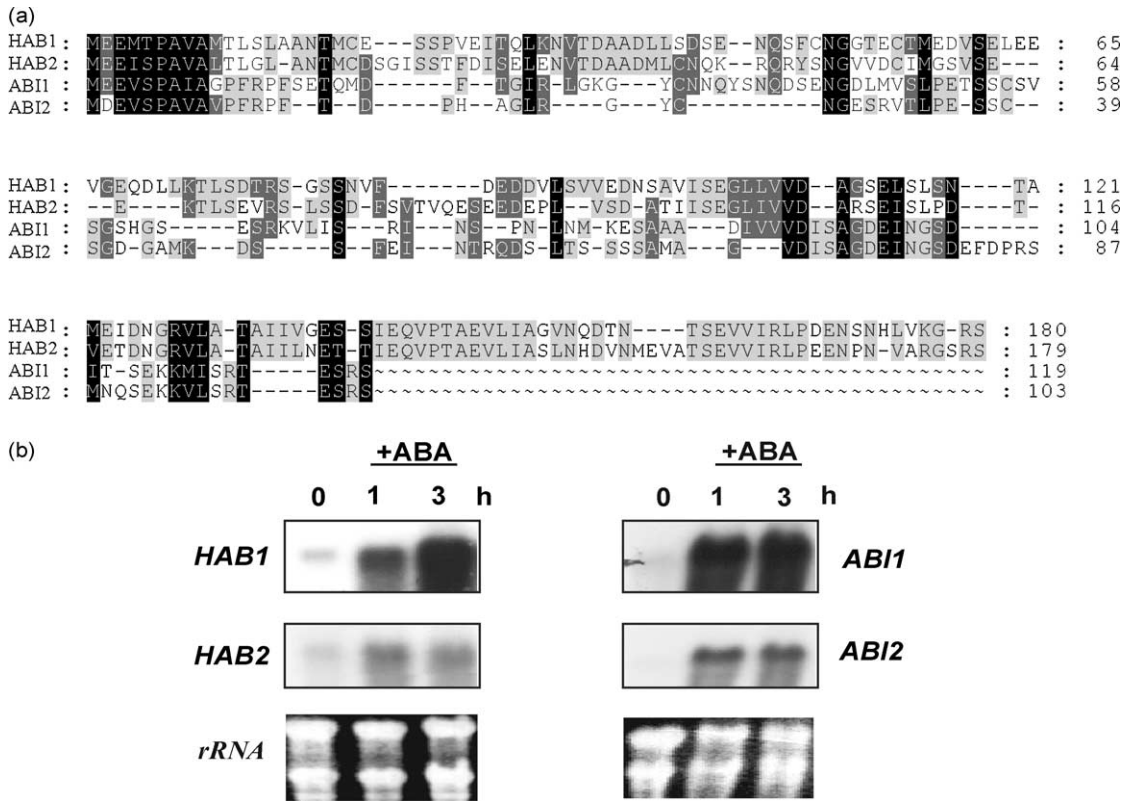

Figure 2. Sequence similarity at the N-terminal extension of *HAB1, HAB2, ABI1* and *ABI2* gene products; ABA-mediated mRNA accumulation.
(a) The N-terminal extensions of the proteins were aligned using the PILEUP program and Genedoc software. Positions with identical amino acid residues or conservative substitutions are highlighted in black, where they match all proteins.
(b) ABA induction of the *HAB1, HAB2, ABI1* and *ABI2* transcripts. A time-course induction at 0, 1 and 3 h after 50 μM ABA treatment is shown. Each track of the blot contained approximately 20 μg total RNA. The loading of the gel was visualised by ethidium bromide staining.

Finally, cluster #5 contains PP2Cs involved in ABA signalling, such as ABI1, ABI2 and PP2CA. Two sub-branches can be distinguished, one including several PP2CA-like proteins and the other including ABI1, ABI2, HAB1 and At1g17550. Pair-wise comparisons further subdivide this latter sub-branch into two pairs of PP2Cs, i.e. ABI1/ABI2 and HAB1/At1g17550 (Figure 1). Indeed, overall amino acid sequence comparison between HAB1 and At1g17550 reveals 75% identity. Moreover, the N-terminal extension of HAB1 exhibits 60% identity to the corresponding one from At1g17550, and some stretches showing amino acid sequence similarity at the N-terminus are apparent in the four PP2Cs (Figure 2a). From these data, a close functional relationship between *HAB1* and *At1g17550* can be predicted. Therefore, we propose to name *At1g17550* as *HAB2*. *ABI1*, *ABI2* and *HAB1* were reported to be upregulated by ABA (Leung *et al*., 1997; Rodriguez *et al*., 1998b). *HAB2* is also upregulated in response to ABA; however, its level of expression is lower than the one observed for *HAB1* (Figure 2b). An analogous situation occurs in the case of *ABI1*/*ABI2*, where the level of expression in response to ABA is notably higher for *ABI1* than *ABI2* (Figure 2b).

Tissue and cellular expression pattern of Pro_{HAB1}:GFP

Using Northern blot analysis, the *HAB1* mRNA was detected previously in root, leaf, stem, flower and silique (Rodriguez *et al*., 1998b). To further extend this analysis, a characterisation of transgenic plants harbouring a transcriptional fusion between the GFP reporter gene and the *HAB1* promoter was carried out. Ten transgenic lines were analysed and they showed similar patterns of GFP expression, which was upregulated by ABA in all the cases (Figure 3i). Expression of Pro_{HAB1}*:GFP* was observed in all plant tissues examined, particularly in key target sites of ABA action, such as guard cells (Figure 3a), seeds (Figure 3b,c), embryo (Figure 3d) and silique (Figure 3e). GFP expression driven by the *HAB1* promoter was also observed in meristematic tissues, including the root meristem (Figure 3g) and shoot apical meristem (Figure 3h). Roots (Figure 3f,g), stem and leaves (data not shown) of Pro_{HAB1}*:GFP* transgenic plants also showed GFP expression. These results, together with previous Northern blot analysis, indicate that *HAB1* is expressed ubiquitously in all plant organs.

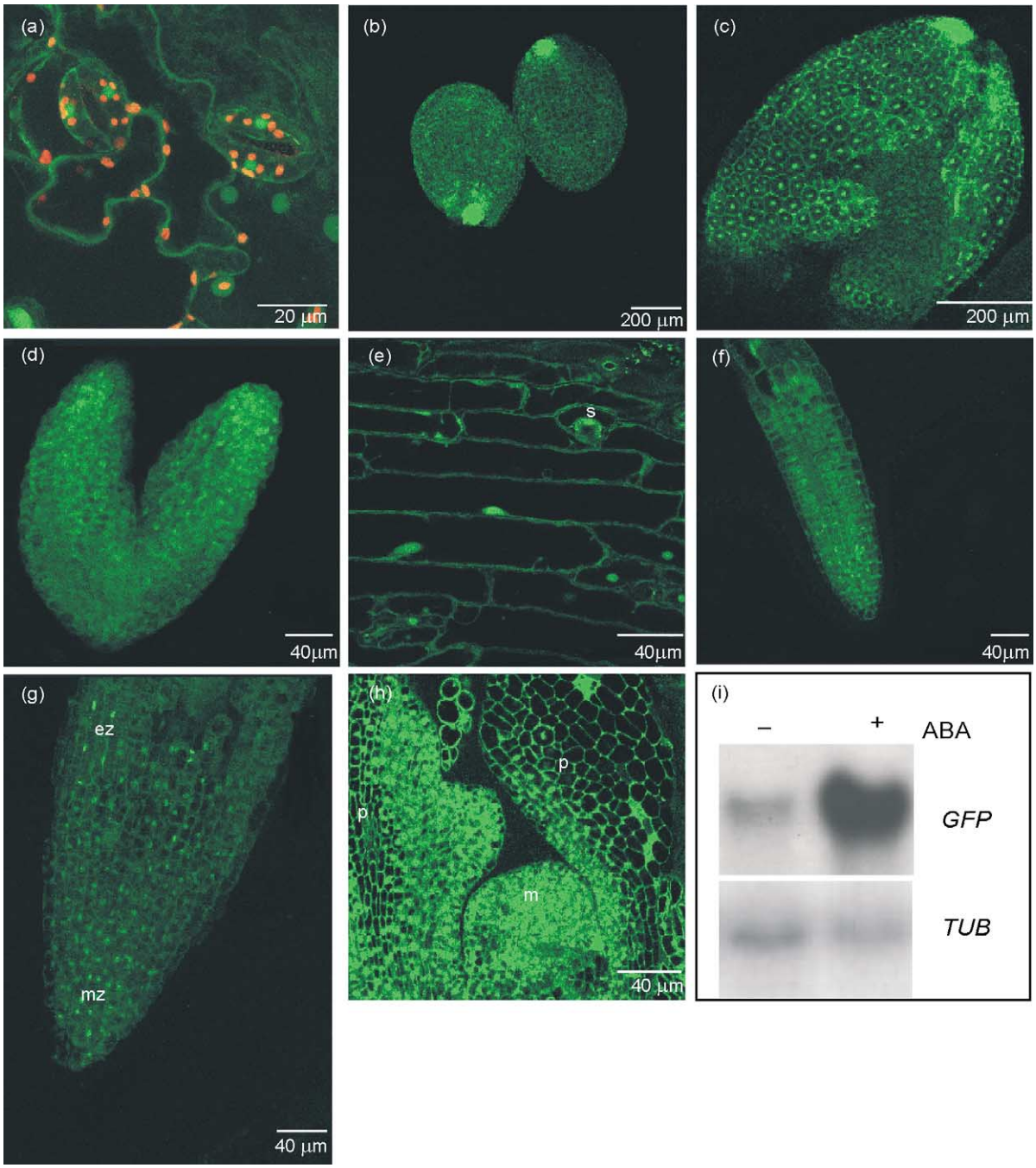

Figure 3. Expression pattern of Pro_{HAB1}:*GFP* in different tissues and cellular types.
Confocal microscopy photographs of transgenic plants harbouring a transcriptional fusion of the *HAB1* promoter and *GFP* reporter gene.
(a) Cotyledon epidermal and guard cells. Abaxial surface of a cotyledon treated with 30 μM ABA.
(b) Seed.
(c) Seed coat.
(d) Embryo at the transition between heart-to-torpedo stage (84 h after flowering).
(e) Silique epidermal and guard cells. s, stoma.
(f–h) Images of 13-day-old seedlings treated with 30 μM ABA. (f) Lateral root. (g) Primary root. mz, meristematic zone; ez, elongation zone. (h) Shoot apex. m, shoot apical meristem; p, leaf primordia.
(i) ABA-mediated upregulation of GFP expression in Pro_{HAB1}:*GFP* transgenic lines. mRNA levels of the indicated genes were determined in transgenic plants by Northern blot analysis using total RNAs isolated from mock-treated (–) or plants treated with 50 μM ABA for 3 h (+).

Construction and characterisation of 35S:HAB1 *lines*

Sequence homology of *HAB1* to *ABI1*/*ABI2* suggests that *HAB1* might be involved in ABA signalling. To investigate *HAB1* function, we generated transgenic *Arabidopsis* plants expressing a *35S:HAB1* transgene. Initial characterisation of the recovered 64 T_1 lines revealed that approximately 70% showed a wilty phenotype under low-humidity conditions (data not shown). Finally, 10 T_3 homozygous lines with high mRNA expression levels of the *35S:HAB1* transgene were recovered. Southern blot analysis of five T_3 homozygous lines revealed the presence of a single insertion of the *35S:HAB1* transgene in addition to the endogenous *HAB1* gene (Figure 4a). The 3 kb *Bgl*II fragment, corresponding to the endogenous *HAB1* gene, showed a higher intensity in the wild-type track because threefold more DNA was loaded in the gel (data not shown). Northern blot analysis showed substantially higher (5–10-fold) expression of the *35S:HAB1* transgene compared to endogenous *HAB1* (Figure 4b). Furthermore, the steady-state levels of the endogenous *HAB1* mRNA were not appreciably altered in the transgenic lines as compared to the wild-type control (Figure 4b).

To determine whether sustained transcriptional upregulation of *HAB1* affects ABA sensitivity, we compared germination and early seedling development of *35S:HAB1* and wild-type seeds in media supplemented with ABA (Figure 4c,d). At 5 days post-stratification, *35S:HAB1* seeds developed green cotyledons on 1 μM ABA medium (Figure 4d), and these seedlings further developed green

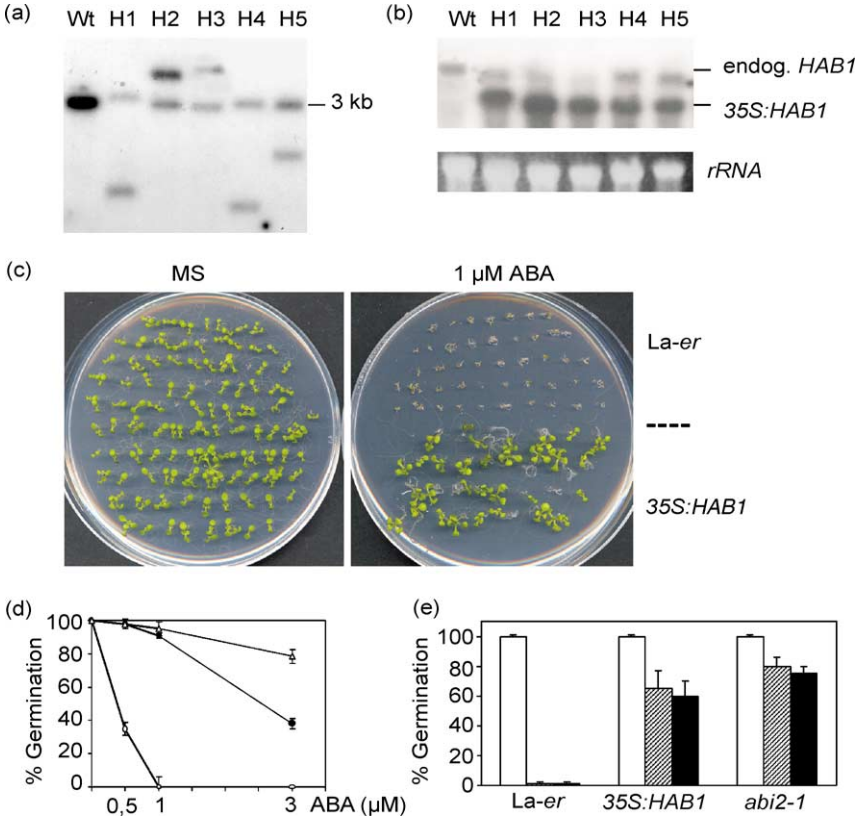

Figure 4. Molecular analysis of *35S:HAB1* transgenic lines.
Germination assays of *35S:HAB1* seeds reveal ABA insensitivity compared to wild-type control.
(a) Southern blot analysis of *Arabidopsis* transgenic lines expressing a *35S:HAB1* transgene. Wt, La-*er* background; H1–H5, transgenic lines harbouring a *35S:HAB1* transgene. Genomic DNA was digested with *Bgl*II, blotted to a nylon membrane and probed with a *HAB1* probe. The 3-kb *Bgl*II fragment generated from the endogenous *HAB1* gene is indicated.
(b) Northern blot analysis of *Arabidopsis* transgenic lines expressing a *35S:HAB1* transgene. Each track of the blot contained approximately 20 μg total RNA prepared from wild type (Wt, La-*er* background) or H1–H5 transgenic plants. The loading of the gel was visualised by ethidium bromide staining. The endogenous *HAB1* gene and the *35S:HAB1* transgene are indicated.
(c) Seed germination and seedling development of wild type and *35S:HAB1* in response to ABA. Wild type (La-*er*) and *35S:HAB1* seeds on MS agar plates supplemented with (right) or without (left) 1 μM ABA. The photograph was taken 5 (MS) or 10 (ABA) days after sowing.
(d) Percentage of seeds that germinated and developed green cotyledons in the presence of the indicated concentrations of ABA. Approximately 200 seeds of either La-*er* (open circle), *35S:HAB1* (filled circle) or *abi2-1* (open triangle) plants were sowed and scored five (0, 0.5 and 1 μM ABA) or 12 days (3 μM ABA) later.
(e) Percentage of seeds that germinated and developed green cotyledons in MS medium (white columns) or medium supplemented with 400 mM mannitol (hatched columns) or 10 μM paclobutrazol (black columns). Seeds were scored 10 days after sowing. Error bars represent ±SD of three independent experiments with more than 100 seeds plated per data point.

leaves (Figure 4c) in contrast to wild-type plants. These data indicate a reduced sensitivity of *35S:HAB1* seeds to ABA with respect to wild type. Finally, at 12 days post-stratification, approximately 40% of *35S:HAB1* seeds were able to germinate and develop green cotyledons on medium supplemented with 3 μM ABA, whereas wild-type seeds did not (Figure 4d). All five transgenic lines showed similar germination phenotypes in the presence of ABA (data not shown).

We wished to further examine the response of *35S:HAB1* seeds in other germination assays. Osmotic stress delays seed germination and arrests early seedling development mainly through ABA action. Indeed, both ABA-insensitive as well as ABA-deficient mutants are able to bypass the delay in germination and growth arrest induced by osmotic stress (Gonzalez-Guzman *et al.*, 2002; Leon-Kloosterziel *et al.*, 1996; Lopez-Molina *et al.*, 2001; Werner and Finkelstein, 1995). Seed germination under 400 mM mannitol led to a severe delay in radicle emergence and growth arrest in wild-type individuals; in contrast, *35S:HAB1* seeds were able to germinate and they developed green cotyledons under such conditions (Figure 4e). An additional seed germination assay was carried out in the presence of paclobutrazol, an inhibitor of GA biosynthesis. GAs and ABA play antagonistic roles in seed germination, and ABA-insensitive (or ABA-deficient) mutants have a lower need for GAs during germination (Koornneef and Karssen, 1994). This requirement was compared in wild-type, *35S:HAB1* and *abi2-1* seeds germinated in a medium containing paclobutrazol. In contrast to wild type, both *35S:HAB1* and *abi2-1* seeds germinated and developed green cotyledons in medium supplemented with 10 μM paclobutrazol, indicating a reduced requirement for GAs at this developmental stage (Figure 4e).

35S:HAB1 *plants show enhanced sensitivity to drought, higher transpiration rate, ABA-resistant growth and diminished expression of ABA-responsive genes*

Stomatal closure and consequent reduction in water loss is a key ABA-controlled process that preserves water under drought conditions. Therefore, the ABA-insensitive *abi1-1* and *abi2-1* mutants are very sensitive to water-stress conditions because of impaired regulation of stomatal closure. We mimicked drought conditions by exposing plants to the drying atmosphere of a flow laminar hood. Figure 5(a) shows that, compared to wild type, *35S:HAB1* and *abi2-1* plants developed a severe wilty phenotype 3 h after exposure to such conditions. This result suggests that *35S:HAB1* plants show a higher transpiration rate than wild-type plants. When measured by the loss of FW of detached rosette leaves, the water loss in wild-type plants was approximately twofold lower than that in *35S:HAB1* and *abi2-1* plants (Figure 5b). Thus, constitutive overexpression of *HAB1* resulted in increased transpiration and reduced tolerance to drought.

ABA has an inhibitory effect on root growth, and accordingly, ABA-insensitive mutants show higher ABA-resistant root growth than wild-type plants (Himmelbach *et al.*, 1998). Figure 5(c) shows that *35S:HAB1* plants had a reduced sensitivity to the ABA-promoted inhibition of root growth as compared to wild-type plants. In presence of 10 μM ABA in the medium, root growth of *35S:HAB1* plants was approximately twofold higher than that of wild-type plants. Additionally, prolonged culture of wild-type plants on 30 μM ABA-containing medium led to growth arrest of the aerial part of the plant and yellowing of the leaves (Figure 5d). In contrast, after 2 weeks in a medium supplemented with 30 μM ABA, *35S:HAB1* plants showed little inhibition of leaf growth and green leaves, and eventually flowered under these conditions (Figure 5d).

Finally, to further establish the role of *HAB1* in ABA signalling, we examined whether the reduction in ABA sensitivity in transgenic plants was accompanied by altered expression of ABA-responsive genes (Figure 5e). *RAB18* is an ABA-inducible gene, whose expression is drastically inhibited both in *abi1-1* and *abi2-1* mutants (Leung *et al.*, 1997). *35S:HAB1* plants also showed a severe reduction in the expression of *RAB18* upon ABA treatment (Figure 5e). Accumulation of *delta 1-pyrroline-5-carboxylate synthase* (*P5CS1*) mRNA is induced by drought, salinity and ABA, and it is reduced by approximately 50% in the *abi1-1* mutant compared to wild-type control (Strizhov *et al.*, 1997). Likewise, *35S:HAB1* plants reduced *P5CS1* transcript levels to about half of those detected in wild-type plants. *RD29A* is a cold-, drought- and ABA-inducible gene that, in addition to the dehydration-responsive element (DRE), contains ABA-responsive elements in its promoter (Shinozaki and Yamaguchi-Shinozaki, 1997). Impaired induction of *RD29A* by ABA was observed in *35S:HAB1* plants compared to wild type. Therefore, the reduced physiological responses to ABA of *35S:HAB1* plants were also correlated with a reduced expression of three ABA-responsive genes.

Identification of a T-DNA insertion mutant of HAB1

A T-DNA insertion mutant of *HAB1* was identified in the Salk collection of T-DNA lines, which corresponds to donor stock number SALK_002104 (http://signal.salk.edu/cgi-bin/tdnaexpress). In order to identify individuals homozygous for the T-DNA insertion, genomic DNA was obtained from kanamycin-resistant seedlings of the SALK_002104 line and was submitted to Southern blot analysis (Figure 6b). Homozygous and hemizygous individuals were thus distinguished (Figure 6b), and plants homozygous for the T-DNA insertion in *HAB1* were selected for further studies. The DNA sequence of the T-DNA flanking region of the SALK_002104 line indicated that the T-DNA insertion lied at

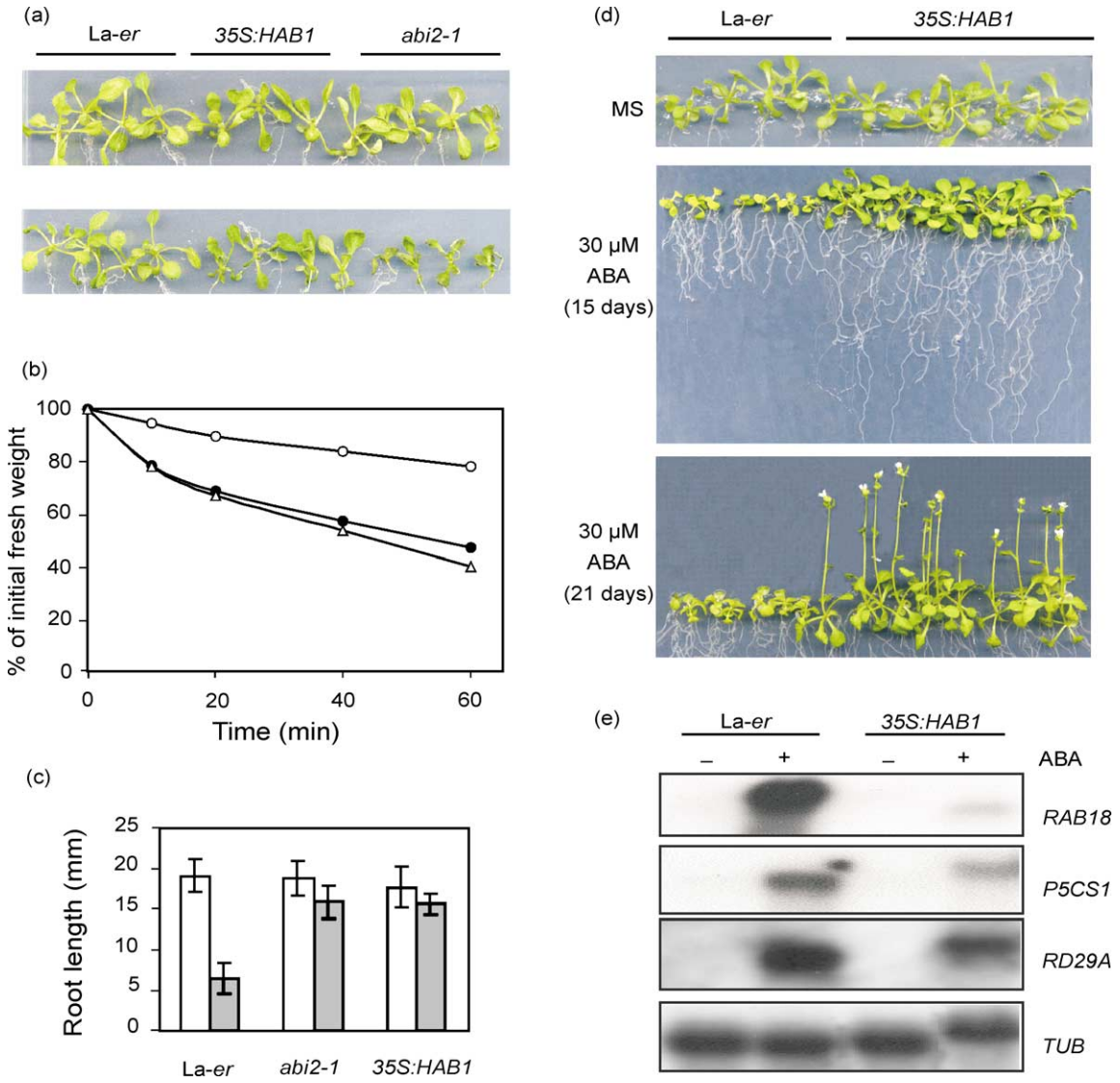

Figure 5. *35S:HAB1* plants show ABA-insensitivity compared to wild-type plants.
(a) Drought hypersensitivity of *35S:HAB1* plants. 15-day-old plants were submitted to the drying atmosphere of a flow laminar hood. A photograph was taken at the beginning of the experiment (top) and 3 h after exposure to such conditions (bottom).
(b) Enhanced transpiration rate of *35S:HAB1* plants compared to wild type. Loss of FW was measured in detached rosette leaves of either La-*er* (open circle), *35S:HAB1* (filled circle) or *abi2-1* (open triangle) plants.
(c) Root growth assay for scoring ABA sensitivity. The root growth was determined after 5 days of the transfer of 5-day-old seedlings onto MS plates containing 10 μM ABA (filled bars) or medium without ABA (open bars). Error bars represent ±SD ($n = 30$).
(d) Growth of La-*er* and *35S:HAB1* plants in medium supplemented with 30 μM ABA. The photographs were taken after 15 days of the transfer of 5-day-old seedlings from MS medium to plates lacking (top) or containing 30 μM ABA (middle). After 21 days on 30 μM ABA-containing medium, *35S:HAB1* plants produced flowers (bottom).
(e) Expression of ABA-regulated genes in *35S:HAB1* plants compared to wild-type control. mRNA levels of the indicated genes were determined by Northern blot analysis using total RNAs isolated from mock-treated plants (−) or plants treated with 50 μM ABA for 3 h (+). Radioautography of X-ray film was 10 h for *RAB18*, 10 h for *P5CS1*, 7 h for *RD29A* and 14 h for *TUB*. Each track of the blot contained approximately 10 μg total RNA. The loading of the gel was quantified by hybridisation with a *TUB* probe.

the end of the third intron of *HAB1* (http://signal.salk.edu/cgi-bin/tdnaexpress). In order to confirm these data, a genomic fragment adjacent to the left border of the T-DNA insertion was isolated by PCR and was sequenced. Sequence analysis confirmed that the T-DNA insertion was localised at nucleotide 1513 of the *HAB1* gene (numbering refers to the ATG start codon). Therefore, the T-DNA insertion lies at the third intron of *HAB1*, only eight nucleotides upstream of the beginning of the fourth exon of *HAB1* (Figure 6a). The 5-kb T-DNA insertion might disrupt splicing or affect the stability of the *HAB1* transcript. In any case, this T-DNA-disrupted allele of *HAB1* is predicted to result at

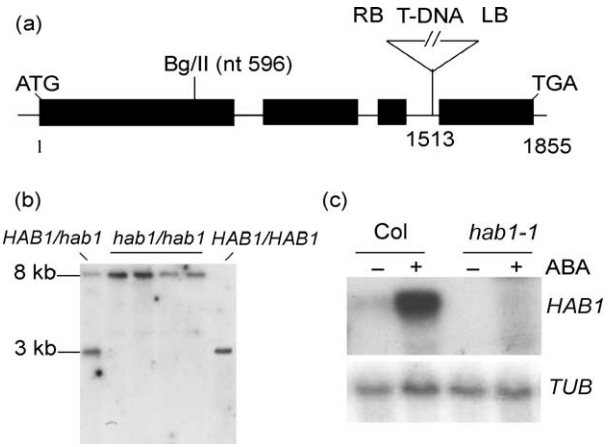

Figure 6. Molecular characterisation of the *hab1-1* mutant.
(a) Map of *HAB1* gene and localisation of the T-DNA insertion in the *hab1-1* allele. The numbering begins at the ATG translation start codon. Exons are represented by black boxes.
(b) Southern blot analysis of genomic DNA from wild-type plants (*HAB1*/*HAB1*) or individuals homozygous (*hab1-1*/*hab1-1*) or hemizygous (*HAB1*/*hab1-1*) for the T-DNA insertion from the SALK_002104 line. Genomic DNA was digested with *Bgl*II, blotted to a nylon membrane and probed with a genomic *HAB1* probe encompassing from nucleotide 596–1855. The 3- and 8-kb *Bgl*II fragments generated from the wild-type or the T-DNA-tagged *HAB1* alleles, respectively, are indicated.
(c) Northern blot analysis of wild-type and *hab1-1* mRNA probed with a radiolabelled fragment of *HAB1* cDNA (nucleotides 1300–1600). Each track of the blot contained approximately 20 μg total RNA isolated from mock-treated plants (−) or plants treated with 50 μM ABA for 3 h (+). The loading of the gel was quantified by hybridisation with a *TUB* probe.

least in the loss of the last 111 amino acids of the HAB1 protein, which includes essential residues for PP2C function (Das *et al.*, 1996; Rodriguez, 1998). Therefore, the SALK_002104 line likely contains a null allele of *HAB1*. Indeed, Northern blot analysis failed to detect a full-length *HAB1* transcript in the *hab1-1* mutant (Figure 6c).

hab1-1 *mutant seeds are ABA-hypersensitive*

Progeny of *hab1-1* homozygous individuals was harvested and, subsequently, seed germination assays were performed (Figure 7). In the absence of exogenous ABA, *hab1-1* mutant seeds showed wild-type germination ratios after 3 days stratification at 4°C. However, in the presence of exogenous ABA, the *hab1-1* mutant showed ABA hypersensitive inhibition of seed germination (Figure 7b; IC_{50} of 0.37 μM ABA for *hab1-1* versus IC_{50} of 0.67 μM ABA for Columbia wild type). Wild-type and *hab1-1* mutant plants were crossed and the resulting F_1 seeds showed wild-type germination ratios on 0.5 μM ABA (data not shown). F_2 seeds showed a segregation of the *hab1-1* phenotype of 115–321 corresponding to a ratio of about 1–3 ($\chi^2 = 0.44$; $P > 0.5$). F_2 ABA-hypersensitive seeds showed kanamycin-resistance and were homozygous for the T-DNA insertion as determined by PCR analysis ($n = 40$). These data suggest that the *hab1-1* mutation is recessive and segregates as a single nuclear locus linked to the T-DNA insertion.

Additional dose–response analyses of germination in media supplemented with increasing concentrations of NaCl (Figure 7c) or mannitol (Figure 7d) were also performed. Compared to wild-type seeds, *hab1-1* mutant shows enhanced inhibition of germination under these conditions. Osmotic stress blocks germination through ABA action; therefore, these results are consistent with the ABA-hypersensitive inhibition of germination observed above. Finally, an enhanced sensitivity to inhibition of germination by paclobutrazol was observed in *hab1-1* mutant compared to wild-type seeds (Figure 7e). This result indicates a higher requirement for GAs during germination in *hab1-1* compared to wild type, which is in agreement with both the antagonistic role of GAs to ABA during germination and the hypersensitive response of *hab1-1* to ABA.

Finally, we transformed *hab1-1* plants with the *HAB1* cDNA under the control of its own promoter. Seeds from three independent *hab1-1*::*HAB1* lines showed wild-type germination in the presence of either 0.5 μM ABA or 250 mM mannitol (Figure 7g). These results confirm that *hab1-1* mutation is responsible for the enhanced response to ABA observed in the mutant seeds.

Transpiration rate in hab1-1

Water loss in detached leaves of *hab1-1* mutant was similar to that of wild-type plants (data not shown), and whole plant transpiration rate was quite similar in wild-type and *hab1-1* plants (Figure 7f). This result is in accordance to previous analyses of water loss in loss-of-function alleles of *ABI1* and *ABI2*, or *PP2CA* antisense plants, which show kinetics of water loss similar to that of wild-type plants (Gosti *et al.*, 1999; Merlot *et al.*, 2001; Tahtiharju and Palva, 2001). Taking into account the complexity of the PP2C subfamily involved in ABA signalling, it is conceivable that loss-of-function of *HAB1* in stomata is masked by the activity of other PP2Cs.

Discussion

The importance of the PP2C class of protein serine/threonine phosphatases in plants is highlighted by the high number of *PP2C* genes (at least 69) found in the *Arabidopsis* genome (Kerk *et al.*, 2002). In contrast, no more than 15 PP2Cs are found in the human genome (Cheng *et al.*, 2000) and only 6 are found in the yeast genome (Stark, 1996). Therefore, with respect to other eukaryotic organisms, plants seem to have greatly expanded the regulatory mechanisms based on PP2C de-phosphorylation. In fungi, mammals and plants, examples are found where PP2Cs act as negative regulators of protein kinase cascades activated

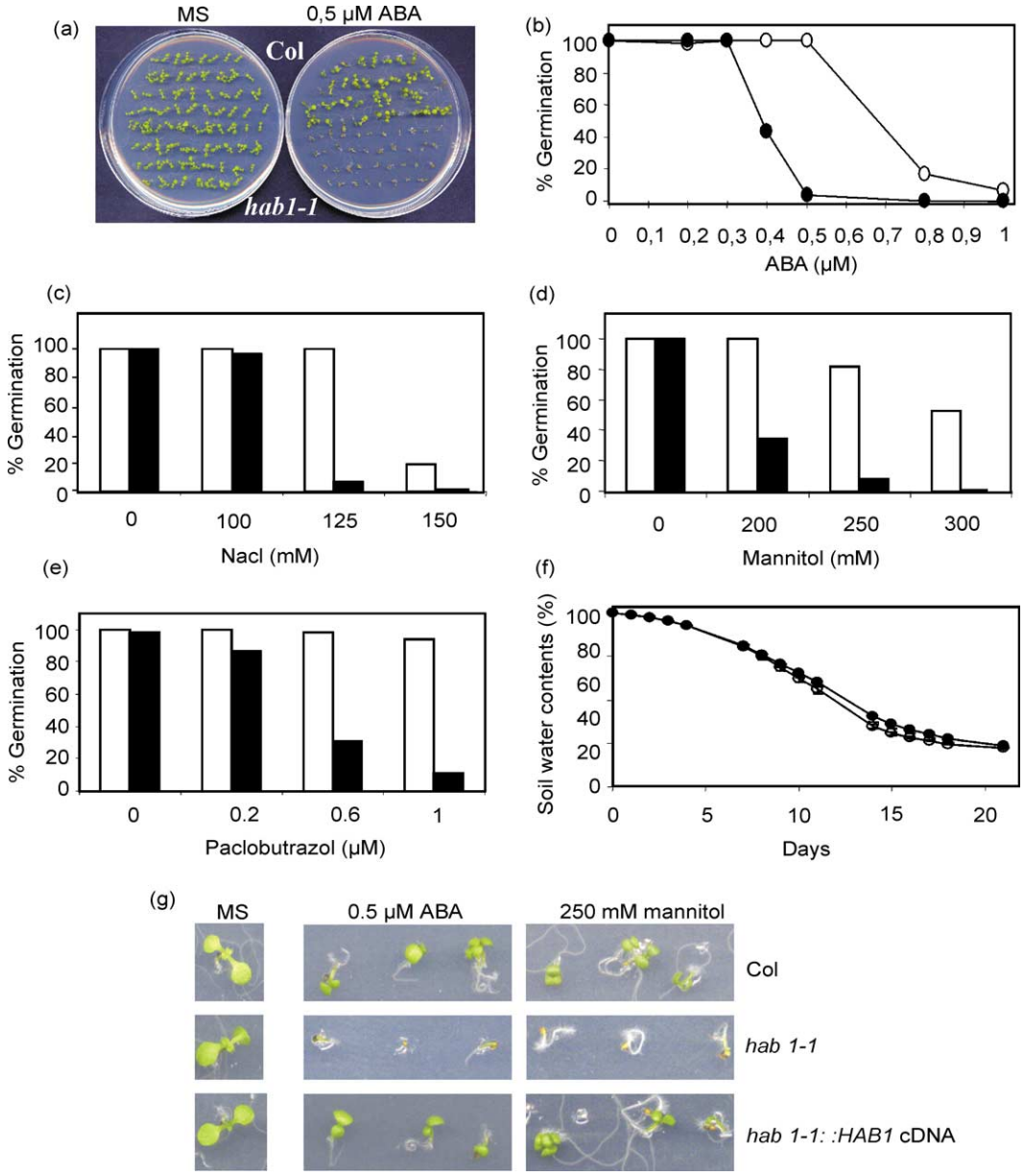

Figure 7. ABA-hypersensitive inhibition of seed germination in *hab1-1* mutant.
(a) Seed germination and seedling development of wild-type and *hab1-1* mutant in response to ABA. Wild-type (Columbia background) and *hab1-1* mutant seeds were sowed on MS agar plates supplemented with (right) or without (left) 0.5 µM ABA. The photograph was taken 10 days after sowing.
(b–e) Percentage of seeds that germinated and developed green cotyledons in the presence of the indicated concentrations of ABA, NaCl, mannitol or paclobutrazol, respectively. Approximately 200 seeds of Col (white symbols) and *hab1-1* mutant (black symbols) plants were sowed and scored 10 days later.
(f) Whole plant transpiration of wild-type (white circle) and *hab1-1* (black circle) mutant plants. Changes in soil water content during drought stress treatment are indicated. SD was less than 6%.
(g) Complementation of the *hab1-1* hypersensitivity to ABA. Seeds from wild-type, *hab1-1* mutant and a representative *hab1-1* transformed line (expressing the *HAB1* cDNA under control of its own promoter) were germinated in MS medium or medium supplemented with either 0.5 µM ABA or 250 mM mannitol. The photograph was taken after 10 (0.5 µM ABA) and 8 (250 mM mannitol) days.

as a result of stress (Rodriguez, 1998; Takekawa *et al.*, 1998; Warmka *et al.*, 2001). Particularly, in plants, the stress-induced MP2C acts as a negative regulator of a MAPK pathway activated by wounding or salt stress (Meskiene *et al.*, 1998, 2003). However, the regulatory role of eukaryotic PP2Cs clearly extends to other processes. For instance, both yeast and human PP2Cs de-phosphorylate cyclin-dependent kinases and, therefore, participate in cell cycle regulation (Cheng *et al.*, 2000). Additional examples of singular functions of PP2Cs are well documented in plants. Thus, *Arabidopsis* KAPP and POLTERGEIST are negative regulators of the CLAVATA pathway, which is

involved in regulation of shoot and floral meristem size (Williams *et al.*, 1997; Yu *et al.*, 2003). Finally, the *Arabidopsis* PP2CA regulates the K^+ channel AKT2 and therefore extends the role of PP2C to control K^+ transport and membrane polarisation (Cherel *et al.*, 2002).

The field of ABA signalling also offers a significant contribution to our understanding of a plant process regulated by PP2Cs. Studies on *abi1* and *abi2* mutants, transient expression experiments, as well as analysis of transgenic *PP2CA* antisense plants, indicate that PP2Cs act as negative regulators of ABA signalling (Gosti *et al.*, 1999; Merlot *et al.*, 2001; Sheen, 1998; Tahtiharju and Palva, 2001). Transient expression studies in maize protoplasts demonstrated that overexpression of *ABI1* and *PP2CA* blocked the induction of a reporter gene driven by an ABA-inducible promoter (Sheen, 1998). The identification and physiological characterisation of loss-of-function alleles (*abi1-1R1–abi1-1R7*) of the *ABI1* gene were crucial to provide *in planta* evidence on the role of ABI1 as a negative regulator of ABA signalling (Gosti *et al.*, 1999). This notion was further supported for *ABI2* by the isolation of the loss-of-function *abi2-1R1* allele and analysis of ABA responses in double mutants *abi1-1R4 abi2-1R1* or *abi1-1R5 abi2-1R1* (Merlot *et al.*, 2001). Recent controversy on the role of ABI1 has arisen from the work of Wu *et al.* (2003), which questions the role of ABI1 as a negative regulator of ABA signalling on the basis that *ABI1* overexpression in *Arabidopsis* does not affect the ABA signalling pathway. However, it seems logical to think that in case ABI1 was a positive regulator of ABA signalling instead of a negative one, *35S:ABI1* lines should show enhanced response to ABA, which was not reported by Wu *et al.* (2003). Additionally, the results of Wu *et al.* (2003) are opposed to those of Sheen (1998), which showed that overexpression of *ABI1* in maize protoplasts led to a blockade of ABA-inducible gene expression. Additional experiments will be required to resolve this controversy.

Analysis of PP2C activity in *abi1-1R5 abi2-1R1* plants revealed that ABI1 and ABI2 contribute to approximately 50% of the ABA-induced PP2C activity (Merlot *et al.*, 2001). These data indicated that additional PP2Cs participate in ABA signalling. Indeed, it has been shown that antisense inhibition of *PP2CA* expression leads to increased sensitivity to ABA during development of frost tolerance and seed germination (Tahtiharju and Palva, 2001). In this context, the features of *HAB1*, namely *ABI1*/*ABI2* sequence similarity and ABA-induced upregulation, made it a likely additional candidate to regulate ABA signalling. Considering the complexity of the plant subfamily of PP2Cs involved in ABA signalling (see cluster #5 in Figure 1) and the potential functional redundancy of these proteins, an overexpression approach was initially chosen. We reasoned that in case *HAB1* was a positive regulator of ABA signalling, introduction of a *35S:HAB1* transgene might lead to enhanced or even constitutive response to ABA. Alternatively, sustained upregulation of *HAB1* might lead to an ABA-insensitive phenotype in case it was a negative regulator of ABA signalling. The analysis of ABA response in *35S:HAB1* lines supports the latter hypothesis. Thus, transpiration assays in *35S:HAB1* T_1 lines indicated enhanced water loss as compared to control plants, as expected for an ABA-insensitive phenotype. This result was confirmed in T_3 plants, which showed an approximately twofold higher transpiration rate than wild-type plants, and consequently an increased sensitivity to drought stress (Figure 5a,b).

Further analysis of ABA-mediated responses both in seeds as well as in vegetative tissues indicated that sustained upregulation of *HAB1* led to a reduced ABA sensitivity (Figures 4 and 5). For instance, root growth assays also revealed a reduced sensitivity to inhibition of growth by ABA in *35S:HAB1* plants compared to wild type (Figure 5c,d). The inhibitory effect of high ABA concentrations on root growth has been attributed to activation of the ethylene response pathway (Beaudoin *et al.*, 2000; Ghassemian *et al.*, 2000). Additionally, a possible link between the ABA growth-control pathway and cell cycle control has been provided by the discovery of cyclin-dependent protein kinase inhibitor (ICK1), which is induced by ABA (Wang *et al.*, 1998). Both root and shoot meristematic activities of *35S:HAB1* plants were remarkably resistant to the inhibitory effect of 30 μM ABA compared to wild-type plants (Figure 5d). Indeed, under such high ABA concentration, the shoot apical meristem of *35S:HAB1* plant is able to switch from vegetative to reproductive growth and to produce flowers (Figure 5d). As enhanced *HAB1* expression attenuates inhibition of growth by ABA, the ABA-mediated upregulation of *HAB1* might contribute to regulation of the cell cycle arrest induced by ABA in meristematic tissues. Finally, the changes in the mRNA levels of three genes responsive to exogenous ABA – *RAB18, P5CS1* and *RD29A* – also reflect a reduced ABA signalling in *35S:HAB1* plants compared to wild-type control (Figure 5e).

In order to complete our study on *HAB1* function, we also analysed a T-DNA insertion mutant of *HAB1*. No gene deletion mutant or T-DNA-disrupted allele of *PP2C* genes involved in ABA signalling had been characterised previously. The T-DNA insertion present in the *hab1-1* allele analysed in this study is predicted to result at least in the loss of the C-terminal 111 amino acid residues of HAB1, which include 5 out of the 11 conserved motifs of the PP2C family (Rodriguez, 1998). Indeed, Northern blot analysis failed to detect a full-length *HAB1* transcript. Therefore, the T-DNA-disrupted *hab1-1* allele is likely to be null and results in the loss of *HAB1* function. ABA dose–response analyses indicated that *hab1-1* seeds were hypersensitive to the inhibition of germination by ABA, as compared to wild type (Figure 7). Accordingly, an increased requirement for GAs during germination was observed in *hab1-1* seeds compared to wild type (Figure 7) and, consequently, *HAB1*

function could play an important physiological role to promote germination. Thus, we suggest that promotion of germination would imply not only a positive action of GAs, but also a negative regulation of ABA signalling by HAB1 activity.

Whereas an enhanced response to ABA in germination was observed for *hab1-1* mutant, its transpiration rate was similar to that of wild-type plants. A similar result was obtained for *abi1-1R1* to *abi1-1R7* revertants, which were ABA-supersensitive in germination, but showed kinetics of water loss similar to wild type (Gosti *et al.*, 1999). Additionally, the loss-of-function *aba2-1R1* mutant displayed wild-type ABA-induced stomatal closing (Merlot *et al.*, 2001), and ABA-mediated drought responses were not affected by inhibition of *PP2CA* expression (Tahtiharju and Palva, 2001). Taking into account the fact that at least four PP2Cs are negative regulators of ABA signalling, a partial functional redundancy might explain the lack of phenotype in transpiration assays for single loss-of-function alleles. Moreover, analysis of the *Arabidopsis* genome reveals a putative paralog of *HAB1*, i.e. *HAB2*. Although these two homologous PP are unlikely to have completely redundant roles and *HAB2* transcriptional upregulation in response to ABA is significantly lower than *HAB1* (Figure 2b), it is possible that HAB2 (or ABI1/ABI2) activity partially masks the loss-of-function of *HAB1*. It will be necessary to test whether simultaneous inactivation of these PP2Cs affects transpiration rate.

The *abi1-1* and *abi2-1* mutants, as well as *35S:HAB1* plants, show a reduced ABA sensitivity. It has been previously suggested (Gosti *et al.*, 1999) that abi1-1 and abi2-1 proteins have dominant negative effects and might act by trapping their endogenous substrates (positive regulators of ABA signalling) in a dead complex. To explain the molecular basis of HAB1 action, it will be required to identify its endogenous substrate. However, according to the phenotype of *35S:HAB1* plants, a simple model can be envisaged, where enhanced HAB1 phosphatase activity attenuates the ABA transduction cascade by de-phosphorylating a positive regulator of ABA signalling. Thus, positive regulators of ABA signalling could be inactivated either through formation of poison complexes (*abi1-1*, *abi2-1*) or by sustained de-phosphorylating activity (*35S:HAB1*). The target of HAB1 is likely different from those of ABI1 and ABI2, as loss of HAB1 function leads to enhanced response to ABA. HAB1 might function either in the same or in a different branch of ABA signalling than ABI1 and ABI2. Characterisation of new loss-of-function alleles of *ABI1* and *ABI2* and subsequent epistasis analyses with *hab1-1* are required to address this question. Additionally, it will help to understand the relative contribution of each PP2C in ABA signalling. Finally, ABA promotes transcriptional upregulation of *ABI1*, *ABI2* and *HAB1*, therefore, leading to enhanced de-phosphorylating activity and attenuation of the signal. As *ABI1*, *ABI2* and *HAB1* expression is itself upregulated by ABA, attenuation of the signal would lead to diminished de-phosphorylating activity, restoring the capacity of ABA response.

Negative regulation of signal transduction is required for a proper control of the complex signalling pathways that operate in a cell. Mechanisms of negative feedback, blockage of downstream signalling or transcriptional repression are a common issue in signalling pathways, particularly in hormone action (McCourt, 1999). With respect to ABA signalling, in addition to *ABI1*, *ABI2*, *PP2CA* and *HAB1*, other genes have been identified as negative regulators of the pathway. Thus, constitutive expression of the *A. thaliana* homeodomain protein 6 (ATHB6) leads to ABA insensitivity in a subset of ABA responses, which suggests that ATHB6 represents a negative transcriptional regulator of the ABA signal (Himmelbach *et al.*, 2002). Interestingly, ATHB6 is the first target described of ABI1 (Himmelbach *et al.*, 2002). The farnesyl transferase β-subunit enhanced response to ABA1 (ERA1) also plays a crucial role as a negative regulator of ABA signalling (Cutler *et al.*, 1996; Pei *et al.*, 1998). The recessive *era1* mutant shows enhanced response to ABA and, therefore, protein farnesylation of certain signalling proteins appears crucial for negative regulation of ABA signalling. The mRNA cap-binding protein ABH1 is a novel modulator of ABA signalling (Hugouvieux *et al.*, 2001). A recessive loss-of-function *abh1* allele shows ABA hypersensitivity, indicating that *ABH1* negatively modulates ABA signalling (Hugouvieux *et al.*, 2001). Finally, the mutant *ade1* exhibits sustained and enhanced levels of an ABA-inducible gene, suggesting a negative regulatory function for this locus (Foster and Chua, 1999). Therefore, a complex negative regulatory mechanism seems to have evolved to reset ABA signalling and to avoid undesirable effects because of sustained activation of the ABA pathway. It remains as a major challenge for the future to identify additional signalling elements linking the known intermediates, particularly the targets of the negative regulators of the pathway.

Experimental procedures

Plant material

Arabidopsis thaliana plants were routinely grown under greenhouse conditions in pots containing a 1 : 3 vermiculite:soil mixture. For *in vitro* culture, seeds were surface-sterilised by treatment with 70% ethanol for 20 min, followed by commercial bleach (2.5%) containing 0.05% Triton X-100 for 10 min and, finally, four washes with sterile distilled water. Stratification of the seeds was conducted for 3 days at 4°C. Afterwards, seeds were sowed on Murashige–Skoog (MS) plates (Murashige and Skoog, 1962) containing solid medium composed of MS basal salts and 1% sucrose, solidified with 1% agar, and the pH was adjusted to 5.7 with KOH before autoclaving. Plates were sealed and incubated in

a growth chamber having a controlled environment at 22°C under a 16-h light/8-h dark photoperiod at 80–100 μE m^{-2} sec^{-1}.

Recombinant constructs and generation of transgenic plants

The coding region of the *HAB1* cDNA was excised from the pSKAtP2C-HA construct (Rodriguez *et al.*, 1998b) using an *Ecl*136II–*Eco*RI double digestion and subcloned into *Sma*I–*Eco*RI doubly digested pBIN121 (Clontech, Palo Alto, USA). The pBIN121-HAB1 construct was transferred to *Agrobacterium tumefaciens* C58C1 (pGV2260; Deblaere *et al.*, 1985) by electroporation, and *Arabidopsis* plants (La-*er* ecotype) were transformed by the floral dip method (Clough and Bent, 1998). Seeds of plants transformed with pBIN121-HAB1 were harvested and plated on kanamycin selection medium to identify T_1 transgenic plants. T_2 seeds, plated on selection medium to assay the segregation ratio, and transgenic lines with a 3 : 1 (resistant/sensitive) ratio were selected. Southern blot analysis was performed to select lines carrying a single T-DNA copy. T_3 progenies, homozygous for the selection marker, were used for further studies.

The 2-kb fragment of the *HAB1* promoter region used in this work was obtained by PCR-mediated amplification from Columbia plants using oligonucleotides FpHAB1: 5′-CAACAGCAATATATG-TATCTACG and RpHAB1: 5′-CCTCCATGGATCCTCCAAAATCAGA-GATTTCC. This latter primer introduces a unique *Bam*HI site around the ATG start codon of the *HAB1* coding sequence. The amplified DNA was cloned into the *Bam*HI site of a pBluescript SK-GFP vector. pBluescript SK-GFP bears unique *Bam*HI and *Nco*I sites in front of the start codon of a promoterless *GFP* coding sequence located upstream of the *nopaline synthase* (*NOS*) terminator. Thus, the recombinant clone (pBluescript SK-Pro_{HAB1}*:GFP*) harboured a transcriptional fusion between the *HAB1* mRNA 5′ untranslated sequence and the *GFP* coding sequence. The complete expression cassette comprising the *HAB1* promoter, the *GFP* coding sequence and the *NOS* terminator was subcloned into *Sac*I–*Sal*I doubly digested pCAMBIA 2300. The resulting construct was named pCAMBIA2300-Pro_{HAB1}*:GFP* and was used to transform *Arabidopsis* plants as described above. The *GFP* reporter gene used in this study (Chiu *et al.*, 1996) was kindly provided by J. Sheen (Boston, USA).

For functional complementation of the *hab1-1* mutant, the *GFP* reporter gene of the pBluescript SK-Pro_{HAB1}*:GFP* construct was replaced with the coding sequence of *HAB1*, generating the pBluescript SK-Pro_{HAB1}*:HAB1* construct. The complete expression cassette comprising the *HAB1* promoter, the *HAB1* coding sequence and the *NOS* terminator was subcloned into *Sac*I–*Sal*I doubly digested pCAMBIA 1300 (HYG^R). The resulting construct was named pCAMBIA1300-Pro_{HAB1}*:HAB1* and used to transform *hab1-1* (KAN^R) plants as described above. Transgenic plants were screened *in vitro* on a MS medium (Sigma M5524) with 20 mg l^{-1} hygromycin B (Sigma H9773, Sigma-Aldrich, St Louis, MO, USA).

Germination assays

To measure ABA sensitivity, seeds were plated on solid medium, composed of MS basal salts, 1% sucrose and increasing concentrations of ABA. To determine sensitivity to inhibition of germination by high osmoticum or paclobutrazol, the medium was supplemented with increasing concentrations of either sodium chloride and mannitol, or paclobutrazol, respectively. In order to score seed germination, the percentage of seeds that had germinated and developed fully green expanded cotyledons was determined.

Root growth and transpiration assays

The root growth assay for scoring ABA sensitivity was performed by measuring root growth after 5 days of the transfer of 5-day-old seedlings onto MS plates containing 10 μM ABA. Kinetics analysis of water loss was performed in detached leaves at the same developmental stage and size from single 3-week-old plants. Five leaves per individual were excised and FW was determined at ambient conditions (25°C and approximately 40% relative humidity (RH)) after the indicated periods of time.

Whole plant transpiration was measured basically as described by Pei *et al.* (1998). Both wild-type and *hab1-1* plants (five individuals per experiment, three independent experiments) were grown under normal watering conditions for 21 days and were then subjected to drought stress by completely terminating irrigation and minimising soil evaporation by covering pots with Saran Wrap. Pots were weighed every day at the same time. Pots containing no plants were subjected to the same treatment to determine the background rate of water loss.

RNA analysis

About 10–12 seven-day-old seedlings were transferred from MS plates to 125-ml flasks containing 25 ml of MS solution and 1% sucrose. The flasks were shaken under cool fluorescent light. After 10 days, seedlings were mock-treated or treated with 50 μM ABA. Plant material was collected and frozen in liquid nitrogen. Total RNA was extracted as described by Gonzalez-Guzman *et al.* (2002), separated on formaldehyde–agarose gels and blotted to a nylon membrane. Blots were hybridised with random-priming ^{32}P-labelled probes. mRNA levels were quantified by PHOSPHORIMAGE analysis of Northern blots using a Bioimaging analyser BAS1500 (Fujifilm España). A full-length cDNA probe for *HAB1* was prepared as described previously by Rodriguez *et al.* (1998b). The *P5CS1* probe was kindly provided by L. Szabados (Institute of Plant Biology, Hungary). The *RAB18*, *RD29A*, tubulin (*TUB*) and *HAB2* probes were prepared by PCR amplification from genomic DNA of wild-type Columbia plants. Primers *RAB18*, *RD29A* and *TUB* have been described previously by Gonzalez-Guzman *et al.* (2002). Primer *HAB2*: 5′-GTGTAATCAGAAAAGACAAAG and 5′-GCCACCTC-CATATTCACATCG.

Molecular characterisation of hab1-1 *allele*

A 158-bp genomic fragment adjacent to the left border of the T-DNA insertion was isolated from *hab1-1* plants by PCR using primers LBb1: 5′-GCGTGGACCGCTTGCTGCAACT and R380: 5′-TCCGGTTCTGGGATCACAT. The amplified product was sequenced on both strands.

Fluorescence microscopy

The fluorescence photographs of plants expressing the *GFP* reporter gene under control of the *HAB1* promoter were taken using a Leica TCS-SL confocal microscope and laser scanning confocal imaging system. For GFP detection, the excitation source was an argon ion laser at 488 nm and emission was observed between 510 and 530 nm. Chloroplast auto-fluorescence was detected between 660 and 700 nm.

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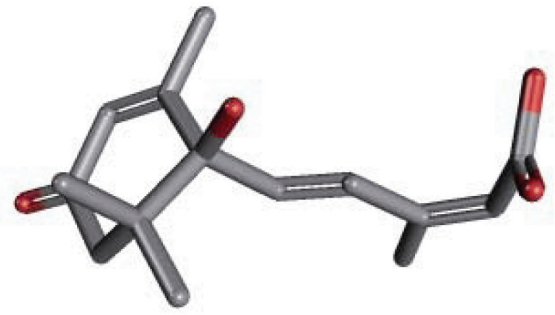

Capítulo II

Enhancement of Abscisic Acid Sensitivity and Reduction of Water Consumption in Arabidopsis by Combined Inactivation of the Protein Phosphatases Type 2C ABI1 and HAB1[1][W]

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Abscisic acid (ABA) plays a key role in plant responses to abiotic stress, particularly drought stress. A wide number of ABA-hypersensitive mutants is known, however, only a few of them resist/avoid drought stress. In this work we have generated ABA-hypersensitive drought-avoidant mutants by simultaneous inactivation of two negative regulators of ABA signaling, i.e. the protein phosphatases type 2C (PP2Cs) ABA-INSENSITIVE1 (ABI1) and HYPERSENSITIVE TO ABA1 (HAB1). Two new recessive loss-of-function alleles of ABI1, *abi1-2* and *abi1-3*, were identified in an Arabidopsis (*Arabidopsis thaliana*) T-DNA collection. These mutants showed enhanced responses to ABA both in seed and vegetative tissues, but only a limited effect on plant drought avoidance. In contrast, generation of double *hab1-1 abi1-2* and *hab1-1 abi1-3* mutants strongly increased plant responsiveness to ABA. Thus, both *hab1-1 abi1-2* and *hab1-1 abi1-3* were particularly sensitive to ABA-mediated inhibition of seed germination. Additionally, vegetative responses to ABA were reinforced in the double mutants, which showed a strong hypersensitivity to ABA in growth assays, stomatal closure, and induction of ABA-responsive genes. Transpirational water loss under drought conditions was noticeably reduced in the double mutants as compared to single parental mutants, which resulted in reduced water consumption of whole plants. Taken together, these results reveal cooperative negative regulation of ABA signaling by ABI1 and HAB1 and suggest that fine tuning of ABA signaling can be attained through combined action of PP2Cs. Finally, these results suggest that combined inactivation of specific PP2Cs involved in ABA signaling could provide an approach for improving crop performance under drought stress conditions.

The plant hormone abscisic acid (ABA) plays a crucial role in plant responses to several abiotic stresses such as drought, salt, and cold, as well as plant growth and development. In vegetative tissues, water stress produced by drought or high osmoticum treatment boosts ABA biosynthesis, leading to a variety of adaptive ABA-mediated responses such as stomatal closure and differential gene expression (Finkelstein et al., 2002; Nambara and Marion-Poll, 2005). ABA signaling in guard cells leads to stomatal closure, which occurs through rapid changes of ion fluxes and osmoregulation (Schroeder et al., 2001; Hetherington and Woodward, 2003). ABA regulation of the transpiration flow through stomatal pores is a crucial response of the plant to water deficit, as exemplified by the wilty phenotype of both ABA-deficient and ABA-insensitive mutants (Zeevaart and Creelman, 1988). Additionally, the ABA-dependent signaling pathway regulates stress-inducible gene expression, leading to a coordinated remodeling of gene expression that affects more than 1,000 genes of the plant transcriptome (Hoth et al., 2002; Seki et al., 2002; Takahashi et al., 2004).

Biochemical and genetic analyses have resulted in the identification of many elements of the ABA signal transduction pathway, although important pieces are still lacking. Recently, the RNA-binding protein FCA has been identified as an ABA-binding receptor with a singular role in flowering control, however, key responses to ABA such as inhibition of seed germination or stomatal response were not affected in the *fca-1* mutant (Razem et al., 2006). Accordingly, FCA appears to be an ABA receptor involved in controlling flowering time but additional ABA receptors must perform ABA perception. Putative candidates might be some plasma membrane receptors, such as RPK1, which is known to be involved in ABA signaling (Osakabe et al., 2005).

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Furthermore, in guard cells several studies have indicated the presence of intracellular ABA receptors (Allan et al., 1994; Schwartz et al., 1994; Schwarz and Schroeder, 1998; Levchenko et al., 2005).

It is well known that a variety of second messengers contribute to the transmission of the ABA signal, which includes Ca^{2+}, cADP-Rib, reactive oxygen species, nitric oxide, phosphoinositides, phosphatidic acid, and sphingosine 1-P (Schroeder and Hagiwara, 1989; Gilroy et al., 1990; McAinsh et al., 1990; Wu et al., 1997; Leckie et al., 1998; Jacob et al., 1999; Lemtiri-Chlieh et al., 2000; Pei et al., 2000; Allen et al., 2001; Ng et al., 2001; Neill et al., 2002; Guo et al., 2003). It is also known that phosphorylation/dephosphorylation events play a crucial role in ABA signaling, which involves a complex network of protein kinases and phosphatases as well as other signal transducers (for review, see Finkelstein et al., 2002). Finally, many transcriptional factors (TFs) of ABA-inducible genes are known. The TFs comprise ABA-responsive element (ABRE)-binding proteins (ABA-INSENSITIVE5 [ABI5]/ABF/AREB/AtbZIP family), ABI3/VP1/B3, ABI4/APETALA2, MYC, MYB, and HD-ZIP domain proteins (Giraudat et al., 1992; Suzuki et al., 1997; Finkelstein et al., 1998; Choi et al., 2000; Finkelstein and Lynch, 2000; Uno et al., 2000; Bensmihen et al., 2002; Himmelbach et al., 2002; Abe et al., 2003). Most of these TFs play a positive role in ABA signaling, but some of them function as repressors of ABA response (Himmelbach et al., 2002; Pandey et al., 2005; Song et al., 2005).

Genetic analyses of ABA signal transduction have identified both negative and positive regulators of ABA signaling (McCourt, 1999; Finkelstein et al., 2002). For instance, recessive mutations leading to ABA hypersensitivity were found in the *era1* (Cutler et al., 1996), *abh1* (Hugouvieux et al., 2001), *fry1* (Xiong et al., 2001b), *hypersensitive to ABA1* (*hab1*; Leonhardt et al., 2004; Saez et al., 2004), *sad1* (Xiong et al., 2001a), and *gcr1* (Pandey and Assmann, 2004) mutants. The intragenic revertants of *abi1-1* and *abi1-1R1* to *R7* also carry recessive mutations that lead to enhanced responsiveness to ABA (Gosti et al., 1999). Loss-of-function mutants generated by RNA interference for the *SOS3-like calcium-binding protein 5* and its interacting *protein kinase 3* were also hypersensitive to ABA (Guo et al., 2002). As loss of function of the above-mentioned genes leads to enhanced ABA responsiveness, their corresponding gene products must represent negative regulators of ABA signaling. On the other hand, recessive mutations leading to reduced ABA sensitivity have been identified in the *abi3* (Giraudat et al., 1992), *abi4* (Finkelstein et al., 1998), *abi5* (Finkelstein and Lynch, 2000), *ost1* (Mustilli et al., 2002), *rcn1* (Kwak et al., 2002), *rpk1* (Osakabe et al., 2005), and the *rbohD/F* double mutants (Kwak et al., 2003). Therefore, these loci point out to positive regulators of ABA signal transduction.

Protein phosphatases type 2C (PP2Cs) were identified as components of ABA signaling pathway from pioneer work with the ABA-insensitive *abi1-1* and *abi2-1* mutants (Koornneef et al., 1984; Leung et al., 1994, 1997; Meyer et al., 1994; Rodriguez, et al., 1998). Currently, at least four Arabidopsis (*Arabidopsis thaliana*) PP2Cs, ABI1, ABI2, PP2CA, and HAB1 (formerly named AtP2C-HA), are known to regulate ABA signaling. Evidence on their role as negative regulators of ABA signaling has been provided by genetic approaches (Gosti et al., 1999; Merlot et al., 2001; Tahtiharju and Palva, 2001; Gonzalez-Garcia et al., 2003; Leonhardt et al., 2004; Saez et al., 2004; Kuhn, et al., 2006; Yoshida et al., 2006). For instance, the recessive T-DNA insertion mutant *hab1-1* shows ABA-hypersensitive inhibition of seed germination and enhanced ABA-mediated stomatal closure (Leonhardt et al., 2004; Saez et al., 2004). *HAB1* is broadly expressed in the plant and strongly induced by ABA (Leonhardt et al., 2004; Saez et al., 2004). Constitutive expression of *HAB1* under a 35S promoter led to reduced ABA sensitivity both in seeds and vegetative tissues, compared to wild-type plants (Saez et al., 2004).

In the case of *ABI1*, recessive alleles were isolated as intragenic revertants of the originally dominant *abi1-1* mutation, and named *abi1-1R1* to *R7* (Gosti et al., 1999). Therefore, these recessive alleles, in addition to the original Gly-180 Asp mutation, carry a second mutation that abolishes the dominant character of the *abi1-1* mutation. The same approach was applied to the dominant mutant *abi2-1*, leading to the identification of the recessive *abi2-1R1* allele (Merlot et al., 2001). It cannot be excluded that intragenic revertants of *abi1-1* still retain some activity (not necessarily an enzymatic one) in the corresponding gene products, even though their in vitro protein phosphatase activity was shown to be negligible (Gosti et al., 1999). Thus, we were interested in the isolation of direct knockout alleles of *ABI1*, namely *abi1-2* and *abi1-3*, to conclusively clarify its role in ABA signaling. Furthermore, double knockout mutants in PP2Cs have not yet been generated and we have analyzed *hab1 abi1* double loss-of-function mutants here to determine whether these PP2Cs are strictly redundant or additive in their functions. Phenotypic analysis of *abi1-2* and *abi1-3* provided new data regarding the role of *ABI1* in ABA-induced stomatal closure, transpiration, and ABA-mediated regulation of gene expression. The phenotypic effect on ABA signaling observed in single *hab1-1*, *abi1-2*, and *abi1-3* mutants was notably reinforced in double mutants, which showed both enhanced responsiveness to ABA and drought avoidance. Thus, these results show a new biotechnological approach to increase plant drought avoidance, i.e. the combined inactivation of PP2Cs involved in ABA signaling.

RESULTS

Identification and Characterization of Knockout Alleles of *ABI1*

Two T-DNA insertion mutants of *ABI1* were identified in the Salk collection (Columbia [Col] background),

corresponding to donor stock numbers SALK_72009 and SALK_76309, and they were named *abi1-2* and *abi1-3*, respectively. Homozygous individuals were identified by PCR and Southern-blot analyses (data not shown). Sequencing of the T-DNA flanking region in *abi1-2* showed that the insertion was localized two nucleotides upstream of the ATG start codon (Fig. 1A). In the case of *abi1-3*, the T-DNA insert was localized 546 nucleotides downstream from the ATG start codon (Fig. 1A). Both T-DNA insertions severely impaired *ABI1* expression, based on reverse transcription (RT)-PCR (Fig. 1B) and quantitative RT-PCR (qRT-PCR) analyses (Fig. 1C). Expression of *HAB1* and *ABI1* in wild type was quite similar to that in *abi1-2*/*abi1-3* and *hab1-1* mutant backgrounds, respectively (Fig. 1C).

Progeny of both *abi1-2* and *abi1-3* homozygous individuals was harvested and different analyses to test their sensitivity to ABA were performed. First, the sensitivity of the mutants to inhibition of seed germination by ABA was analyzed (Fig. 2A). In the absence of exogenous ABA, *abi1-2* and *abi1-3* mutant seeds showed a germination ratio similar to wild type. However, in the presence of exogenous ABA, both the *abi1-2* and *abi1-3* mutants showed ABA-hypersensitive inhibition of seed germination (Fig. 2A; Supplemental Fig. 1). F_1 seeds that were hemizygous for the T-DNA insertion present either in *abi1-2* or *abi1-3* showed wild-type germination on 0.5 μM ABA. In the next generation, F_2 seeds showed an ABA-hypersensitive phenotype in approximately a 1:3 proportion (112 hypersensitive:313 wild type, $\chi^2 = 0.42$, $P > 0.5$ for *abi1-2*; 121 hypersensitive:319 wild type, $\chi^2 = 1.4$, $P > 0.1$ for *abi1-3*). Finally, F_2 ABA-hypersensitive seedlings showed linkage between the ABA-hypersensitive phenotype and the presence of a homozygous T-DNA insertion in *ABI1* as determined by PCR analysis ($n = 40$). Taken together, these data indicate that both the *abi1-2* and *abi1-3* mutations are recessive and segregate as a single nuclear locus linked to the T-DNA insertion present in the *ABI1* gene. The ABA inhibitory concentration to achieve 50% inhibition (IC_{50}) of seed germination was approximately 2-fold lower for *abi1-2* and *abi1-3* than for the wild type (0.35, 0.37, and 0.67 μM ABA, respectively; Supplemental Fig. 1).

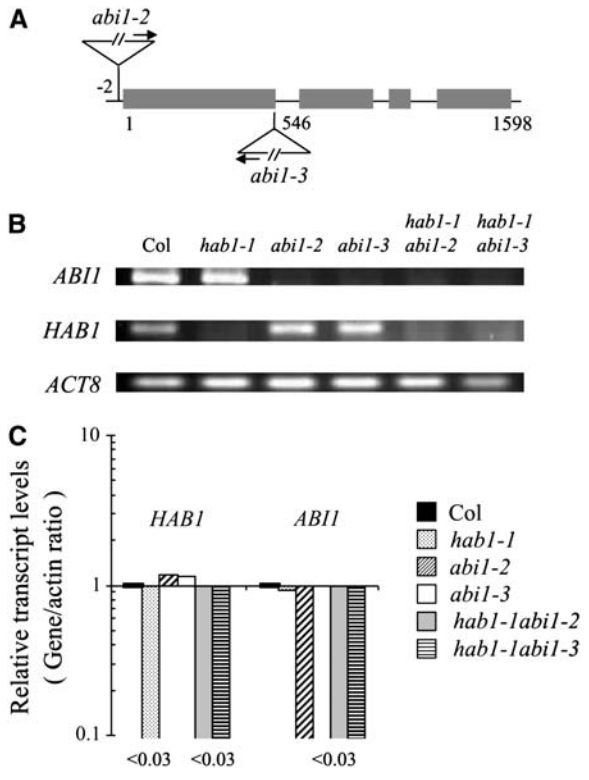

Figure 1. Map of *abi1-2* and *abi1-3* mutants. *ABI1* and *HAB1* transcript levels in wild type, *hab1-1*, *abi1-2*, *abi1-3*, and double *hab1-1 abi1-2*/*hab1-1 abi1-3* mutants. A, Scheme of the *ABI1* gene and localization of the T-DNA insertions in *abi1-2* and *abi1-3* mutants. The numbering begins at the ATG translation start codon. The T-DNA left border primer (LBpROK2) that was used to localize the T-DNA insertion is indicated by an arrow. B, RT-PCR analysis shows absence of full-length transcripts of *ABI1* or *HAB1* in genotypes containing either the *abi1-2*/*abi1-3* or *hab1-1* alleles, respectively. PCR reactions were performed as indicated in "Materials and Methods" and amplification of *β-actin-8* was used as control. Samples were taken for analysis after 25 PCR cycles. C, Expression of *HAB1* and *ABI1* in wild type was similar to that in *abi1-2*/*abi1-3* and *hab1-1* mutants, respectively.

Sensitivity to osmotic stress was also analyzed. ABA plays a critical role promoting inhibition of both seed germination and early seedling growth under high osmoticum (Gonzalez-Guzman et al., 2002). Thus, whereas ABA-hypersensitive mutants are generally more sensitive than wild type to the inhibition of seed germination promoted by osmotic stress (Saez et al., 2004), both ABA-deficient and ABA-insensitive mutants are more tolerant to osmotic stress at this stage (Leon-Kloosterziel et al., 1996; Gonzalez-Guzman et al., 2002). Dose-response analyses of germination and early growth in media supplemented with increasing concentrations of NaCl or mannitol were performed for *abi1-2* and *abi1-3* (Fig. 2, B and C). Both *abi1-2* and *abi1-3* mutants showed higher inhibition of germination and early growth by osmotic stress than wild-type seeds.

Generation and Analysis of *hab1-1 abi1-2* and *hab1-1 abi1-3* Double Mutants

Sequence similarity analysis of the Arabidopsis *PP2C* gene family reveals a branch composed by four members: *ABI1*, *ABI2*, *HAB1*, and *HAB2* (Saez et al., 2004). *ABI1* and *HAB1* appear to play a predominant role over *ABI2* and *HAB2*, respectively, according to their mRNA expression levels and mutant phenotype (Merlot et al., 2001; Leonhardt et al., 2004; Saez et al., 2004; Kuhn, et al., 2006; A. Saez, N. Robert, J. I. Schroeder, and P. L. Rodriguez, unpublished data). Double loss-of-function phenotypes in plant PP2Cs have not yet been analyzed in knockout mutants. To unravel a possible functional redundancy between *ABI1* and *HAB1*, we decided to generate double mutant lines that contained knockout alleles of both genes. To this end we crossed the previously described

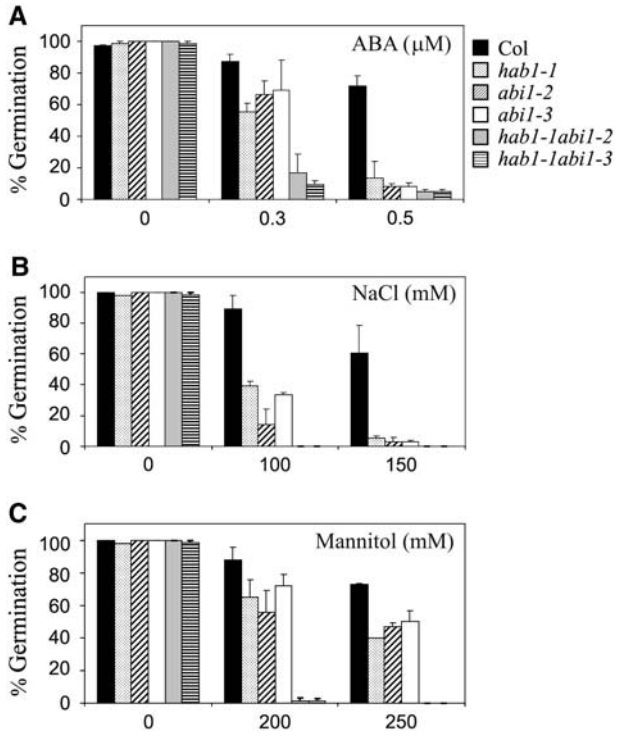

Figure 2. ABA-hypersensitive germination inhibition of *hab1-1*, *abi1-2*, *abi1-3*, and double *hab1-1 abi1-2*/*hab1-1 abi1-3* mutants as compared to wild-type seeds. A to C, Percentage of seeds that germinated and developed green cotyledons in the presence of the indicated concentrations of ABA, NaCl, and mannitol. Approximately 200 seeds of each genotype were sowed on each plate and scored for germination and early growth 10 d later. Values are averages ± SD for three independent experiments.

hab1-1 mutant with either *abi1-2* or *abi1-3*. PCR (data not shown) and RT-PCR analyses (Fig. 1B) of the resulting F_2 population allowed the identification of *hab1-1 abi1-2* and *hab1-1 abi1-3* double mutants, and their response to ABA was analyzed in germination, growth, and transpiration assays.

Analysis of germination and early seedling growth in media supplemented with 0.3 μM ABA indicated an enhanced responsiveness to ABA of the double mutants as compared to the single parental mutants (Fig. 2A; Supplemental Fig. 1). Thus, the IC_{50} of ABA in seed germination was 0.18 μM for the double mutants versus 0.35 and 0.37 μM for *abi1-2* and *abi1-3*, respectively. In agreement with this result, the double mutants were particularly sensitive to inhibition of germination and early growth promoted by both NaCl and mannitol (Fig. 2, B and C). Thus, a concentration of 100 mM NaCl practically abolished germination of the double mutants, whereas 15% to 40% germination was still observed in the single parental mutants (Fig. 2B). Likewise, 200 mM mannitol leads to almost complete inhibition of germination for the double mutants, whereas more than 50% germination is still observed in the single parental mutants (Fig. 2C).

ABA has an inhibitory effect on plant growth when the medium is supplemented with micromolar concentrations of the hormone. For instance, the ABA-insensitive mutants *abi1-1* and *abi2-1* and *35S:HAB1* plants show ABA-resistant growth compared to wild-type plants (Leung et al., 1994, 1997; Meyer et al., 1994; Rodriguez, et al., 1998; Saez et al., 2004). In contrast, the recessive *abi1-1R1* to *R7* alleles were more sensitive to ABA inhibition of root growth than Landsberg *erecta* wild type (Gosti et al., 1999). Figure 3 shows that both *abi1-2* and *abi1-3* displayed enhanced sensitivity to ABA-mediated growth inhibition than wild-type plants. After 10 d in 10 μM ABA, both *abi1-2* and *abi1-3* plants showed yellowing and impaired growth of both leaves and roots. Under these conditions, the *hab1-1* mutant also showed reduced growth as compared to wild-type plants, although growth was inhibited less in *hab1-1* than in *abi1-2* and *abi1-3* mutants (Fig. 3). Finally, both double mutants showed a dramatic growth inhibition in medium supplemented with 10 μM ABA, and they were markedly more sensitive to ABA than the single parental mutants (Fig. 3).

Enhanced ABA-Induced Stomatal Closing and Reduced Water Loss of the *hab1-1 abi1-2* and *hab1-1 abi1-3* Double Mutants

ABA signaling, by regulating stomatal aperture, plays a crucial role to reduce water loss under water shortage. Different analyses were performed to evaluate responses in wild type and the different mutant backgrounds (Fig. 4). Thus, short-term water-loss assays were performed by evaluating the decline in fresh weight of detached leaves (Verslues et al., 2006). The single loss-of-function *abi1-2* and *abi1-3* mutants, as well as *hab1-1*, did not exhibit significant differences in the transpiration rate of detached leaves compared to wild type (Fig. 4A). In contrast, combined inactivation of *HAB1* and *ABI1* resulted in a phenotype of reduced water loss in both double mutants (Fig. 4A).

To further analyze stomatal responses to ABA in the mutants, direct measurements of stomatal closing were performed (Fig. 4B). ABA-induced stomatal closing was assayed in the single *abi1-2* and *hab1-1* mutants, as well as in the double mutant *hab1-1 abi1-2* (Fig. 4B). Stomatal aperture measurements indicated that *abi1-2*, *hab1-1*, and double mutant *hab1-1 abi1-2* were hypersensitive to ABA-induced stomatal closing in the range of 10 to 100 nM ABA. Moreover, the response of the double mutant *hab1-1 abi1-2* to 10 nM ABA was more sensitive as compared to the single parental mutants (Fig. 4B). Similar results to those obtained for *abi1-2* and double mutant *hab1-1 abi1-2* were obtained for *abi1-3* and double mutant *hab1-1 abi1-3*, respectively (Supplemental Fig. 2).

The *era1*, *abh1*, and *gcr1* mutants display enhanced ABA-induced stomatal closing and reduced water loss as compared to wild-type plants (Pei et al., 1998; Hugouvieux et al., 2001; Pandey and Assmann, 2004).

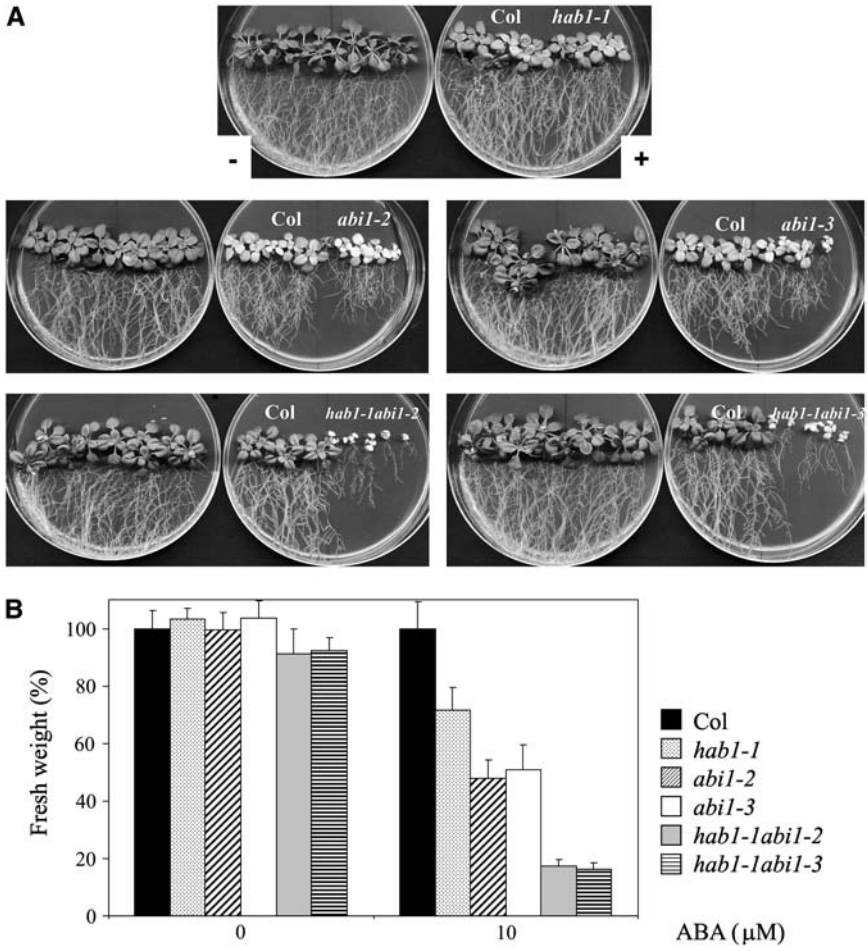

Figure 3. ABA-hypersensitive growth inhibition of *hab1-1*, *abi1-2*, *abi1-3*, and double *hab1-1 abi1-2*/*hab1-1 abi1-3* mutants as compared to wild-type plants. A, Growth of the different mutants and wild type in medium supplemented (+) or not (−) with 10 μM ABA. The photographs were taken after 12 d of the transfer of 5-d-old seedlings from Murashige and Skoog medium to plates lacking or containing 10 μM ABA. B, Percentage of fresh weight from the different mutants as compared to wild type. The percentage was calculated with respect to the fresh weight of wild type in Murashige and Skoog medium either lacking or containing 10 μM ABA. Fresh weight of wild type was reduced by 35% in plates supplemented with ABA as compared to medium lacking ABA. Values are averages ± SD (n = 30).

Therefore, we examined water loss of the different genetic backgrounds described here. Water-loss data were obtained, under greenhouse conditions, after exposing 21-d-old plants to drought stress by completely terminating irrigation and minimizing soil evaporation. Figure 4D shows that after 14 d without watering, wild-type plants wilted and many rosette leaves yellowed. In contrast, *hab1-1 abi1-2* and *hab1-1 abi1-3* double mutant plants did not show symptoms of wilting and they had turgid green rosette leaves. A limited improvement was observed under these conditions in single mutants (Fig. 4D), although far from the phenotype observed in the double mutants. Water loss was estimated by comparing fresh and turgid weight of rosette leaves after 12 d without watering (Fig. 4C). Under these experimental conditions, where the plants were submitted to a long period of drought, the single *hab1-1*, *abi1-2*, and *abi1-3* mutants showed a reduced water loss as compared to wild type (Fig. 4C). Detached-leaf water-loss assays are likely not sensitive enough as to detect such variations (Kuhn et al., 2006), which are apparent after long periods of drought. Thus, whereas wild-type plants exhibited a marked water loss under these conditions, the ABA-hypersensitive mutants exhibited a reduced water loss, particularly in the case of the *hab1-1 abi1-2* and *hab1-1 abi1-3* double mutants.

Enhanced Expression of ABA-Inducible Genes in PP2C Mutants as Compared to Wild Type

The effect of the isolated single and double *hab1* and *abi1* loss-of-function mutations was analyzed on ABA-regulated gene expression. To this end, we used qRT-PCR to analyze the expression of the ABA- and drought-responsive *RAB18*, *P5CS1*, *RD29B*, *KIN1*, *RD29A*, and *RD22* genes, in wild type, single, and double mutants. These gene markers have been widely used to monitor the ABA and stress response pathways in plants (Kurkela and Franck, 1990; Lang and Palva, 1992; Yamaguchi-Shinozaki and Shinozaki, 1994; Strizhov et al., 1997; Abe et al., 2003). In general, in the absence of ABA or stress treatments, these gene markers show a low expression, which is strongly upregulated in response to the inductive signal.

Interestingly, in the absence of exogenous ABA treatment, the double *hab1-1 abi1-2* and *hab1-1 abi1-3* mutants showed approximately 2-fold higher mRNA levels of some gene markers (*RAB18*, *RD29A*, and *RD29B*) as compared to Col wild type (Table I). In the

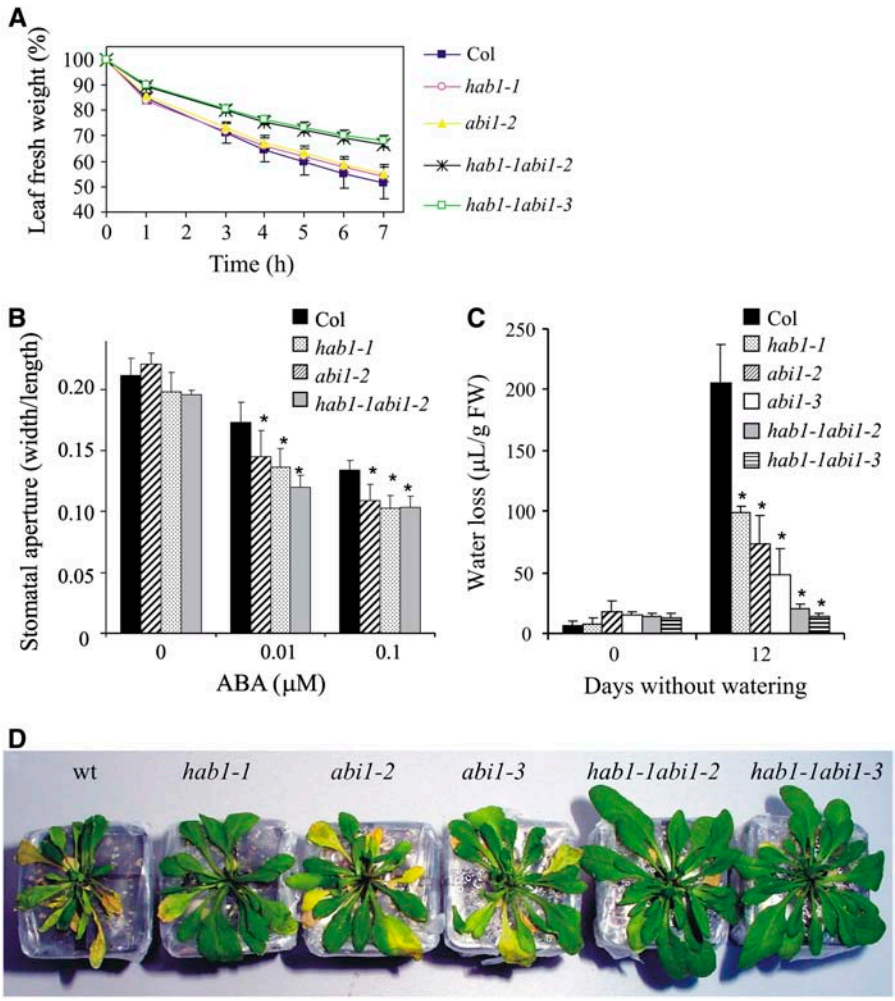

Figure 4. Reduced water loss of double *hab1-1 abi1-2*/*hab1-1 abi1-3* mutants as compared to wild type or single parental mutants. A, Detached-leaves water-loss assays show reduced water loss in double *hab1-1 abi1-2*/*hab1-1 abi1-3* mutants. Five leaves per individual at the same developmental stage and size from 21-d-old plants were excised and fresh weight was determined after submitting the leaves to the drying atmosphere of a flow laminar hood ($n = 4$). Results for *abi1-2* and *abi1-3* were almost identical. B, ABA-induced stomatal closing is ABA hypersensitive in *hab1-1*, *abi1-2*, and double mutant *hab1-1 abi1-2* as compared to wild-type plants. Stomatal apertures were measured 2 h and 30 min after addition of 0.01 or 0.1 μM ABA. Data represent the average of three independent experiments ± SEM (n = 30–40 stomata per experiment). C, Quantification of water loss in 5-week-old plants after 12 d without watering. Data shown are the average amounts of water loss measured in 10 leaves (μL/g fresh weight) collected from four different plants. Asterisks in B and C indicate $P < 0.01$ (Student's t test) when data was compared from mutant and wild type. D, Enhanced drought tolerance of double *hab1-1abi1-2*/*hab1-1 abi1-3* mutants with respect to wild type or single parental mutants. Photograph was taken 14 d after water was withheld. Shoot was cut to better show the effect of drought on rosette leaves.

case of single mutants and under control conditions, only the *RD29B* marker was 2-fold up-regulated in all the single mutants. Upon ABA treatment, as a general trend, induction by ABA was higher in the mutants than in wild type. This enhanced response to ABA was particularly apparent in the double mutants for gene markers that contain ABRE but no typical drought-responsive element (DRE) at the promoter, such as *RAB18*, *RD29B*, and *P5CS1* (between 4- and 8-fold higher expression level than wild type). Gene markers that contain both DRE and ABRE elements *KIN1* and *RD29A*, were also hyperinduced by ABA in the double mutants, although to a lower level (2- to 3-fold). Finally, ABA-mediated induction of *RD22*, which lacks both ABRE and DRE consensus sequences at its promoter, was also up-regulated.

DISCUSSION

In this work, we report the identification and characterization of two new *ABI1* recessive alleles, *abi1-2* and *abi1-3*, as well as *hab1-1 abi1-2* and *hab1-1 abi1-3* double mutants. The knockout *abi1-2* and *abi1-3* mutants (Col background) showed enhanced ABA sensitivity in germination and growth assays, which is in agreement with previous results reported for intragenic revertants of *abi1-1* (Landsberg *erecta* background). ABA-induced stomatal closing was also ABA hypersensitive in *abi1-2* and *abi1-3* (Supplemental Fig. 2) in the range of 10 to 100 nM, in contrast to the recessive *abi1-1R4* allele, which showed a wild-type response at 100 nM ABA (Merlot et al., 2001). This discrepancy might be due to the different genetic background of each mutant or might reflect that *abi1-1R4* is not a knockout mutation. In spite of the enhanced response to ABA in stomatal closure assays, water-loss measurements in detached-leaf assays did not reveal significant differences with respect to wild type for single mutants. This may be due to the finding that detached-leaf water-loss assays to a degree reflect differences in stomatal apertures of wild type compared to a mutant at the beginning of drought experiments rather than later wilting-induced signaling events (Kuhn et al., 2006). In intact plants after a longer drought period, both *abi1-2* and *abi1-3* showed reduced water loss as compared to wild type (Fig. 4C). Finally, both *abi1-2* and *abi1-3* showed an enhanced up-regulation of some

Table I. *Enhanced expression of ABA-inducible genes in PP2C mutants with respect to wild type*

Numbers indicate the induction level of the stress-responsive genes under mock or ABA treatment (10 μM for 3 h) in wild type and mutants. Values are the expression level reached in each mutant genotype with respect to the wild type (value 1). qRT-PCR analyses were made in triplicate on RNA samples obtained from mock-treated plants or plants treated once with 10 μM ABA.

	Genotype					
	RAB18	*KIN1*	*RD22*	*P5CS1*	*RD29a*	*RD29b*
Mock						
Col	1	1	1	1	1	1
hab1-1	0.9	0.8	1.5	0.9	2.7	2.0
abi1-2	1.1	1.0	1.4	1.1	1.3	2.6
abi1-3	0.8	0.7	1.1	0.8	1.0	2.6
hab1-1 abi1-2	2.1	1.6	2.1	1.7	2.0	2.5
hab1-1 abi1-3	2.3	1.2	1.6	1.9	2.0	2.7
ABA treatment						
Col	1	1	1	1	1	1
hab1-1	2.6	1.7	2.5	3.0	3.5	2.1
abi1-2	3.7	1.6	1.7	2.6	1.7	2.4
abi1-3	2.7	1.5	1.5	2.0	1.5	1.3
hab1-1 abi1-2	6.0	2.7	3.0	6.4	2.2	3.9
hab1-1 abi1-3	8.6	3.6	3.1	6.0	2.2	4.9

ABA- and drought-inducible genes compared to wild type, although to a modest level (1.5- to 3-fold). In general, a similarly enhanced response to ABA was observed in the *hab1-1* mutant, except that ABA-mediated inhibition of growth was stronger in both *abi1-2* and *abi1-3* than *hab1-1*, indicating that *ABI1* plays a predominant role in this particular response to ABA. Finally, these phenotypes conclusively indicate that *ABI1* is a global negative regulator of ABA signaling. We speculate that the reduced sensitivity to ABA observed in the dominant *abi1-1* allele might be due to the formation of an inactive complex between abi1-1 and one of its substrates (Gosti et al., 1999), which might be a master positive regulator of ABA signaling. In both *abi1-2* and *abi1-3* recessive mutants the putative target of ABI1 might be hyperactive in response to ABA; conversely, it would be inactivated by the effect of the *abi1-1* dominant allele.

Previous studies have not analyzed double knockout mutants in plant PP2Cs. An *abi1-1R4 abi2-1R1* double mutant was more responsive to ABA than the single parental mutants (Merlot et al., 2001). Combined inactivation of *HAB1* and *ABI1* in the *hab1-1 abi1-2* and *hab1-1 abi1-3* double mutants led to an additive ABA hypersensitivity compared to the single parental mutants. Thus, the IC_{50} for ABA-mediated inhibition of germination was 2-fold lower in the double mutants than in single parental mutants. The double mutants were also more sensitive than single parental mutants to inhibition of germination and early growth mediated by osmotic stress. Imposing osmotic stress at the seedling stage leads to increased ABA biosynthesis and consequently to early growth arrest (Lopez-Molina et al., 2001; Gonzalez-Guzman et al., 2004). Thus, whereas in adult plants ABA plays a crucial role to coordinate the various aspects of the low water potential response to allow plant survival, in seeds and seedlings ABA action is mainly focused to prevent germination and to arrest seedling growth. Interestingly, lowering the osmotic potential of the media by using 200 mM mannitol (−0.5 MPa) had a limited effect on wild type or single mutants, but practically abolished early growth of the double mutants (Fig. 2C). According to the dramatic effect of the combined loss-of-function phenotype, ABI1 and HAB1 must cooperate to negatively regulate ABA signaling at the seed and seedling stage. Another PP2C, PP2CA, was recently shown to strongly and negatively regulate ABA signaling during germination (Kuhn, et al., 2006; Yoshida et al., 2006). The ABA-mediated seed germination phenotype of *pp2ca* or *hab1-1 abi1-2*/*hab1-1 abi1-3* mutants was apparent even though HAB1 and ABI1, or PP2CA, respectively, were functional. Therefore, at least two branches of ABA signaling (or not completely redundant functions of these proteins) appear to exist during seed germination, and the impairing of any of them leads to strong ABA hypersensitivity.

In addition to enhanced ABA-mediated inhibition of seed germination, vegetative responses to ABA were superinduced in the double mutant compared to single parental mutants. For instance, inhibition of growth upon prolonged culture in medium supplemented with ABA was particularly dramatic in *hab1-1 abi1-2* and *hab1-1 abi1-3* double mutants. Transpiration water loss was also noticeably reduced in the double mutants, either measured as detached-leaf assays or after a long period of drought. Finally, ABA-inducible gene expression was notably up-regulated in the double mutants compared to single parental mutants, particularly for those stress-responsive genes mostly regulated through an ABA-dependent pathway, such as *RAB18*, *RD29B*, and *P5CS1*. Taken together, these results indicate partially overlapping functions for HAB1 and ABI1 as negative regulators of ABA signaling, although a predominant role for ABI1 in growth control can be deduced from the ABA-mediated growth-inhibition phenotype observed in *abi1-2* and *abi1-3*. Additionally, these results reveal fine modulation of ABA signaling through the combined action of HAB1 and ABI1 and suggest that different degrees of ABA sensitivity can be engineered in plants through PP2C modulation of the ABA signal transduction pathway.

ABA biosynthetic and signaling pathways can be considered as potential targets to improve plant performance under drought. Thus, it has been demonstrated that transgenic plants producing high levels of ABA display improved growth under drought stress than wild type (Iuchi et al., 2001; Qin and Zeevaart, 2002). Priming of ABA biosynthesis can be obtained by direct overexpression of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in the biosynthetic pathway (Iuchi et al., 2001; Qin and Zeevaart, 2002), or through the use of chemicals that accelerate ABA accumulation

(Jakab et al., 2005). Alternatively, mutants affected in ABA signal transduction might also show an enhanced ABA response leading to stress-tolerant phenotypes.

Many examples of ABA-hypersensitive mutants have been reported (Finkelstein et al., 2002); however, in spite of the critical role of ABA to coordinate plant response to drought, a general correlation between enhanced response to ABA and drought tolerance has not been well established. Thus, although some mutants (i.e. *era1*, *abh1*, and *gcr1*) with enhanced response to ABA have been shown to cause reduced water consumption (Pei et al., 1998; Hugouvieux et al., 2001; Pandey and Assmann, 2004), many examples of mutants that do not match this assertion are known. For instance, the *fry1* and *sad1* mutants, which show ABA-hypersensitive inhibition of seed germination and superinduction of ABA-responsive genes, have compromised tolerance to drought stress (Xiong et al., 2001a, 2001b). Likewise, the *calcineurin B-like 9*, the *calcineurin B-like-interacting protein kinase*, and the *APETALA2-like ABA repressor 1* mutants, which display ABA hypersensitivity and enhanced expression of ABA signaling genes, do not correlate with stress-tolerance phenotypes (Kim et al., 2003; Pandey et al., 2004, 2005). Therefore, superinduction of ABA- and stress-inducible genes in ABA-hypersensitive mutants does not appear to be sufficient to induce drought avoidance. A differential feature of the *era1*, *abh1*, and *gcr1*, as well as *hab1-1 abi1-2*/*hab1-1 abi1-3* double mutants is an enhanced response to ABA in stomata and reduced water loss. Thus, an important consideration for engineering drought avoidance by enhancing ABA responses may include amplifying the molecular mechanisms through which ABA closes stomata. Prospecting of fully or partially sequenced plant genomes from other plants than Arabidopsis reveals the presence of gene products that are likely orthologous to the PP2Cs involved in ABA signaling in Arabidopsis, such as ABI1 and HAB1. Therefore, based on the results presented here, we suggest that silencing in crop plants of genes encoding PP2Cs with similar roles to ABI1 and HAB1 may provide a new biotechnological approach to enhance drought avoidance mechanisms.

A major advance in the study of ABA effect on stomatal closure and opening has been recently reported by Mishra et al. (2006). This work shows that ABA signaling bifurcates at ABI1 and the heterotrimeric G-protein α-subunit GPA1 to regulate ABA-mediated stomatal closure and inhibition of stomatal opening. In this work, an *abi1* knockout line (*abi1-ko*, corresponding to SALK_076309, here named *abi1-3*) was used to show a genetic interaction with the *phospholipase Dα1* mutant (*pldα1*). Thus, whereas the single mutant *pldα1* abolished both ABA promotion of stomatal closure and ABA inhibition of stomatal closure, the double mutant *pldα1 abi1-ko* remained insensitive to ABA in the ABA inhibition of stomatal closing response, but was sensitive to ABA for promotion of stomatal closure. This result suggests that inhibition of stomatal opening by ABA is not governed through ABI1, whereas ABI1 inhibits ABA promotion of stomatal closure. The results further suggest that PLDα1 is not needed for ABA-induced stomatal closing when ABI1 is deleted. These findings are interesting in light of these and other recent findings that several PP2Cs function as negative regulators of ABA signaling (Leonhardt et al., 2004; Saez et al., 2004; Kuhn et al., 2006; Yoshida et al., 2006), but deletion of the ABI1 PP2C is sufficient to restore PLDα1-independent ABA-induced stomatal closing in *pldα1* (Mishra et al., 2006). Finally, we show here that the *abi1-2* and *abi1-3* knockout lines show enhanced ABA-induced stomatal closing. The fact that the *abi1-3* line reported by Mishra et al. (2006) did not show an ABA-hypersensitive phenotype in the stomatal closure response can likely be explained because a high dose (50 μM) of ABA was assayed.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) plants were routinely grown under greenhouse conditions in pots containing a 1:3 vermiculite-soil mixture. For in vitro culture, seeds were surface sterilized by treatment with 70% ethanol for 20 min, followed by commercial bleach (2.5% sodium hypochlorite) containing 0.05% Triton X-100 for 10 min, and finally, four washes with sterile distilled water. Stratification of the seeds was conducted in the dark at 4°C for 3 d. Then, seeds were sowed on Murashige and Skoog (1962) plates composed of Murashige and Skoog basal salts, 0.1% 2-[*N*-morpholino]ethanesulfonic acid, 1% agar, and 1% Suc. The pH was adjusted to 5.7 with potassium hydroxide before autoclaving. Plates were sealed and incubated in a controlled environment growth chamber at 22°C under a 16-h light, 8-h dark photoperiod at 80 to 100 μE m^{-2} s^{-1}.

Mutant Identification by PCR Screening

Two lines containing a single T-DNA insertion in *ABI1* were identified in the SALK T-DNA collection (SALK_72009 and SALK_76309; Alonso et al., 2003) and obtained from the Nottingham Arabidopsis Stock Center (http://nasc.nott.ac.uk). To identify individuals homozygous for the T-DNA insertion, genomic DNA was obtained from kanamycin-resistant seedlings and submitted to PCR genotyping using the following *ABI1* primers: line SALK_72009, 5′-AGGAAACCCTTATTGAAATTC and 5′-CTCTGTTCTGCTGATCATCT; line SALK_76309, 5′-CCGGCCCTCGAGATGATCAGCAGAACAGAGAGT and 5′-CCGGCCCTCGAGTCAGTTCAAGGGTTTGCT. As T-DNA left border primer of the pROK2 vector, we used LBpROK2 (5′-GCCGATTTCGGAACCACCATC).

To generate the *hab1-1 abi1-2* and *hab1-1 abi1-3* double mutants, we transferred pollen of either *abi1-2* or *abi1-3* to the stigmas of emasculated flowers of *hab1-1*. The resulting F_2 individuals were genotyped by PCR for the presence of homozygous *hab1-1* (Saez et al., 2004), *abi1-2*, and *abi1-3* alleles (see above).

Germination Assays

To measure ABA sensitivity, seeds were plated on solid medium composed of Murashige and Skoog basal salts, 1% Suc, and increasing concentrations of ABA. To determine sensitivity to inhibition of germination by high osmoticum the medium was supplemented with increasing concentrations of either sodium chloride or mannitol, respectively. To score seed germination, the percentage of seeds that had germinated and developed fully green expanded cotyledons was determined.

Growth and Stomatal Aperture Assays

The ABA-resistant growth was scored by weighting whole plants after 12 d of the transfer of 5-d-old seedlings onto Murashige and Skoog plates supplemented with 10 μM ABA. Data were obtained for three independent

experiments, each done with 15 plants. For assays of ABA-induced stomatal closing, leaves of 5- to 6-week-old plants were used. Measurements were performed on epidermal peels, which were first incubated for 2 h and 30 min in stomatal opening buffer containing 10 mM KCl, 7.5 mM iminodiacetic acid, and 10 mM MES/Tris, pH 6.2, at 20°C. Then, they were incubated for 2 h and 30 min in the same buffer supplemented or not with 10 and 100 nM ABA. Data were expressed as the average of four experiments where 30 to 40 stomata were measured for each one.

Drought Stress and Water-Loss Assays

Two different water-loss assays were performed. Short-term assays were performed in detached leaves at the same developmental stage and size from 21-d-old plants. Five leaves per individual were excised and fresh weight was determined after submitting the leaves to the drying atmosphere of a flow laminar hood. Kinetics analysis of water loss was performed and represented as the percentage of initial fresh weight at each time point.

Long-term assays were performed after removing watering in plants maintained under greenhouse conditions. To this end, plants (10 individuals per experiment, three independent experiments) were grown under normal watering conditions for 21 d and then subjected to drought stress by completely terminating irrigation and minimizing soil evaporation by covering pots with plastic Saran Wrap film. Ten leaves from each plant were removed at the time points indicated. Subsequently, leaves were weighted, incubated in demineralized water for 3 h, and weighed again. The difference in weight was considered as water loss.

RNA Analyses

Plants were grown on Murashige and Skoog plates supplemented with 1% Suc. After 7 d, approximately 30 to 40 seedlings were either mock or 10 μM ABA treated. After 3 h, plant material was collected and frozen in liquid nitrogen. Total RNA was extracted using a Qiagen RNeasy plant mini kit and 1 μg of the RNA solution obtained was reverse transcribed using 0.1 μg oligo(dT)$_{15}$ primer and Moloney murine leukemia virus reverse transcriptase (Roche) to finally obtain a 40 μL cDNA solution. qRT-PCR amplifications and measurements were performed using an ABI PRISM 7000 sequence detection system (Perkin-Elmer Applied Biosystems). The sequences of the primers used for PCR amplifications were the following ones: for *HAB1* (At1g72770), forward 5′-AACTGCTGTTGTTGCCTTG and reverse 5′-GGTTCTGGTCT-TGAACTTTCT; for *ABI1* (At4g26080), forward 5′-ATGATCAGCAGAAC-AGAGAGT and reverse 5′-TCAGTTCAAGGGTTTGCT; for *KIN1* (At5g15960), forward 5′-GCTGGCAAAGCTGAGGAGAA and reverse 5′-TTCCCGCCTG-TTGTGCTC; for *RD29A* (At5g52310), forward 5′-GTCCAAAGTTAC-TGATCC-CAC and reverse 5′-CTTCATATCAAAATCATGACT; for *P5CS1* (At2g39800), forward 5′-TTTATGGTGCTATAGATCACA and reverse 5′-GAATGTCCT-GATGGGTGTAAAC; for *RAB18* (At5g66400), forward 5′-ATG GCG TCT TACCAGAACCGT and reverse 5′-CCAGATCCGGAGCGGTGAAGC; for *RD29B* (At5g52300), forward 5′-ATG GAG TCA CAG TTG ACA CGT CC and reverse 5′-GAG ATA GTC ATC TTC ACC ACC AGG; for *RD22* (At5g25610), forward 5′-ATG GCG ATT CGG CTT CCT CTG ATC and reverse 5′-GAC ATT CAT TTC TTT CCC GCG AAC; and for *β-actin-8* (At1g49420), forward 5′-AGTGGTCGTACAACCGGTATTGT and reverse 5′-GAGGATAGCATGTGGAAGTGAGAA.

qRT-PCR amplifications were monitored using the Eva-Green fluorescent stain (Biotium). Relative quantification of gene expression data was carried out using the $2_T^{-\Delta\Delta C}$ or comparative C_T method (Livak and Schmittgen, 2001). Expression levels were normalized using the C_T values obtained for the *β-actin-8* gene. The presence of a single PCR product was further verified by dissociation analysis in all amplifications. All quantifications were made in triplicate on RNA samples obtained from plants treated once with ABA.

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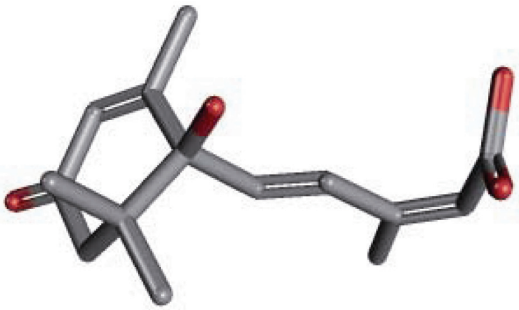

Capítulo III

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HAB1–SWI3B Interaction Reveals a Link between Abscisic Acid Signaling and Putative SWI/SNF Chromatin-Remodeling Complexes in *Arabidopsis* [C][W]

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Abscisic acid (ABA) has an important role for plant growth, development, and stress adaptation. HYPERSENSITIVE TO ABA1 (HAB1) is a protein phosphatase type 2C that plays a key role as a negative regulator of ABA signaling; however, the molecular details of HAB1 action in this process are not known. A two-hybrid screen revealed that SWI3B, an *Arabidopsis thaliana* homolog of the yeast SWI3 subunit of SWI/SNF chromatin-remodeling complexes, is a prevalent interacting partner of HAB1. The interaction mapped to the N-terminal half of SWI3B and required an intact protein phosphatase catalytic domain. Bimolecular fluorescence complementation and coimmunoprecipitation assays confirmed the interaction of HAB1 and SWI3B in the nucleus of plant cells. *swi3b* mutants showed a reduced sensitivity to ABA-mediated inhibition of seed germination and growth and reduced expression of the ABA-responsive genes *RAB18* and *RD29B*. Chromatin immunoprecipitation experiments showed that the presence of HAB1 in the vicinity of *RD29B* and *RAB18* promoters was abolished by ABA, which suggests a direct involvement of HAB1 in the regulation of ABA-induced transcription. Additionally, our results uncover SWI3B as a novel positive regulator of ABA signaling and suggest that HAB1 modulates ABA response through the regulation of a putative SWI/SNF chromatin-remodeling complex.

INTRODUCTION

The phytohormone abscisic acid (ABA) is a key regulator of plant growth and development as well as plant responses to decreased water availability. A fast mechanism to adjust ABA levels and respond to changing environmental cues is the hydrolysis of glucose-conjugated ABA (Lee et al., 2006). Additionally, water stress leads to the accumulation of ABA through enhanced expression of ABA biosynthetic genes, mainly *9-cis-epoxycarotenoid dioxygenase3* (Nambara and Marion-Poll, 2005; Barrero et al., 2006). ABA triggers a variety of adaptive responses, such as stomatal closure and differential gene expression, which are crucial for plant survival under stress conditions (Schroeder et al., 2001; Nambara and Marion-Poll, 2005).

Decades of research in ABA signaling have resulted in the identification of many elements of the ABA signal transduction pathway, including both negative and positive regulators (reviewed in Finkelstein et al., 2002; Himmelbach et al., 2003; Israelsson et al., 2006). Under water stress, ABA signaling leads to coordinated remodeling of gene expression, which affects more than ~5% of the plant transcriptome (Huang et al., 2007). Downstream nuclear effects of ABA are mediated by different transcription factors (TFs) that play a positive role in ABA signaling, which comprise ABA-responsive element binding proteins (ABI5/ABF/AREB/bZIP family) (Choi et al., 2000; Finkelstein and Lynch, 2000; Uno et al., 2000; Bensmihen et al., 2002), *Arabidopsis thaliana* ABI3 and maize (*Zea mays*) VP1 TFs of the B3 domain family (McCarty et al., 1991; Giraudat et al., 1992), the ABI4 TF from the APETALA2 domain family (Finkelstein et al., 1998), and ATMYC2 and ATMYB2 TFs (Abe et al., 2003). Some TFs that function as transcriptional repressors of ABA response have also been described (Himmelbach et al., 2002; Pandey et al., 2005; Song et al., 2005). In eukaryotes, the packaging of DNA into chromatin implies that both transcriptional activators and repressors work together with large multisubunit complexes that remodel nucleosomes to regulate gene expression (Carrozza et al., 2003; Smith and Peterson, 2005). Two general classes of chromatin-modifying factors can be distinguished, those that covalently modify the N-terminal tails of histone proteins and those that utilize ATP hydrolysis to remodel or reposition nucleosomes (Carrozza et al., 2003; Smith and Peterson, 2005). The first class includes protein complexes that acetylate or deacetylate Lys residues present in the N termini of histone proteins (histone acetyltransferases) and histone deacetylases. The second class of factors is composed of ATP-dependent chromatin-remodeling complexes, which alter nucleosome structure or positioning. Among them, the yeast SWI/SNF complex was the first one to be described (Cairns et al., 1994; Peterson et al., 1994). In addition to the ATPase Swi2/Snf2, it contains a central core composed of three additional polypeptides, Swi3, Snf5, and

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Swp73, which are required for the assembly and activity of the complex (Cairns and Kingston, 2000; Smith and Peterson, 2005; Yang et al., 2007). Some reports of chromatin-modifying factors that affect ABA responses have been published (Song et al., 2005; Sridha and Wu, 2006); however, taking into account the deep impact of ABA on the regulation of gene expression and the many TFs involved in this process, we can envisage that many elements in this field are yet to be discovered.

Protein phosphatase type 2Cs (PP2Cs) were identified as key components of ABA signaling from pioneering work with the ABA-insensitive *abi1-1* and *abi2-1* mutants (Koornneef et al., 1984; Leung et al., 1994; Meyer et al., 1994; Leung et al., 1997; Rodriguez et al., 1998a). Currently, at least six *Arabidopsis* PP2Cs, namely ABI1, ABI2, PP2CA/AHG3, ABA-HYPERSENSITIVE GERMINATION1 (AHG1), HYPERSENSITIVE TO ABA1 (HAB1), and HAB2, are known to regulate ABA signaling. Genetic approaches indicate that these PP2Cs are negative regulators of ABA signaling (Gosti et al., 1999; Merlot et al., 2001; Tahtiharju and Palva, 2001; Gonzalez-Garcia et al., 2003; Leonhardt et al., 2004; Saez et al., 2004, 2006; Kuhn et al., 2006; Yoshida et al., 2006b; Nishimura et al., 2007). Although interacting partners for some of these PP2Cs have been described (Cherel et al., 2002; Guo et al., 2002; Himmelbach et al., 2002; Ohta et al., 2003; Miao et al., 2006; Yang et al., 2006; Yoshida et al., 2006a), the overall knowledge of their targets and their role in ABA signaling is far from complete. In this work, we have pursued a two-hybrid approach using the PP2C HAB1 as bait to identify putative interacting preys. Interestingly, a prevalent interacting partner of HAB1 was found to be the SWI3B protein, which is an *Arabidopsis* homolog of the SWI3 core subunit of SWI/SNF chromatin-remodeling complexes (Sarnowski et al., 2002; Zhou et al., 2003). These complexes, already characterized in yeast, *Drosophila*, and mammals, have not yet been biochemically characterized in plants, although genome analysis suggests that *Arabidopsis* contains the active components required to form such complexes (Farrona et al., 2004; Sarnowski et al., 2005). Thus, in *Arabidopsis*, four SWI3-like proteins (i.e., SWI3A, SWI3B, SWI3C, and SWI3D) have been identified (Sarnowski et al., 2002; Zhou et al., 2003) as well as other putative components of SWI/SNF complexes (Brzeski et al., 1999; Farrona et al., 2004; Bezhani et al., 2007). Current data on loci that encode putative components of SWI/SNF chromatin-remodeling complexes show that they operate as modifiers of transcriptional or epigenetic regulation in plant growth and development (Kwon and Wagner, 2007). Our data provide a link between a component of the ABA signaling pathway and a putative component of SWI/SNF chromatin-remodeling complexes and, therefore, suggest that these complexes are also involved in the hormonal response to abiotic stress.

RESULTS

Identification of SWI3B as a HAB1-Interacting Partner

A yeast two-hybrid screen was used to identify proteins that interact with the PP2C HAB1. Preliminary experiments revealed that full-length HAB1 fused to the GAL4 DNA binding domain (GBD) resulted in the activation of *HIS3* and *ADE2* reporters from the AH109 yeast strain used in this study (see Supplemental Figure 1 online). N-terminal truncation of some clade A PP2Cs (Schweighofer et al., 2004) is required to reduce their potential to activate transcription (Himmelbach et al., 2002; this work). Indeed, the N-terminal 1 to 180 amino acid residues either from HAB1 (see Supplemental Figure 1 online) or from the closely related PP2C HAB2, when fused to the GBD, generated a powerful transcriptional activator. Thus, only the catalytic region (amino acid residues 179 to 511) of the PP2C HAB1 (ΔNHAB1) was used as a bait to screen an *Arabidopsis* expression library containing random cDNAs fused to the GAL4 activation domain (GAL) in the pACT2 vector (Nemeth et al., 1998). This N-terminal truncation of HAB1 showed approximately twofold higher phosphatase activity than full-length HAB1 (Figure 1B). From 10^6 colonies screened, 20 positive clones that showed autotrophic growth in medium lacking both adenine and His were selected. Sequence analysis of the recovered pACT2 clones revealed that 11 of the 20 putative interacting preys contained the full-length cDNA from *SWI3B*. Therefore, these results indicate that AtSWI3B is a prevalent HAB1-interacting partner in a two-hybrid screening.

The Interaction of HAB1 and SWI3B Requires a Functional PP2C Catalytic Domain and Maps to the N-Terminal Half of SWI3B

Protein domain analysis using the PFAM database of global domain hidden Markov models and different pattern and profile searches in Expasy (http://www.expasy.org) served to identify SWIRM (48 to 136), SANT (224 to 272), and Leu zipper domains (399 to 452) in the SWI3B amino acid sequence, in agreement with previous findings from Sarnowski et al. (2005). Additionally, we could identify a ZZ zinc finger domain (Cys-x_2-Cys motifs plus a conserved YDL motif) between amino acid residues 169 and 208. A similar ZZ zinc finger domain was identified in *Arabidopsis* SWI3C by Hurtado et al. (2006). In order to determine specific regions of SWI3B involved in the interaction with ΔNHAB1, different deletions of the *SWI3B* coding sequence in the prey vector pACT2 were generated. Previously, we confirmed that a combination of the empty pGBT9 plasmid and pACT2-*SWI3B* did not activate transcription of the *HIS3* and *ADE2* reporter genes (Figure 1A); moreover, none of the deletion constructs activated transcription in the absence of bait protein interactors. In combination with the bait construct pGBT9-*ΔNHAB1*, the deletion constructs C1 and C2 activated transcription of the reporter genes to the same levels as full-length *SWI3B* (Figure 1A). This result mapped the HAB1-interacting domain to the first 220 amino acid residues of SWI3B. In agreement with this result, the prey construct N1 did not activate the reporter genes in the growth assay. Further attempts to delimit the minimal region of SWI3B that interacted with ΔNHAB1 failed, as additional deletions affecting the N-terminal half of SWI3B (SWIRM and ZZ prey constructs) eliminated the interaction with ΔNHAB1.

In order to clarify the specificity of the interaction, we examined whether other SWI3-like proteins from *Arabidopsis* showed interaction with ΔNHAB1. In contrast with SWI3B, none of the SWI3A, SWI3C, or SWI3D proteins interacted with ΔNHAB1

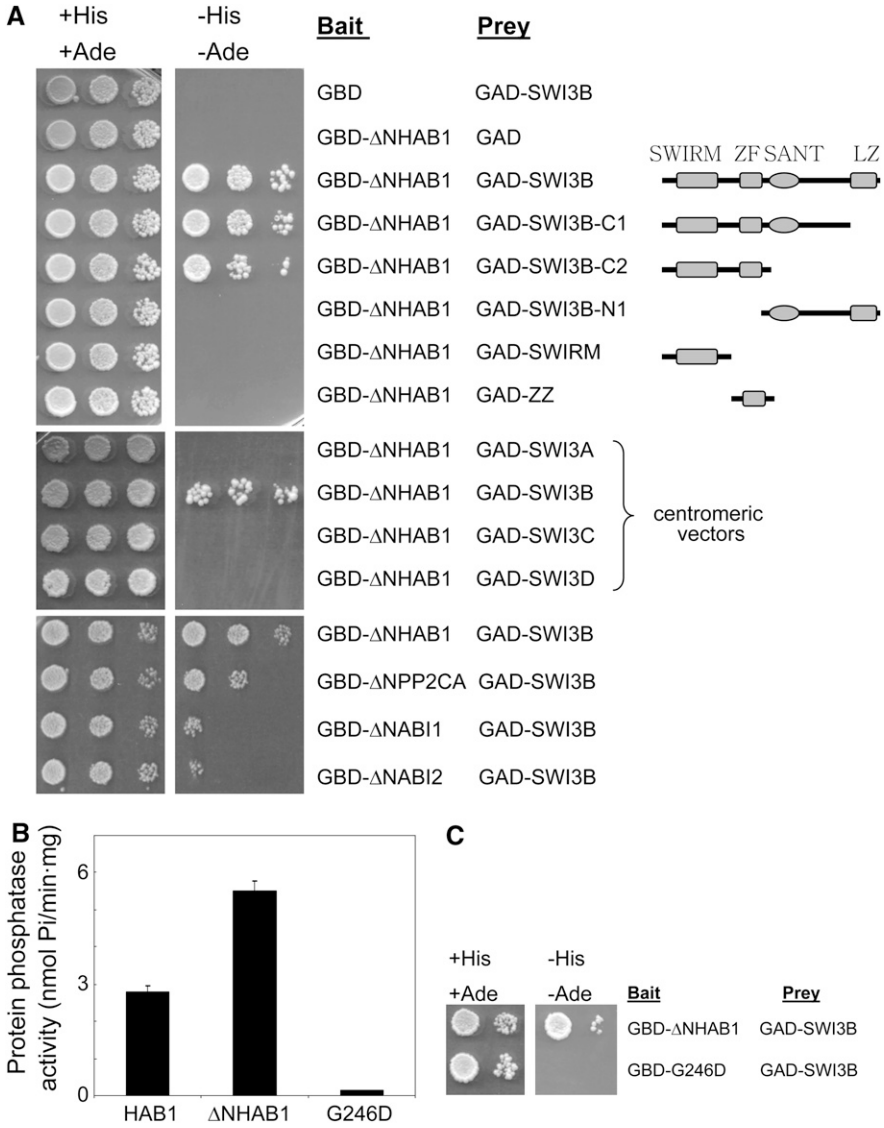

Figure 1. HAB1 and SWI3B Interact in a Yeast Two-Hybrid Assay.

Interaction was determined by growth assay on medium lacking His and adenine. Dilutions (10^{-1}, 10^{-2}, and 10^{-3}) of saturated cultures were spotted onto the plates.

(A) Top, interaction assay with ΔNHAB1 as bait (fused to the GBD) and either full-length or different deletions of SWI3B as putative preys (fused to the GAD). Schemes of SWI3B domains and the different protein deletions are shown. Deletions C1 and C2 lacked C-terminal amino acid residues 346 to 469 and 221 to 469, respectively. Deletion N1 lacked N-terminal amino acid residues 1 to 220. GAD-SWIRM and GAD-ZZ comprised amino acid residues 1 to 140 and 134 to 220, respectively. Middle, interaction assay with SWI3A, SWI3B, SWI3C, and SWI3D as putative preys. Bottom, interaction assay with ΔNPP2CA, ΔNABI1, and ΔNABI2 as baits and SWI3B as prey.

(B) Protein phosphatase activity of MBP-HAB1, MBP-ΔNHAB1, and MBP-G246D ΔNhab1 fusion proteins. Values are averages ± SE from three independent experiments.

(C) Interaction assay with ΔNHAB1 and G246D ΔNhab1 as baits and SWI3B as prey.

(Figure 1A, middle). This result highlights the remarkable functional diversification previously described for the four SWI3-like proteins from *Arabidopsis* (Sarnowski et al., 2005; Hurtado et al., 2006). HAB1 belongs to a group of PP2Cs (clade A; Schweighofer et al., 2004) in which six of the identified genes are associated with ABA signaling. Gene expression data and genetic analysis indicate that HAB1, PP2CA, ABI1, and ABI2 play a predominant role in ABA signaling in both seeds and vegetative tissue (Saez et al., 2004, 2006; Kuhn et al., 2006; http://www.genevestigator.ethz.ch). Therefore, we generated N-terminal truncations of PP2CA, ABI1, and ABI2 fused to GBD and their interaction with SWI3B was examined (Figure 1A, bottom). ΔNPP2CA, ΔNABI1,

and ΔNABI2 were able to interact with SWI3B, although it was apparent in the growth assay that the interaction was weaker than that observed for ΔNHAB1. All fusion proteins were expressed at similar levels, as verified by protein gel blot analysis using antibodies against the GAD and GBD. Finally, in order to examine the role of the catalytic PP2C domain in the interaction with SWI3B, a point-mutated version of HAB1 that replaced Gly-246 for Asp (G246D hab1) was introduced in the two-hybrid test. The Gly-246 is localized in a conserved motif from eukaryotic PP2Cs, and its replacement by Asp interferes with Mg^{2+} binding and strongly impairs PP2C activity (Leung et al., 1994; Meyer et al., 1994). Indeed, both G246D hab1 (Robert et al., 2006) and G246D ΔNhab1 (Figure 1B) show <3% in vitro activity than the wild type. Interestingly, G246D ΔNhab1 did not interact with SWI3B in the two-hybrid assay, indicating that a functional catalytic domain of HAB1 is required for its interaction with SWI3B (Figure 1C).

Subcellular Localization of HAB1 and SWI3B

To determine the subcellular localization of HAB1 and SWI3B proteins in plant cells, we performed in vivo targeting experiments in tobacco (*Nicotiana benthamiana*). To this end, *35S:HAB1-GFP* and *35S:SWI3B-GFP* constructs were generated and delivered into leaf cells of tobacco by *Agrobacterium tumefaciens* infiltration (Voinnet et al., 2003). Coexpression of bZIP63-YFP^N and bZIP63-YFP^C served as a positive control for localization of a nuclear protein (Walter et al., 2004). In the case of SWI3B, strong green fluorescent protein (GFP) fluorescence was observed in the nucleus of tobacco cells, whereas HAB1 localized in both the nucleus and the cytosol (Figure 2A). Similar results were obtained when the coding region of *HAB1* was fused to the C-terminal end of *GFP* (see Supplemental Figure 2 online). Finally, ABA treatment did not modify the subcellular localization of either HAB1-GFP or SWI3B-GFP under our experimental conditions (Figure 2A; see Supplemental Figure 2 online).

In addition to using GFP fusions, we examined the subcellular localization of HAB1 by standard biochemical techniques. To this end, we generated transgenic lines (in a *hab1-1* background) that expressed a double hemagglutinin (HA) epitope–tagged version of HAB1 (HAB1-dHA) under the control of the *HAB1* native promoter. HAB1-dHA efficiently complemented the ABA-hypersensitive phenotype of *hab1-1* in germination assays (Saez et al., 2004). HAB1-dHA could be detected in both the cytosolic and nuclear fractions (Figure 2B). Washing of the nuclei/organelles with a buffer containing 0.5% Triton X-100 released a significant amount of HAB1-dHA (W fraction), which we assume to be of nuclear origin according to the localization of HAB1-GFP and GFP-HAB1 fusions. Additionally, a fraction of HAB1-dHA was associated with the nuclear insoluble fraction, which contains the major histones and is mostly composed of chromatin (Poveda et al., 2004; Cho et al., 2006). Finally, HAB1-dHA was also detected in a nuclear soluble fraction that was obtained by rupture of the nuclei and extraction with a buffer containing 0.4 M NaCl. An estimation of the cytosolic:nuclear HAB1 ratio was made based on protein blot analysis with the anti-HA antibody. Figure 2C shows an approximately threefold difference in HAB1 abundance between the cytosolic and combined nuclear fractions. Taking into account the additional fourfold enrichment during the nuclei isolation process before protein gel loading, the HAB1 cytosolic:nuclear ratio was ~12:1. Treatment with 50 μM ABA for 1 h did not substantially modify this ratio (Figure 2C).

In Planta Interaction between HAB1 and SWI3B

Bimolecular fluorescence complementation (BiFC) assays were used to detect the interaction between HAB1 and SWI3B in plant cells. To this end, HAB1 was translationally fused to the C-terminal 84–amino acid portion of yellow fluorescent protein (YFP^C) in the pSPYCE vector, which generated a HAB1-epitope HA-YFP^C fusion protein (Figure 3B). For the other partner, the N-terminal half of SWI3B was translationally fused to the N-terminal 155–amino acid portion of yellow fluorescent protein (YFP^N) in the pSPYNE vector, which generated a SWI3B-epitope myc-YFP^N fusion protein (Figure 3B). The corresponding constructs were codelivered into leaf cells of tobacco by *Agrobacterium* infiltration and, as a result, fluorescence was observed in the nucleus of tobacco cells (Figure 3A, left). No fluorescence signal was observed when pSPYCE-*HAB1* vector was codelivered with pSPYNE or when pSPYNE-*SWI3B* was codelivered with pSPYCE. Moreover, in agreement with the previous finding in the two-hybrid assay, introduction of the G246D mutation in the sequence of HAB1 abolished the interaction with SWI3B in the BiFC assay (Figure 3A, right).

In addition to the observed BiFC fluorescent signal, we confirmed the interaction by coimmunoprecipitation of HAB1 and SWI3B in tobacco protein extracts prepared from the BiFC assay described above (Figure 3B). HAB1 and SWI3B can be coimmunoprecipitated, as we could detect SWI3B in the immunocomplex precipitated with an antibody to epitope HA, which pulls down the HAB1-HA-YFP^C fusion protein (Figure 3B). By contrast, introduction of the G246D mutation in the sequence of HAB1 prevented the coimmunoprecipitation of SWI3B (Figure 3B). Thus, results from two in planta assays support the interaction between HAB1 and SWI3B.

Finally, BiFC assays showed that PP2CA, ABI1, and ABI2 were able to interact with SWI3B in the nucleus of tobacco cells (Figure 3C). Expression of fusion proteins was verified by protein gel blot analysis using antibodies against the epitope HA and peptide comprising amino acids 3 to 17 of GFP (anti-GFP^N) (Figure 3D). ABA treatment (50 μM for 1 h) did not change the interaction of the PP2Cs and SWI3B. However, complex formation in BiFC is essentially irreversible, which prevents the imaging of changes in the protein association state (Fricker et al., 2006).

swi3b Mutants Show a Reduced Sensitivity to ABA and Reduced Expression of RD29B and RAB18

The *swi3b-1* and *swi3b-2* knockout mutants (Figure 4A) were previously reported to be embryo-lethal (Sarnowski et al., 2005); therefore, we decided to examine heterozygous mutants for phenotypic effects. Phenotypic effects caused by gene haploinsufficiency (monoallelic expression and heterozygosis) have been described in mutants affected in diverse components of the chromatin-remodeling machinery (Bultman et al., 2000; Roberts et al., 2000; Alarcon et al., 2004; David et al., 2006).

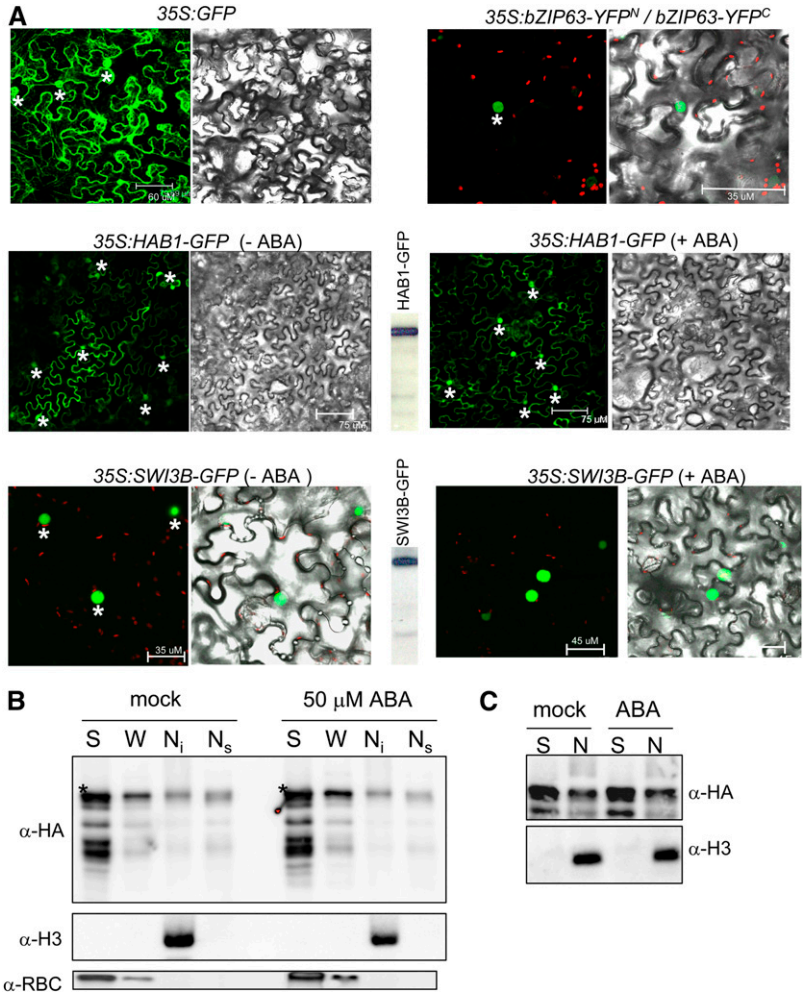

Figure 2. HAB1 Localizes at Both Cytosol and Nucleus.

(A) Subcellular localization of HAB1 and SWI3B in *Agrobacterium*-infiltrated tobacco leaves. Epifluorescence and bright-field images of epidermal leaf cells infiltrated with a mixture of *Agrobacterium* suspensions harboring the indicated constructs and the silencing suppressor p19. BiFC-induced bZIP63 dimerization served to identify the nuclei of tobacco epidermal cells (asterisks). The expression of the proteins is demonstrated by immunodetection with anti-GFP for HAB1-GFP and SWI3B-GFP (center, between panels). Treatment with 50 μM ABA for 1 h (+ABA) did not change the subcellular localization of both HAB1-GFP and SWI3B-GFP.

(B) Biochemical fractionation of HAB1-dHA (full-length protein marked with asterisks). Plant material was obtained from the *hab1-1::ProHAB1-HAB1-dHA* transgenic line after mock treatment or treatment with 50 μM ABA for 1 h. The soluble cytosolic fraction (S), nuclei/organelles wash fraction (W), nuclear insoluble fraction (Ni), and nuclear soluble fraction (Ns) were analyzed using anti-HA, anti-histone 3 (H3), and anti–ribulose-1,5-bis-phosphate carboxylase/oxygenase (RBC) antibodies.

(C) Relative amount of HAB1-dHA in the soluble cytosolic (S) and nuclear (N) fractions after mock treatment or treatment with 50 μM ABA for 1 h.

For instance, heterozygous mice that have a single copy of either *BRG1* (the mammalian orthologous gene of the yeast Swi2/Snf2 ATPase) or *SNF5* (a core component of the SWI/SNF complex) are predisposed to different tumors, indicating that a full dosage of both BRG1 and SNF5 is required for proper control of gene expression and tumor suppression (Bultman et al., 2000; Roberts et al., 2000). Therefore, we decided to analyze ABA responsiveness in the progeny of *Arabidopsis swi3b* +/− seedlings, which represents an ~2:1 mixture of heterozygous and wild-type seeds (Sarnowski et al., 2005). Thus, the progeny from *swi3b-1* and *swi3b-2* heterozygous plants were analyzed to score ABA-mediated inhibition of germination and growth. These assays revealed a reduced sensitivity to ABA of *swi3b* +/− seeds and seedlings compared with the wild type (Figures 4B and 4C). This phenotype was particularly apparent in growth assays, as after 10 d in 10 μM ABA both *swi3b-1* and *swi3b-2* +/− seedlings showed ~80 to 90% higher weight than wild-type seedlings (Figure 4C). By contrast, water-loss assays did not show significant differences between wild-type and *swi3b-1* and *swi3b-2* +/− plants (see Supplemental Figure 3A online). Nevertheless, since ~50% reduction in the expression of *SWI3B* (see Supplemental Figure 3B online) led to reduced sensitivity to ABA in germination

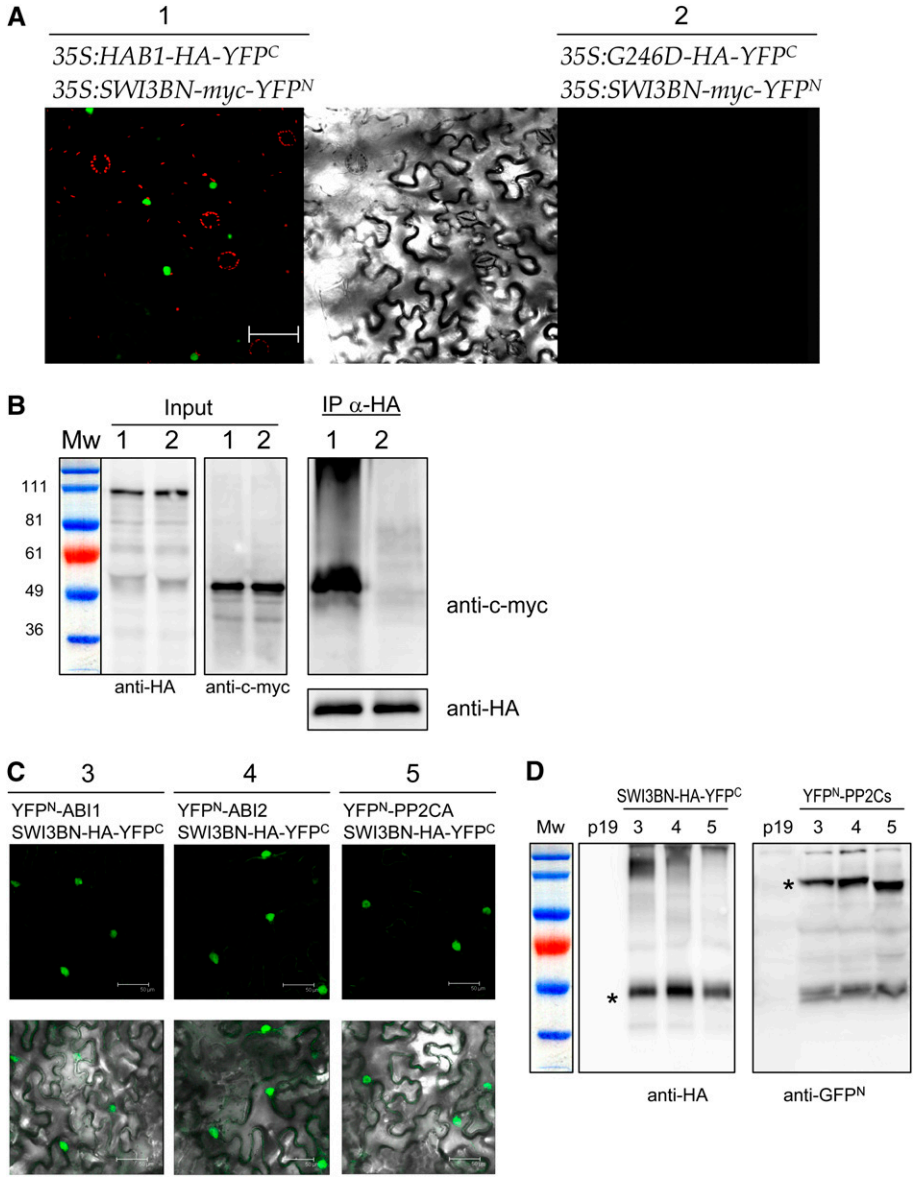

Figure 3. BiFC Visualization and Coimmunoprecipitation Experiments Show Interaction between HAB1 and SWI3B in the Nucleus of Tobacco Leaves.

(A) Introduction of the G246D substitution into HAB1 abolishes the interaction with SWI3B. Epifluorescence and bright-field images of epidermal leaf cells infiltrated with a mixture of *Agrobacterium* suspensions harboring constructs HAB1-HA-YFP^C/SWI3BN-myc-YFP^N (panel 1) or G246D-HA-YFP^C/SWI3BN-myc-YFP^N (panel 2) and the silencing suppressor p19. The bar corresponds to 75 μm. Green color corresponds to YFP, whereas red color is generated by chlorophyll fluorescence.

(B) Coimmunoprecipitation assay demonstrates the interaction between HAB1 and SWI3B in planta. Protein extracts obtained from tobacco leaves infiltrated with *Agrobacterium* suspensions harboring constructs HAB1-HA-YFP^C/SWI3BN-myc-YFP^N (lanes 1) or G246D-HA-YFP^C/SWI3BN-myc-YFP^N (lanes 2) were analyzed using anti-HA or anti-c-myc antibodies. Input levels of epitope-tagged proteins in crude protein extracts (20 μg of total protein) were analyzed by immunoblotting. Immunoprecipitated epitope HA–tagged proteins were probed with anti-c-myc antibodies to detect coimmunoprecipitation of SWI3BN-myc-YFP^N with HAB1-HA-YFP^C.

(C) BiFC assays show the interaction of ABI1, ABI2, and PP2CA with SWI3B in the nucleus of tobacco leaves. Cells were infiltrated with a mixture of *Agrobacterium* suspensions harboring constructs SWI3BN-HA-YFP^C/YFP^N-ABI1 (panel 3), SWI3BN-HA-YFP^C/YFP^N-ABI2 (panel 4), or SWI3BN-HA-YFP^C/YFP^N-PP2CA (panel 5) and the silencing suppressor p19.

(D) Protein gel blot analysis demonstrates the expression of SWI3BN-HA-YFP^C and the corresponding YFP^N-PP2Cs (asterisks). Protein extracts obtained from tobacco leaves infiltrated with *Agrobacterium* suspensions harboring the silencing suppressor p19 and constructs SWI3BN-HA-YFP^C/YFP^N-ABI1 (lane 3), SWI3BN-HA-YFP^C/YFP^N-ABI2 (lane 4), SWI3BN-HA-YFP^C/YFP^N-PP2CA (lane 5), or p19 alone (lane p19) were analyzed using anti-HA or anti-GFP^N antibodies.

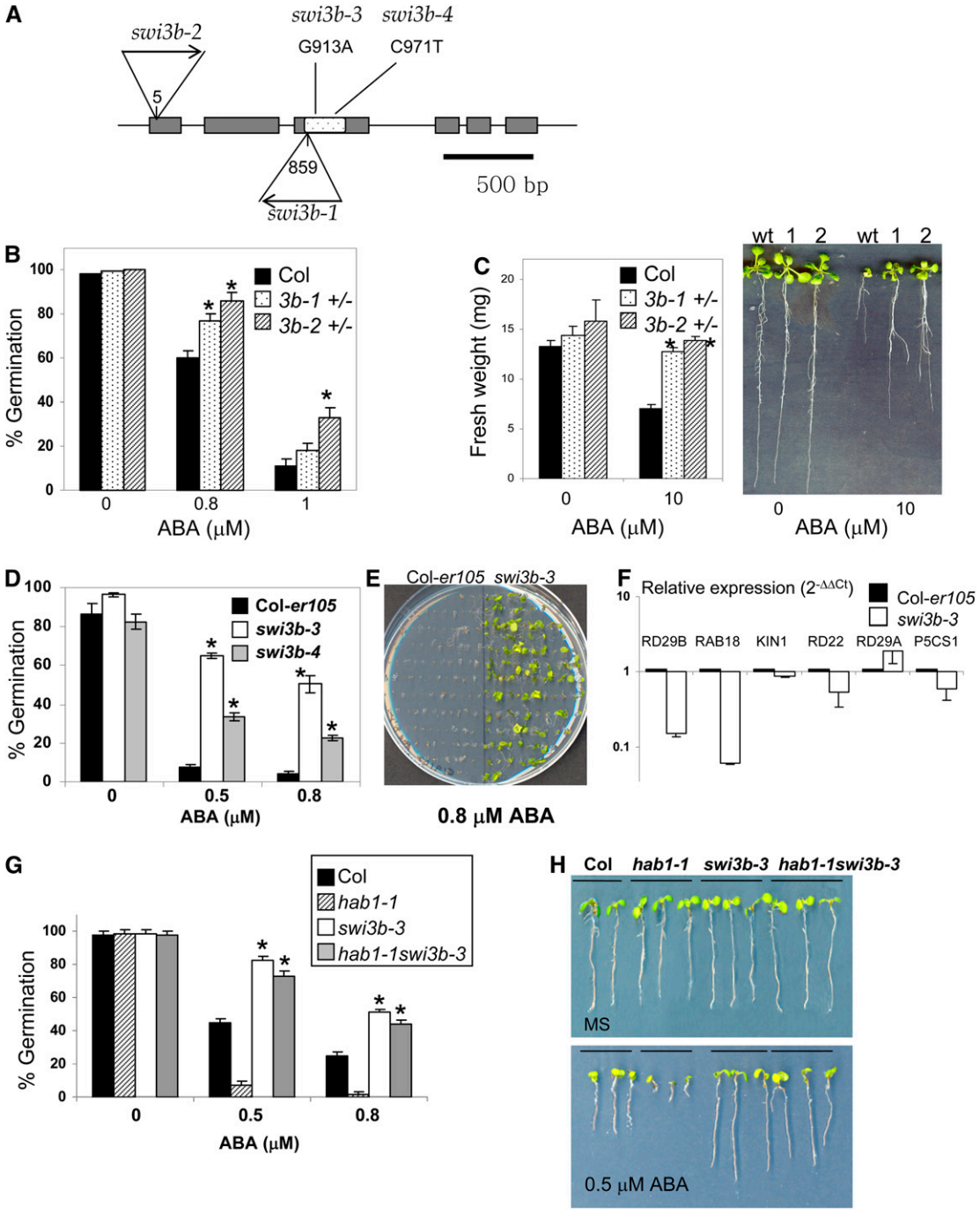

Figure 4. *swi3b* Mutants Show Reduced Sensitivity to ABA-Mediated Inhibition of Germination and Growth.

(A) T-DNA insertions in the *swi3b-1* and *swi3b-2* alleles and localization of ethyl methanesulfonate–induced mutations in *swi3b-3* and *swi3b-4* alleles. The numbering begins at the ATG translation start codon. The gray boxes represent exons. The SANT domain is spotted within the third exon.
(B) ABA effects on germination in the progeny of *swi3b-1* and *swi3b-2* heterozygous plants. The percentage of seeds that germinated and developed green cotyledons in the presence of the indicated concentrations of ABA is shown. Values are averages ± SD for three independent experiments (n = 200 seeds per experiment). * $P < 0.01$ (Student's t test) when comparing data from each genotype and the wild type in the same assay conditions.
(C) Reduced sensitivity of *swi3b-1* and *swi3b-2* heterozygous (+/−) seedlings to ABA-mediated growth inhibition. Fresh weight was measured in 12-d-old seedlings grown in MS medium either lacking or containing 10 μM ABA. Values are averages ± SD for three independent experiments (n = 20 seedlings per experiment). Representative seedlings of Col (wild type [wt]), *swi3b-1* +/− (1), and *swi3b-2* +/− (2) were removed from medium lacking or containing 10 μM ABA and rearranged on agar plates (at right).

and growth assays, these results suggest that *SWI3B* is a positive regulator of ABA signaling.

Further evidence of the role of SWI3B in ABA signaling was obtained through the analysis of point mutations in *swi3b* alleles that were recovered by the *Arabidopsis* TILLING (for targeting-induced local lesions in genomes) program (http://tilling.fhcrc.org:9366/home.html) in a Columbia (Col)–*er105* background. Thus, two new *swi3b* alleles were identified, *swi3b-3* and *swi3b-4*, which resulted in the substitution of Asp-245 by Asn and Ser-264 by Phe, respectively. Both Asp-245 → Asn and Ser-264 → Phe mutations are localized in the SANT domain of SWI3B and, according to SIFT (for sorting intolerant from tolerant) software analysis, are predicted to affect protein function (SIFT score < 0.05) (Ng and Henikoff, 2001). Analysis of ABA-mediated inhibition of germination in *swi3b-3* and *swi3b-4* revealed that both mutants showed a reduced sensitivity to ABA in this assay compared with the Col-*er105* background (where TILLING mutants were originated) (Figure 4D). In particular, the *swi3b-3* mutant also showed a reduced sensitivity to ABA-mediated inhibition of early growth (Figure 4E). These results, together with those of *swi3b-1* and *swi3b-2* +/− seedlings, show that SWI3B is a positive regulator of ABA signaling that mediates the ABA response in seeds and vegetative tissue.

Additionally, we wondered whether SWI3B might play a role in the regulation of gene expression in response to ABA. SWI3B is a putative core component of SWI/SNF complexes, and chromatin remodelers have a well-established role in transcriptional regulation. Therefore, real-time quantitative polymerase chain reaction (RT-qPCR) was used to analyze the expression of the ABA-responsive *RD29B*, *RAB18*, *KIN1*, *RD22*, *RD29A*, and *P5CS1* genes in the wild type and the *swi3b-3* mutant (Figure 4F). In general terms, these gene markers show low expression in the absence of ABA or stress treatment, which is upregulated in response to the inductive signal. Upon ABA treatment, expression of *RD29B* and *RAB18* in *swi3b-3* was 15 and 6%, respectively, of that found in the wild type, whereas expression of the other gene markers did not differ more than twofold in both genotypes. Thus, SWI3B appears to regulate a subset of ABA-inducible genes, whereas its function seems to be partially dispensable or redundant for the expression of other ABA-responsive genes. Finally, to further characterize the genetic relationship between the ABA-hypersensitive locus *hab1-1* and the ABA-insensitive locus *swi3b-3*, we generated a *hab1-1swi3b-3* double mutant. Analysis of ABA-mediated inhibition of germination (Figure 4G) and early seedling growth (Figure 4H) revealed that *hab1-1swi3b-3* showed an ABA-insensitive phenotype, in contrast with *hab1-1*, which indicates that SWI3B is epistatic to HAB1; therefore, HAB1 functions upstream of SWI3B in the ABA signaling pathway. In addition to reduced sensitivity to ABA in the assays described above, the *swi3b-3* allele showed both impaired vegetative and reproductive growth (see Supplemental Figure 4 online), which likely reflects the key role of SWI3B in plant growth and development as a core component of diverse SWI/SNF complexes (Zhou et al., 2003; Sarnowski et al., 2005; Bezhani et al., 2007). In agreement with this role, combination of the *swi3b-3* and *swi3b-2* alleles was embryo-lethal (see Supplemental Figure 4 online).

The Presence of HAB1 in the Vicinity of the ABA-Responsive RD29B and RAB18 Promoters Is Abolished by ABA

The interaction of HAB1 and SWI3B as well as the phenotype of *swi3b* mutants suggest that HAB1 modulates ABA response through the regulation of a putative SWI/SNF chromatin-remodeling complex. In order to analyze the presence of HAB1 in plant chromatin and the putative influence of ABA on it, we performed chromatin immunoprecipitation (ChIP) experiments. To this end, we used the *hab1-1* transgenic line complemented by HAB1-dHA described above and demonstrated that HAB1-dHA could be immunoprecipitated using a monoclonal antibody to HA peptide (Figure 5A). ChIP experiments were performed on formaldehyde–cross-linked chromatin extracted from either *hab1-1* or *hab1-1::ProHAB1-HAB1-dHA* plants. Genomic DNA fragments that coimmunoprecipitated with HAB1-dHA were analyzed by RT-qPCR (Figures 5B to 5D). To this end, we used different primer pairs that covered the promoters of the ABA-responsive genes *RD29B* and *RAB18* as well as a control gene, *β-ACT8*, which is not responsive to ABA (Saez et al., 2006). Aliquots of the total chromatin input were previously used to provide a quantitative measurement of the DNA input present in each sample, and DNA amounts present in ChIP precipitates were measured using cycle threshold values from RT-qPCR

Figure 4. (continued).

(D) Reduced sensitivity to ABA-mediated inhibition of seed germination in *swi3b-3* and *swi3b-4* mutants compared with Col-*er105*. The percentage of seeds that showed radicle emergence at 96 h after seed stratification is shown. Values are averages ± SD for three independent experiments (n = 200 seeds per experiment). Asterisks are as described for **(B)**.

(E) Reduced sensitivity to ABA-mediated inhibition of early growth in the *swi3b-3* mutant compared with Col-*er105* in medium supplemented with 0.8 μM ABA. The photograph was taken at 18 d after sowing.

(F) Reduced expression of ABA-inducible genes in *swi3b-3* compared with Col-*er105*. Values are expression levels reached in the mutant with respect to Col-*er105* (value 1) as determined by RT-qPCR analyses. Expression of gene markers was analyzed in 7-d-old seedlings grown in medium supplemented with 0.3 μM ABA. Values are averages ± SD for three independent experiments (n = 30 to 40 seedlings per experiment).

(G) The *swi3b-3* phenotype is epistatic to *hab1-1*. The percentage of seeds that showed radicle emergence at 96 h after seed stratification is shown. Values are averages ± SD for three independent experiments (n = 200 seeds per experiment). * $P < 0.01$ (Student's t test) when comparing data from *swi3b-3* and the wild type, or *hab1-1swi3b-3* and *hab1-1*, in the same assay conditions.

(H) Reduced sensitivity to ABA-mediated inhibition of early growth in *swi3b-3* and the *hab1-1swi3b-3* double mutant. Photographs were taken at 7 d (MS) and 11 d (0.5 μM ABA) after sowing. As in **(C)**, plants were removed from growth medium and rearranged on plates for photography.

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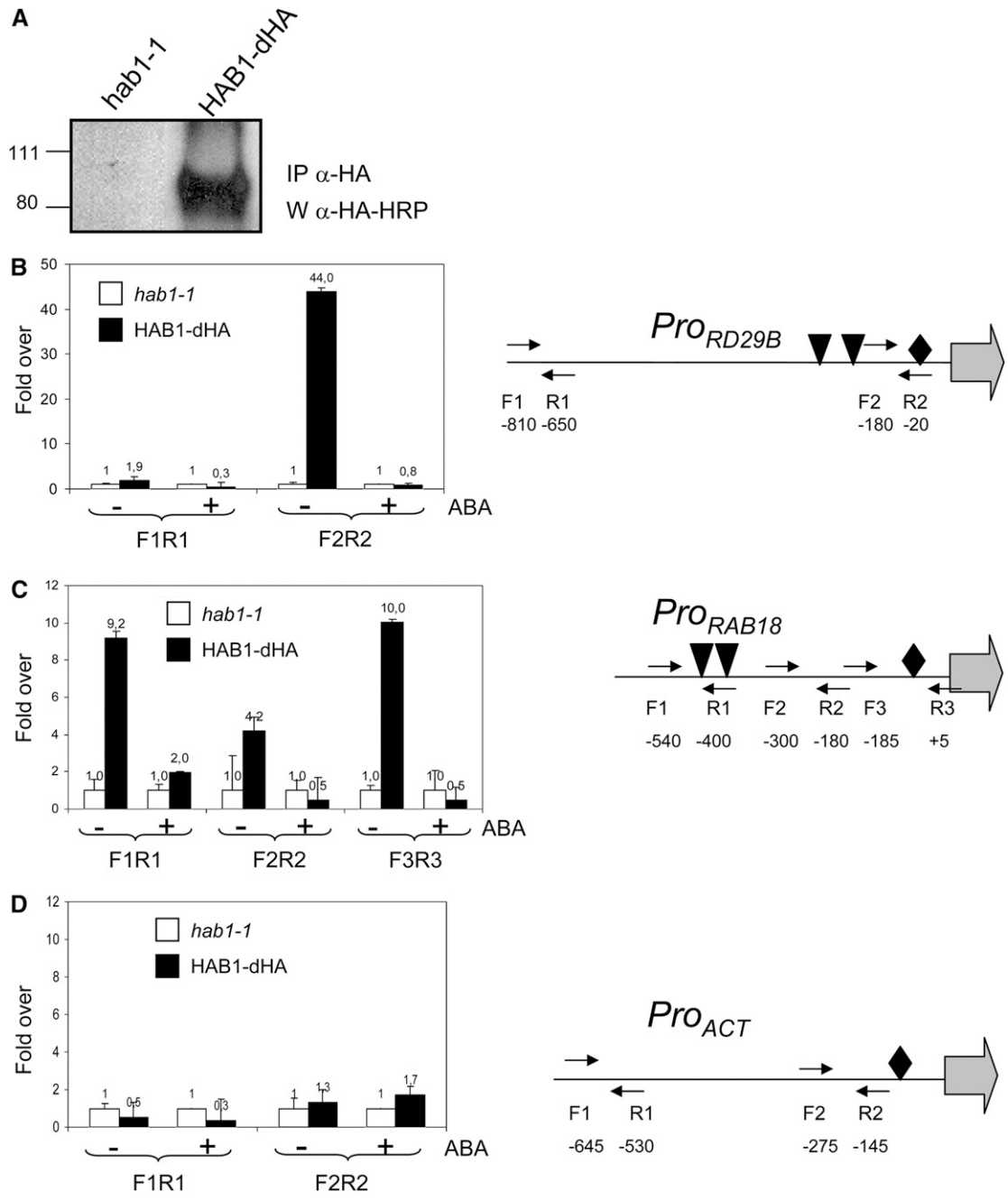

Figure 5. The Presence of HAB1 in the Vicinity of the ABA-Responsive *RD29B* and *RAB18* Promoters Is Abolished by ABA.

(A) Immunoprecipitated samples (IP anti-HA) were subjected to immunoblot analysis (W anti-HA-HRP).
(B) to **(D)** ChIP assays of the *RD29B*, *RAB18*, and *ACT8* promoters in *hab1-1* or *hab1-1::ProHAB1-HAB1-dHA* plants. Genomic DNA fragments that coimmunoprecipitated with HAB1-dHA were analyzed by RT-qPCR using primers of the *RD29B* **(B)**, *RAB18* **(C)**, and *ACT8* **(D)** promoters. Results are presented as ratios of the amount of DNA immunoprecipitated from HAB1-dHA samples to that from the *hab1-1* control (value set to equal 1). The positions of the ABA-responsive elements (triangles) and TATA boxes (diamonds) in the sequences of the different promoters are indicated, as well as the primers used for RT-qPCR analysis. The numbering refers to the ATG translation start codon, and the beginnings of the open reading frames are indicated by arrows.

amplification curves (see Methods). Figures 5B and 5C show that the amounts of *RD29B* and *RAB18* promoter DNAs immunoprecipitated from the HAB1-dHA transgenic plants were over 40- and 10-fold higher, respectively, than that precipitated from *hab1-1* control plants, whereas the amount of *β-ACT8* promoter DNA immunoprecipitated was very similar in both cases (Figure 5D). Interestingly, HAB1 was enriched in those regions of the *RD29B* and *RAB18* promoters that were close to ABA-responsive elements and TATA boxes, and after treatment with 50 μM ABA for 1 h the presence of HAB1 in these regions was abolished (Figures 5B and 5C). *35S-HAB1* lines showed reduced expression of ABA-inducible genes compared with the wild type (Saez et al., 2004); conversely, the *hab1-1* loss-of-function mutant showed twofold higher expression of ABA-inducible genes than the wild type (Saez et al., 2006). These data, together with ChIP results, suggest that HAB1 might repress ABA-induced transcription through direct chromatin interaction and that ABA treatment seems to release such inhibition.

DISCUSSION

Both gain-of-function and loss-of-function phenotypes of the PP2C HAB1 are consistent with a role as a negative regulator of ABA signaling (Leonhardt et al., 2004; Saez et al., 2004). Thus, whereas constitutive expression of *HAB1* (*35S:HAB1*) led to reduced ABA sensitivity in both seeds and vegetative tissues, the recessive *hab1-1* mutant showed ABA-hypersensitive inhibition of seed germination and growth, enhanced ABA-mediated stomatal closure, and enhanced expression of ABA-responsive genes (Leonhardt et al., 2004; Saez et al., 2004, 2006). The ABA-hypersensitive phenotype of *hab1-1* was strongly reinforced when combined with a loss-of-function allele of *ABI1* (Saez et al., 2006). A critical aspect to improve our knowledge on HAB1 function and its role in ABA signaling is the identification of its interacting partners.

Physical Interaction of HAB1 and SWI3B

A two-hybrid assay revealed a strong interaction between the HAB1 catalytic domain and *SWI3B* (Figure 1A). Serial deletions of SWI3B mapped the interacting domain to the N-terminal half of the protein. Thus, both the SWIRM and ZZ zinc finger domains appeared to be required for the interaction, as deletion of either of them abolished the interaction (Figure 1A). The SWIRM (for SWI3-RSC-MOIRA) domain is a small α-helical domain of ~85 amino acid residues found in chromosomal proteins and is predicted to mediate protein–protein interactions in the assembly of chromatin/protein complexes (Aravind and Iyer, 2002; Da et al., 2006). The ZZ zinc finger domain is also likely involved in mediating specific protein–protein interactions with transcriptional adaptors and activators (Ponting et al., 1996). Interestingly, the C-terminal half of SWI3B, which contains both the SANT (for SWI3-ADA2-NCoR-TFIIIB) and Leu zipper domains, was dispensable for the interaction with HAB1 (Figure 1A). However, the mutations found in the *swi3b-3* and *swi3b-4* alleles provide evidence for the importance of the SANT domain for SWI3B function. The equivalent C-terminal half of SWI3C constituted the region that interacted with the ATPase BRAHMA (Hurtado et al., 2006), which also interacted weakly with SWI3B; therefore, it seems likely that such a region plays a similar role in SWI3B. The SANT domain included in this region is structurally related to the Myb DNA binding domain; however, there is no evidence that the SANT domain directly contacts DNA. Instead, SANT domains may directly bind the N-terminal histone tails (Mohrmann and Verrijzer, 2005). Finally, it is suggested that the Leu zipper functions as a homodimerization and heterodimerization domain (Mohrmann and Verrijzer, 2005).

The HAB1 mutant allele G246D ΔNhab1, which had <3% in vitro PP2C activity than the wild type, did not interact with SWI3B. The G246D substitution affects the catalytic center of the PP2C, and according to the crystal structure of human PP2C (Das et al., 1996) such a mutation is expected to disturb the metal-coordinating residues Asp-243 and Gly-244 with concomitant reduction in catalytic activity. An alternative possibility has been postulated by Robert et al. (2006), who suggested that hab1^{Gly246Asp} might show enhanced affinity for its substrate and therefore enhanced dephosphorylating capacity. However, using casein as a substrate, the in vitro PP2C activity of hab1^{Gly246Asp} was severely reduced compared with that of the wild type, as was the case for G246D ΔNhab1. Additionally, the equivalent Gly-180→Asp abi1-1 and Gly-168→Asp abi2-1 mutant proteins did not show enhanced affinity (just the opposite) for their interacting partners, ATHB6/OST1 and SOS2/Prefibrillin, respectively (Himmelbach et al., 2002; Ohta et al., 2003; Yang et al., 2006; Yoshida et al., 2006a). In all of these cases, including the interaction of HAB1 and SWI3B, it appears that a functional catalytic PP2C is required for binding of the different targets.

The interaction of HAB1 and SWI3B was confirmed in planta through BiFC and coimmunoprecipitation assays (Figure 3). HAB1 is localized in both nucleus and cytosol; however, the

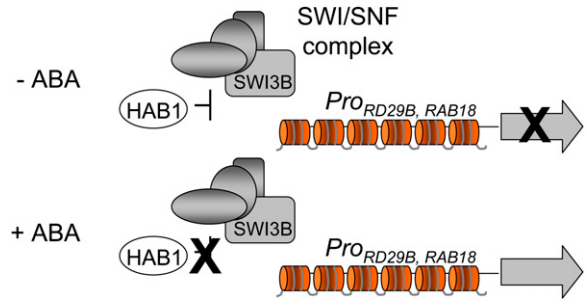

Figure 6. A Model for the Involvement of HAB1, SWI3B, and a Putative Plant SWI/SNF Complex in the Regulation of Plant Transcriptional Response to ABA on the Chromatin Template.

HAB1 is a negative regulator of ABA signaling that interacts with SWI3B, which is a positive regulator of ABA signaling. SWI3B must play a key role as a core subunit of an unidentified SWI/SNF complex, which is predicted to regulate nucleosomal structure in response to ABA. In the absence of ABA, HAB1 is localized in the vicinity of the *RAB18* and *RD29B* promoters and negatively regulates the expression of these genes. ABA inhibits HAB1 and releases its inhibitory effect on a putative SWI/SNF complex. [See online article for color version of this figure.]

BiFC assay clearly identified SWI3B as a nuclear target of HAB1. Interestingly, most of the targets previously identified for clade A PP2Cs were not nuclear proteins (Cherel et al., 2002; Guo et al., 2002; Ohta et al., 2003; Miao et al., 2006; Yang et al., 2006; Yoshida et al., 2006a). However, in the case of ABI1, it is supposed that the interaction with the TF ATHB6 must be nuclear (Himmelbach et al., 2002). Additionally, recent results reveal a nuclear localization signal at the very end of the C-terminal domain of ABI1 that is required for regulating ABA-dependent gene expression (Moes et al., 2008). Inspection of the C-terminal amino acid sequences of HAB1, ABI2, and PP2CA also reveals a similar short region enriched in basic amino acids (see Supplemental Figure 5 online). Additionally, the sequences of HAB1 and ABI2 display a second region that contains two positively charged clusters separated by a short linker region (see Supplemental Figure 5 online). The nuclear interaction of PP2CA, ABI1, and ABI2 with SWI3B found in BiFC assays might be physiologically relevant to regulating ABA signaling. However additional experiments (e.g., ChIP analysis) will be required to confirm the presence of these PP2Cs in plant chromatin and specifically in ABA-regulated promoters. Finally, it is noteworthy that previously described SWI3B-interacting partners connect SWI3B with different components of putative SWI/SNF complexes and, intriguingly, with the RNA and ABA binding protein FCA (Razem et al., 2006). In addition to FCA, six different SWI3B-interacting proteins have been described, namely SWI3A, SWI3C, SWI3D, BRM, SYD, and BSH, which are putative components of SWI/SNF complexes (Sarnowski et al., 2002, 2005; Bezhani et al., 2007). Analysis of the ABA response in mutants affected in these genes will be required for the identification of additional components of SWI/SNF complexes involved in ABA signaling.

Role of HAB1, SWI3B, and a Putative SWI/SNF Complex in ABA Signaling

No SWI/SNF complex has been biochemically purified in plants, although comparative genome analysis indicates that plants encode a remarkably high number of potential components of such a complex (Sarnowski et al., 2005). In yeast, *Drosophila*, and mammals, it is well known that an important subset of highly inducible genes requires a SWI/SNF complex as a transcriptional activator (Mohrmann and Verrijzer, 2005). It has been reported previously that *hab1-1* mutants show twofold higher expression of ABA-responsive genes than wild-type plants (Saez et al., 2006), whereas *35S:HAB1* plants show reduced expression of ABA-inducible genes (Saez et al., 2004); therefore, HAB1 negatively regulates the expression of these genes. HAB1 is localized in both nucleus and cytosol and, therefore, could influence ABA signaling at different steps. ChIP experiments reveal the presence of HAB1 in the vicinity of the ABA-responsive *RAB18* and *RD29B* promoters, and ABA treatment eliminates HAB1 from these regions (Figure 5). These results, taken together with the negative effect of HAB1 on the expression of ABA-inducible genes, strongly suggest a direct regulatory effect of HAB1 on ABA-mediated transcriptional regulation. Thus, the presence of HAB1 in the vicinity of ABA-responsive promoters correlates with the inhibition of their transcription under basal conditions, whereas ABA-mediated removal of HAB1 from these regions appears to be required for full induction of them (Figure 6). In this context, both the HAB1–SWI3B interaction and the impaired upregulation by ABA of *RAB18* and *RD29B* in *swi3b-3* suggest that HAB1 might regulate a putative SWI/SNF complex targeted to some ABA-responsive promoters (Figure 6). The phenotypes described in this work for *swi3b-1* and *swi3b-2* +/− seedlings as well as *swi3b-3* and *swi3b-4* mutants are consistent with SWI3B acting as a positive regulator of ABA signaling. Taking into account the opposed roles of HAB1 and SWI3B in this signaling pathway, it is reasonable to postulate that HAB1 negatively regulates SWI3B function, modulating its role as a positive regulator of ABA signaling. Alternatively, SWI3B might anchor HAB1 to a putative SWI/SNF complex, where the phosphatase activity of HAB1 might dephosphorylate a component required for proper function of the chromatin remodeler. Taking into account that the presence of HAB1 in the vicinity of the ABA-responsive *RD29B* and *RAB18* promoters is abolished by ABA (Figure 5), we speculate that ABA must inhibit HAB1 function, which releases its inhibitory effect on a yet unknown SWI/SNF complex involved in the transcriptional activation of ABA-responsive genes (Figure 6). Finally, it will be an exciting challenge for the future understanding of how the dynamic structure of the chromatin is modulated in response to ABA to regulate gene expression as well as to characterize the cell signaling events that lead to chromatin remodeling.

METHODS

Plant Material

Arabidopsis thaliana (ecotype Col) and tobacco (*Nicotiana benthamiana*) plants were routinely grown under greenhouse conditions in pots containing a 1:3 perlite:soil mixture. For in vitro culture, *Arabidopsis* seeds were surface-sterilized by treatment with 70% ethanol for 20 min, followed by commercial bleach (2.5%) containing 0.05% Triton X-100 for 10 min, and finally, four washes with sterile distilled water. Stratification of the seeds was conducted during 3 d at 4°C. Afterward, seeds were sown on Murashige and Skoog (MS) plates containing solid medium composed of MS basal salts and 1% sucrose, solidified with 1% agar, and pH was adjusted to 5.7 with KOH before autoclaving. Plates were sealed and incubated in a controlled-environment growth chamber at 22°C under a 16-h-light/8-h-dark photoperiod at 80 to 100 $\mu E \cdot m^{-2} \cdot s^{-1}$.

The *swi3b-1* (Koncz_2208) and *swi3b-2* (GABI_302G08) alleles are T-DNA mutants in the Col background. They were kindly provided by G. Rios and have been described previously (Sarnowski et al., 2005). TILLING mutants were obtained through the *Arabidopsis* TILLING project, which performed a high-throughput reverse genetic screening to identify ethyl methanesulfonate–induced mutations in the Col-*er105* background (Till et al., 2003). As a result, two alleles were identified, *swi3b-3* and *swi3b-4*, which showed changes with SIFT score < 0.05 and, therefore, were predicted to be deleterious to the gene product (Ng and Henikoff, 2001). These mutants were backcrossed once with Col-*er105*, and F2 homozygous mutants were genotyped by PCR amplification and DNA sequencing using the primers F1261 and R1560. In the case of *swi3b-3*, a second backcross was done with Col, and F2 *swi3b-3* mutants lacking the *er105* mutation were selected. In order to generate the *hab1-1swi3b-3* double mutant, we transferred pollen of *swi3b-3* (Col background) to the stigmas of emasculated flowers of *hab1-1* (Col background). The resulting F2 individuals were genotyped by PCR for the presence of the double mutant.

Yeast Two-Hybrid Screening

The HAB1 coding sequence was excised from a pSK-HAB1 construct (Rodriguez et al., 1998b) using *Eco*RI-*Sal*I double digestion and subcloned into *Eco*RI-*Sal*I doubly digested pGBT9 to generate an in-frame fusion with the GBD. To generate the HAB1 deletion, the HAB1 sequence encoding the catalytic PP2C region (amino acid residues 179 to 511; ΔNHAB1) was amplified by PCR and blunt-end-cloned into the *Eco*RV site from pBluescript SK+ (Stratagene). The ΔNHAB1 coding sequence was excised with *Eco*RI-*Sal*I and subcloned into pGBT9. The pGBT9-*ΔNHAB1* bait was transformed into the yeast strain AH109 (BD Biosciences). An oligo(dT)-primed cDNA library prepared in plasmid pACT2 using mRNA from an *Arabidopsis* cell suspension was kindly provided by K. Salchert (Nemeth et al., 1998). Yeast host AH109 carrying the pGBT9-*ΔNHAB1* bait was transformed with 100 μg of DNA from the pACT2 cDNA library, then the cells were plated on SCD medium lacking Leu and Trp. Approximately 10^6 clones were obtained, and upon plating in SCD medium lacking Leu, Trp, His, and adenine, 20 clones containing putative interacting preys were selected. Yeast DNA was recovered and electroporated into *Escherichia coli* strain MC1065. pACT2 clones containing putative interacting preys were sequenced and retransformed into yeast strain AH109 carrying either the empty vector pGBT9 or pGBT9-*ΔNHAB1* bait in order to verify true positives.

The PP2CA cDNA was obtained from the ABRC (clone M76G17STM). The PP2CA sequence encoding the catalytic PP2C region (amino acid residues 90 to 399) was amplified using the primers FDNPP2CA and RPP2CA. The PCR product was cloned into the pCR8/GW/TOPO entry vector (Invitrogen), and the ΔNPP2CA-coding sequence was excised with *Eco*RI-*Sal*I and subcloned into pGBT9. The ABI1 and ABI2 cDNAs were kindly provided by Erwin Grill and have been described previously (Meyer et al., 1994; Rodriguez et al., 1998a). The ABI1 sequence encoding the catalytic PP2C region (amino acid residues 122 to 433) was excised using *Eco*RI-*Pst*I double digestion and subcloned into pGBKT7 to generate pGBKT7-*ΔNABI1*. The ABI2 sequence encoding the catalytic PP2C region (amino acid residues 96 to 423; ΔNHAB2) was excised using *Sca*I-*Sal*I double digestion and subcloned into pGBT9 to generate pGBT9-*ΔNABI2*.

Construction of Plasmids

pACT2-SWI3B-C1 was generated from the pACT2-SWI3B full-length cDNA recovered from the two-hybrid screening through *Xho*I digestion and subsequent religation. pACT2-SWI3B-C2, pACT2-SWI3B-N1, pACT2-SWIRM, and pACT2-ZZ were generated through PCR-mediated amplification using the following primer pairs, respectively: FATG and R660, F661 and R1410, FATG and R420, and F400 and R660. Constructs that express fusion proteins between the GAD and SWI3A, SWI3B, SWI3C, or SWI3D in the centromeric vector pPC86 were kindly provided by J.C. Reyes (CABIMER), and they have been described by Hurtado et al. (2006). Protein fusion between the GBD and ΔNHAB1 were generated in the multicopy vector pGBT9 for the yeast two-hybrid screening or the centromeric vector pDBLeu for targeted interaction assays with SWI3-like proteins. The G246D mutation was introduced into the pGBT9-*ΔNHAB1* construct through replacement of a *Bgl*II-*Eco*RV fragment of HAB1 with a PCR-mutagenized version (see below).

Constructs to investigate the subcellular localization of HAB1 and SWI3B were generated in Gateway-compatible vectors. To this end, the coding sequences of HAB1 and SWI3B were PCR-amplified using the following primer pairs, respectively: FBamHI and Rno-stop, and FATG and R1407no-stop. The PCR products were cloned into the pCR8/GW/TOPO entry vector (Invitrogen) and recombined by LR reaction into the pMDC83 destination vector (Curtis and Grossniklaus, 2003).

Constructs to investigate in planta interaction using BiFC assays were made in the pSPYNE-35S and pSPYCE-35S vectors (Walter et al., 2004) as well as the Gateway vector pYFP^{N}43 (kindly provided by A. Ferrando, Universidad de Valencia). The coding sequences of HAB1 and G246D hab1 were excised from pCR8/GW/TOPO constructs using double digestion with *Bam*HI-*Stu*I and subcloned into pSPYCE doubly digested *Bam*HI-*Sma*I. The N-terminal half of SWI3B was excised from a pSK-SWI3B construct using double digestion with *Bam*HI-*Dra*I and subcloned into pSPYNE and pSPYCE doubly digested *Bam*HI-*Sma*I. Constructs in which the basic Leu zipper TF bZIP63 is cloned in pSPYNE-35S and pSPYCE-35S were kindly provided by J. Kudla (University of Münster). The coding sequences of ABI1, ABI2, and PP2CA were PCR-amplified and cloned into pCR8/GW/TOPO and recombined by LR reaction into the pYFP^{N}43 destination vector.

Expression and Purification of MBP-HAB1, MBP-ΔNHAB1, and MBP-G246D ΔNhab1

The coding region of the HAB1 cDNA (Rodriguez et al., 1998b) was PCR-amplified using the primers FSphI and RSphISacI. The PCR product was cloned subsequently into the *Eco*RV site of pBluescript SK (Stratagene), generating pSK-HAB1. Next, an *Eco*RI-*Sal*I DNA fragment was excised from pSK-HAB1 and subcloned into the pMal-c2 vector (New England Biolabs). In order to obtain an N-terminal deletion of HAB1 (ΔNHAB1), a *Hin*dIII DNA fragment encompassing the amino acid residues 116 to 511 was excised from pSK-HAB1 and subcloned into the pMal-c2 vector. HAB1 cDNA was mutagenized by PCR in order to engineer a G246D substitution (Ho et al., 1989). To this end, the following oligonucleotides were used as primers: FPCR1 (5′-TATGATGGTCATGACGGCCATA-AGGTT-3′), in which the codon for Gly-246 (GGA) was changed to Asp (GAC), RATT380, FATTATG, and RPCR2. Once the pMalc2-based constructs were verified by sequencing, expression of recombinant MBP-HAB1, MBP-ΔNHAB1, and MBP-G246D ΔNhab1 was induced with 1 mM isopropylthio-β-galactoside in *E. coli* DH5α cells. The fusion proteins were purified by amylose affinity chromatography according to the manufacturer's instructions (New England Biolabs).

PP2C Activity Assays

Phosphatase activity was measured using ^{33}P-labeled casein as a substrate. Dephosphorylated casein (P-4765; Sigma-Aldrich) was ^{33}P-labeled with bovine heart cAMP-dependent protein kinase (P-5511; Sigma-Aldrich) in a 500-μL reaction volume containing 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM DTT, 60 μM cAMP, 50 μM unlabeled ATP, and 0.1 μCi/μL [γ-^{33}P]ATP. The radiolabeled casein was precipitated with 20% trichloroacetic acid, and after two washings with 10% trichloroacetic acid, the casein was dissolved in 200 mM Tris-HCl, pH 7.6. Phosphatase assays were performed in a 50-μL reaction volume containing 20 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM DTT, and ~10,000 cpm of ^{33}P-labeled casein. After incubation for 30 min at 30°C, the reaction was stopped with 100 μL of 20% trichloroacetic acid, samples were centrifuged, and the release of ^{33}Pi in the supernatant was determined by scintillation counting.

Transient Protein Expression in Tobacco

Experiments were performed basically as described by Voinnet et al. (2003). The different binary vectors described above were introduced into *Agrobacterium tumefaciens* C58C1 (pGV2260) (Deblaere et al., 1985) by electroporation, and transformed cells were selected on Luria-Bertani plates supplemented with kanamycin (50 μg/mL). Then, they were grown in liquid Luria-Bertani medium to late exponential phase and cells were harvested by centrifugation and resuspended in 10 mM MES-KOH, pH 5.6, containing 10 mM $MgCl_2$ and 150 μM acetosyringone to an OD_{600} of 1. These cells were mixed with an equal volume of *Agrobacterium* C58C1

(pCH32 35S:p19) expressing the silencing suppressor p19 of *Tomato bushy stunt virus* (Voinnet et al., 2003) so that the final density of *Agrobacterium* solution was ~1. Bacteria were incubated for 3 h at room temperature and then injected into young fully expanded leaves of 4-week-old tobacco plants. Leaves were examined after 3 to 4 d with a Leica TCS-SL confocal microscope and a laser scanning confocal imaging system. Samples for immunoblot and immunoprecipitation assays were harvested, frozen in liquid nitrogen, and stored at −80°C.

Germination and Growth Assays

To measure ABA sensitivity, seeds (~200 seeds per experiment) were plated on solid medium composed of MS basal salts, 1% sucrose, and increasing concentrations of ABA. In order to score seed germination, the percentage of seeds that had germinated and developed fully green expanded cotyledons was determined. ABA-resistant growth from *swi3b* +/− heterozygous seedlings (~20 seedlings per experiment) was scored by weighing whole plants after 12 d of the transfer of 5-d-old seedlings grown on 0.5 μM ABA onto MS plates supplemented with 10 μM ABA. Heterozygous individuals from the *swi3b-1* or *swi3b-2* progeny were identified by their hygromycin or sulfadiazine resistance, respectively.

Protein Extraction, Protein Blot Analysis, and Immunoprecipitation

Protein extracts for immunodetection experiments were prepared from either tobacco leaves infiltrated with *Agrobacterium* or transgenic lines from *Arabidopsis*. Plant material (~200 mg) for protein gel blot analysis was directly extracted in 2× Laemmli buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 2% mercaptoethanol, and 0.001% bromphenol blue), and proteins were run on a 10% SDS-PAGE gel and analyzed by immunoblotting. Plant material (~1 g) for immunoprecipitation experiments was extracted in 3 volumes of PBS supplemented with 1 mM EDTA, 0.05% Triton X-100, and protease inhibitor cocktail (Roche). Protein concentration in each lysate was adjusted to the same value, and equal volumes of lysates (1 mL) were incubated with 1 μg/mL anti-HA high-affinity rat monoclonal antibody (clone 3F10; Roche) for 4 h at 4°C. After incubation, 20 μL of protein G–agarose beads (Roche) was added to precipitate the antigen/antibody complex. The protein G–agarose beads were collected after 1 h of incubation at 4°C by centrifugation and washed three times with extraction buffer. The antigen/antibody complex was eluted by boiling in Laemmli buffer and run on a 10% SDS-PAGE gel. Proteins immunoprecipitated with anti-HA antibodies were transferred onto Immobilon-P membranes (Millipore) and probed with either anti-HA-peroxidase or anti-c-myc-peroxidase conjugate (Roche), and detection was performed using the ECL advance protein gel blotting detection kit (GE Healthcare). The imaging of the chemiluminiscent signal was achieved using a highly efficient cooled CCD camera system (LAS-3000 luminiscent image analyzer from Fuji Photo Film). The signal intensities of the digitalized images were quantified using Image-Gauge version 4.0 software (Fuji Photo Film) according to the manufacturer's conditions. Immunodetection of GFP fusion proteins was performed with an anti-GFP monoclonal antibody (clone JL-8; Clontech) as primary antibody and ECL anti-mouse peroxidase (GE Healthcare) as secondary antibody. A rabbit antibody against peptide comprising amino acids 3 to 17 of GFP (anti-GFP^N) was employed to detect YFP^N fusion proteins (G1544; Sigma-Aldrich).

RNA Analyses

Plants were grown on MS plates supplemented with 1% sucrose either in the absence or presence of 0.3 μM ABA. After 7 d, ~30 to 40 seedlings were collected and frozen in liquid nitrogen. Total RNA was extracted using a Qiagen RNeasy plant mini kit, and 1 μg of the RNA solution obtained was reverse-transcribed using 0.1 μg of oligo$(dT)_{15}$ primer and Moloney murine leukemia virus reverse transcriptase (Roche), to finally obtain a 40-μL cDNA solution. RT-qPCR amplifications and measurements were performed using an ABI PRISM 7000 sequence detection system (Perkin-Elmer Applied Biosystems). RT-qPCR amplifications were monitored using the Eva-Green fluorescent stain (Biotium). Relative quantification of gene expression data was performed using the $2^{-\Delta\Delta C_T}$ (or comparative C_T) method (Livak and Schmittgen, 2001). Expression levels were normalized using the C_T values obtained for the *β-actin8* gene. The presence of a single PCR product was further verified by dissociation analysis in all amplifications. All quantifications were made in triplicate on RNA samples obtained from three independent experiments. The sequences of the primers used for PCR amplifications are indicated at Supplemental Table 1 online.

Generation of Epitope HA-Tagged HAB1 Transgenic Lines

The pBluescriptSK-*ProHAB1:HAB1* construct was described by Saez et al. (2004). Two copies of the HA epitope sequence encoding YPYDVPDYA were cloned at the C-terminal sequence of *HAB1* cDNA in the construct mentioned above. The complete expression cassette comprising the *HAB1* promoter, the double HA epitope–tagged *HAB1* coding sequence, and the *NOS* terminator was subcloned into *Sac*I-*Sal*I doubly digested pCAMBIA 1300 (hygromycin-resistant). The resulting construct was named pCAMBIA1300-*ProHAB1:HAB1-dHA* and used to transform *hab1-1* (kanamycin-resistant) plants as described by Saez et al. (2004). Transgenic plants were screened in vitro on MS medium (M5524; Sigma-Aldrich) with 20 mg/L hygromycin B (H9773; Sigma-Aldrich).

Biochemical Fractionation of Epitope HA-Tagged HAB1

This protocol is based on fractionation techniques described by Bowler et al. (2004), Poveda et al. (2004), and Cho et al. (2006). Rosette leaves from 3- to 4-week-old plants were mock-treated or treated with 50 μM ABA for 1 h, harvested, and frozen in liquid nitrogen. Next, plant material was ground in lysis buffer (20 mM Tris-HCl, pH 7.4, 25% glycerol, 20 mM KCl, 2 mM EDTA, 2.5 mM $MgCl_2$, 250 mM sucrose–containing protease inhibitor cocktail [Roche], and 1 mM phenylmethylsulfonyl fluoride [PMSF]). The lysate was filtered through four layers of Miracloth and centrifuged at 1000*g* for 10 min at 4°C to pellet the nuclei. The soluble cytosolic fraction was removed, and the pellet was washed in nuclei resuspension buffer, 20 mM Tris-HCl, 25% glycerol, 2.5 mM $MgCl_2$, and 0.5% Triton X-100. After centrifugation at 1000*g* for 30 s at 4°C, a nuclear pellet was obtained, which was resuspended in 5 volumes of medium salt buffer (Bowler et al., 2004), 20 mM Tris-HCl, 0.4 M NaCl, 1 mM EDTA, 5% glycerol, 1 mM 2-mercaptoethanol, 0.1% Triton X-100, 0.5 mM PMSF, and protease inhibitor cocktail (Roche) and then frozen and thawed. After incubation with gentle mixing for 15 min at 4°C, the nuclear insoluble fraction, containing the major nuclear protein histones, was precipitated by centrifugation at 10,000*g* for 10 min, whereas the supernatant contained the nuclear soluble fraction. Detection of HAB1 was performed using anti-HA peroxidase conjugate (Roche). The purity of the different fractions was demonstrated using rabbit antibodies against histone H3 (Abcam) and ribulose-1,5-bisphosphate carboxylase.

ChIP

The ChIP protocol described here is a variation of the previously published protocols from Johnson et al. (2002) and Pascual-Ahuir et al. (2006). A transgenic line of Arabidopsis expressing a double HA epitope–tagged HAB1 in a *hab1-1* background was used as starting plant material. In parallel, plant material from the *hab1-1* mutant was used as a control for the experiment. Rosette leaves from 3- to 4-week-old plants were

mock-treated or treated with 50 μM ABA for 1 h and then harvested and immersed in buffer A (0.4 M sucrose, 10 mM Tris, pH 8, 1 mM EDTA, 1 mM PMSF, and 1% formaldehyde) under vacuum for 10 min. Gly was added to a final concentration of 0.1 M, and incubation was continued for an additional 5 min under vacuum. Next, the plant material was washed with TBS (20 mM Tris-HCl, pH 8, and 150 mM NaCl) and frozen in liquid nitrogen. Cross-linked material (~1 g) was ground with a mortar with pestle, after which it was resuspended in 1 mL of ice-cold lysis buffer (50 mM HEPES/KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate, 0.1% SDS, and 1 mM PMSF) and transferred to a 2-mL screw-cap vial. Approximately 0.5 mL of zirconia/silica beads was added, and plant material was disrupted at 4°C for 10 min in the Mini Bead Beater 8 (Biospec Products; maximal speed, three rounds of 1 min). The lysate was collected into a 1.5-mL microtube and centrifuged for 1 min at 4°C. The pellet was collected and washed once in 1 mL of ice-cold lysis buffer. Next, the pellet was resuspended in 0.5 mL of cold lysis buffer and sonicated three times for 20 s (Branson Sonifier; output, 50%; needle, 5). Sonication resulted in the fragmentation of the chromatin into soluble pieces in the range of 300 to 500 bp. After centrifugation for 30 min at 4°C, the supernatant containing the soluble chromatin fragments (chromatin input) was transferred to 1.5-mL microtubes and stored at −80°C.

To immunoprecipitate HAB1-dHA cross-linked to chromatin fragments, samples were incubated with 10 μg/mL anti-HA high-affinity rat monoclonal antibody (clone 3F10; Roche) for 30 min on a roller at room temperature. In the meantime, protein G–agarose beads were washed twice with lysis buffer, 25 μL was added to each sample, and the incubation was continued for 60 min. The agarose beads were recovered by centrifugation and then washed with 1 mL of each of the following buffers: 2× lysis buffer, 2× lysis buffer and 0.5 M NaCl, 1× buffer B (10 mM Tris-HCl, pH 8, 0.25 M LiCl, 1 mM EDTA, 0.5% Nonidet P-40, and 0.5% deoxycholate), and 1× TE (10 mM Tris-HCl, pH 8, and 1 mM EDTA). The immunocomplexes were eluted from the beads by incubation for 10 min at 65°C in 250 μL of buffer containing 50 mM Tris-HCl, pH 8, 10 mM EDTA, and 1% SDS. After centrifugation, the supernatant was transferred to a microtube containing 250 μL of TE buffer and 20 μg of Pronase (Roche), and the samples were incubated for 1 h at 42°C followed by 5 h at 65°C to reverse formaldehyde-induced cross-links. In addition to the immunoprecipitated samples, aliquots (50 μL) of the total chromatin input that were not subjected to immunoprecipitation were also treated with Pronase and de-cross-linked to provide a quantitative measurement of the DNA input present in each sample. Finally, genomic DNA fragments were purified by the addition of 50 μL of 4 M LiCl and extraction with 300 μL of phenol:chloroform:isoamyl alcohol and ethanol precipitation (adding 20 μg of glycogen as carrier). DNA pellets were washed with 70% ethanol, dissolved in 100 μL of TE buffer, and stored at −20°C. RT-qPCR was used to determine the amounts of genomic DNA immunoprecipitated in the ChIP experiment. The sequences of the primers used for PCR amplifications are indicated at Supplemental Table 1 online.

Accession Numbers

The Arabidopsis Genome Initiative locus identifiers for *HAB1* and *SWI3B* are *At1g72770* and *At2g33610*, respectively. *RD29B*, *RAB18*, *KIN1*, *RD22*, *RD29A*, and *P5CS1* correspond to *At5g52300*, *At5g66400*, *At5g15960*, *At5g25610*, *At5g52310*, and *At2g39800*, respectively.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. The N-Terminal Region of HAB1 Has Transcriptional Activation Function in Yeast When Fused to the Gal4 DNA Binding Domain.

Supplemental Figure 2. Treatment with 50 μM ABA for Different Periods of Time (5 min to 1 h) Does Not Change the Subcellular Localization of GFP-HAB1.

Supplemental Figure 3. Detached Leaves Water Loss Assays Do Not Show Significant Differences between Wild-Type and *swi3b-1* and *swi3b-2* +/− Plants.

Supplemental Figure 4. Reduced Vegetative and Reproductive Growth of the *swi3b-3* Mutant.

Supplemental Figure 5. Putative Nuclear Localization Signals in HAB1, PP2CA, and ABI2.

Supplemental Table 1. Primers Used for PCR Amplifications.

Supplemental Data Set 1. Text File of the Alignment Corresponding to Supplemental Figure 5.

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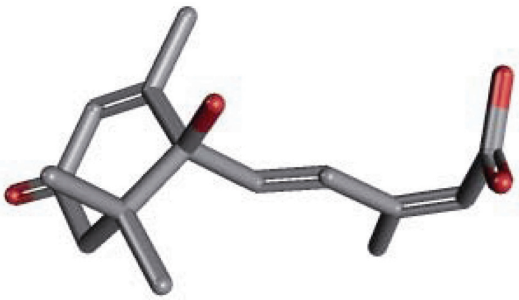

Capítulo IV

Triple Loss of Function of Protein Phosphatases Type 2C Leads to Partial Constitutive Response to Endogenous Abscisic Acid[1][C][W][OA]

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The phytohormone abscisic acid (ABA) is a key regulator of plant growth and development as well as plant responses to situations of decreased water availability. Protein phosphatases type 2C (PP2Cs) from group A, which includes the ABI1/HAB1 and PP2CA branches, are key negative regulators of ABA signaling. Specifically, HAB1, ABI1, ABI2, and PP2CA have been shown to affect both seed and vegetative responses to ABA. To further understand their contribution to ABA signaling and to unravel possible genetic interactions and functional redundancy among them, we have generated different combinations of double and triple mutants impaired in these PP2Cs. Interestingly, *hab1-1pp2ca-1* and *abi1-2pp2ca-1* double mutants showed reduced water loss and enhanced resistance to drought stress, which further supports the role of PP2CA in vegetative responses to ABA. Two triple *hab1-1abi1-2abi2-2* and *hab1-1abi1-2pp2ca-1* mutants were generated, which showed an extreme response to exogenous ABA, impaired growth, and partial constitutive response to endogenous ABA. Thus, transcriptomic analysis revealed a partial up-regulation/down-regulation of a subset of ABA-responsive genes in both triple mutants in the absence of exogenous ABA. Comparison of ABA responses in the different *pp2c* mutants showed that a progressive increase in ABA sensitivity could be obtained through combined inactivation of these PP2Cs. These results indicate that ABA response is finely tuned by the integrated action of these genes, which is required to prevent a constitutive response to endogenous ABA that might have a deleterious effect on growth and development in the absence of environmental stress.

Protein phosphorylation/dephosphorylation events in abscisic acid (ABA) signaling involve several known protein kinases and phosphatases (Finkelstein et al., 2002; Hirayama and Shinozaki, 2007; Verslues and Zhu, 2007). For instance, the guard cell-specific protein kinase AAPK from *Vicia faba* and the orthologous OST1/SnRK2.6 regulate ABA-induced stomatal closure (Li et al., 2000; Mustilli et al., 2002; Yoshida et al., 2002). The Arabidopsis (*Arabidopsis thaliana*) genome contains 10 SnRK2s; among them, SnRK2.2, SnRK2.3, and SnRK2.6/OST1 are key regulators of ABA signaling. Both SnRK2.2 and SnRK2.3 regulate ABA responses in germination, growth, and gene expression, whereas SnRK2.6/OST1 specifically regulates stomatal aperture (Mustilli et al., 2002; Fujii et al., 2007). Another protein kinase involved in ABA signaling is PKABA1, which is induced by ABA and suppresses GA-inducible gene expression in barley (*Hordeum vulgare*) aleurone layers (Gomez Cadenas et al., 1999). In addition to the above-described calcium-independent protein kinases, several calcium-dependent protein kinases that belong either to the calcium-dependent protein kinases or to the SnRK3/CIPK family mediate ABA signaling (Sheen, 1996; Guo et al., 2002; Kim et al., 2003; Mori et al., 2006; Cheong et al., 2007).

On the other hand, protein phosphatases type 2A (PP2As) and type 2C (PP2Cs) are involved in the regulation of ABA signaling. Disruption of the PP2A regulatory subunit RCN1 confers ABA insensitivity in Arabidopsis, which suggests that RCN1 functions as a positive transducer of ABA signaling (Kwak et al., 2002). Recent results have reported that a catalytic

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subunit of PP2A functions as a negative regulator of ABA signaling (Pernas et al., 2007). According to the classification of Schweighofer et al. (2004), 76 Arabidopsis genes qualify as members of the big PP2C family. Among them, group A contains most of the PP2Cs that are associated with ABA signaling, which are separated in two subgroups (i.e. the ABI1 and PP2CA branches). The pioneering evidence for the involvement of PP2Cs in the regulation of ABA signaling process was provided by the identification of the ABA-insensitive *abi1-1* and *abi2-1* mutants and the cloning of the corresponding loci (Koornneef et al., 1984; Leung et al., 1994, 1997; Meyer et al., 1994; Rodriguez, et al., 1998). Currently, genetic evidence indicates that at least six Arabidopsis PP2Cs, namely, ABI1, ABI2, PP2CA/AHG3, AHG1, HAB1, and HAB2, act as negative regulators of ABA signaling (Gosti et al., 1999; Merlot et al., 2001; Tahtiharju and Palva, 2001; Gonzalez-Garcia et al., 2003; Leonhardt et al., 2004; Saez et al., 2004, 2006; Kuhn et al., 2006; Yoshida et al., 2006; Nishimura et al., 2007). AHG1 and PP2CA/AHG3 appear to play an essential role for ABA signaling in germination and postgermination growth (Kuhn et al., 2006; Yoshida et al., 2006; Nishimura et al., 2007), but the *ahg1-1* mutant has no ABA-related phenotype in adult plants (Nishimura et al., 2007). The analysis of a double *ahg1-1ahg3-1* mutant suggests that AHG1 has specific functions in seed development and germination, shared partially with PP2CA/AHG3 (Nishimura et al., 2007). Kuhn et al. (2006) showed that PP2CA/AHG3 plays an essential role for ABA signaling both in seed and vegetative tissue, as the *pp2ca* mutant alleles showed ABA hypersensitivity in germination, growth, and stomatal closure assays. Conversely, *35S:PP2CA* expression caused ABA insensitivity in seed germination and ABA-induced stomatal closure assays (Kuhn et al., 2006). Previous work of Sheen (1998) showed that PP2CA can block ABA-mediated gene induction when transiently overexpressed in protoplasts. These results suggest that PP2CA function might be related to other PP2Cs from the ABI1 branch; however, genetic interactions among both branches have not been investigated.

Single reduction/loss-of-function alleles from ABI1, ABI2, and HAB1 produced phenotypic effects on ABA signaling to a different extent and it was apparent from double mutant analyses that some functional redundancy occurs among them (Merlot et al., 2001; Saez et al., 2006). For instance, inactivation of both HAB1 and ABI1 led to a stronger response to ABA than that found in either *hab1-1* or *abi1-2* monogenic mutants. A similar trend was obtained by combination of the recessive *abi1-1R4* and *abi2-1R1* alleles (Merlot et al., 2001). Combined inactivation of close members of a gene family is usually required to unravel possible functional genetic redundancy and to establish a functional hierarchy among them. This fact has been particularly evident in hormonal signaling pathways (Hua and Meyerowitz, 1998; Kwak et al., 2003; Higuchi et al., 2004; Achard et al., 2006; Iuchi et al., 2007). Alternatively, gain-of-function approaches can circumvent genetic redundancy, as deduced from the global ABA-insensitive phenotype found in the dominant mutants *abi1-1D* and *abi2-1D*, as well as the transgenic lines *35S:HAB1*, *35S:PP2CA*, and *35S:hab1Gly-246Asp* (Koornneef et al., 1984; Saez et al., 2004; Kuhn et al., 2006; Robert et al., 2006).

In this article, we have combined loss-of-function mutations in the *ABI1*, *ABI2*, *HAB1*, and *PP2CA* genes to unravel their contributions to ABA signaling. The *hab1-1*, *abi1-2*, and *pp2ca-1* alleles have been described previously (Leonhardt et al., 2004; Saez et al., 2004, 2006; Kuhn et al., 2006) and in this article we have identified a loss-of-function allele for *ABI2*, named *abi2-2*. Different combinations of mutations were generated (Table I) and, as a result, two triple mutants, *hab1-1abi1-2abi2-2* and *hab1-1abi1-2pp2ca-1*, were generated, which showed an extreme response to exogenous ABA and a partial constitutive response to endogenous ABA. Additionally, *hab1-1pp2ca-1* and *abi1-2 pp2ca-1* double mutants showed stronger responses to ABA than single parental mutants, which reveals a genetic interaction between both branches of PP2Cs for the regulation of ABA signaling.

RESULTS

Generation and Analysis of Double *hab1-1pp2ca-1* and *abi1-2pp2ca-1* Mutants

Seeds of *pp2ca-1* are strongly ABA-hypersensitive, whereas *pp2ca-1* seedlings show only a moderated enhanced sensitivity to ABA-mediated inhibition of root growth and loss of fresh weight of detached rosette leaves in *pp2ca-1* was similar to wild type (Kuhn et al., 2006). We wondered whether combination of the *pp2ca-1* mutation with either *hab1-1* or *abi1-2* might reinforce ABA responses as it was described previously for the double *hab1-1abi1-2* mutant (Saez et al., 2006). Therefore, the corresponding double mutants were generated and their ABA response analyzed. ABA-mediated inhibition of seed germination was stronger in the *pp2ca-1* single mutant than in *hab1-1* and *abi1-2* single mutants (Fig. 1A), which is in agreement with previous results from Yoshida et al. (2006). Interestingly, both double *hab1-1pp2ca-1* and

Table I. *List of T-DNA mutants used in this work*

The *hab1-1*, *abi1-2*, and *pp2ca-1* single and double *hab1-1abi1-2* mutants have been described previously (Saez et al., 2004, 2006; Kuhn et al., 2006). The *abi2-2* mutant was obtained from the NASC. Different crosses were performed to generate the double and triple mutants mentioned in the table.

Single Mutants	Double Mutants	Triple Mutants
hab1-1 SALK2104	*hab1-1abi1-2*	*hab1-1abi1-2pp2ca-1*
abi1-2 SALK72009	*abi1-2abi2-2*	*hab1-1abi1-2abi2-2*
pp2ca-1 SALK28132	*hab1-1pp2ca-1*	
abi2-2 SALK15166	*abi1-2pp2ca-1*	

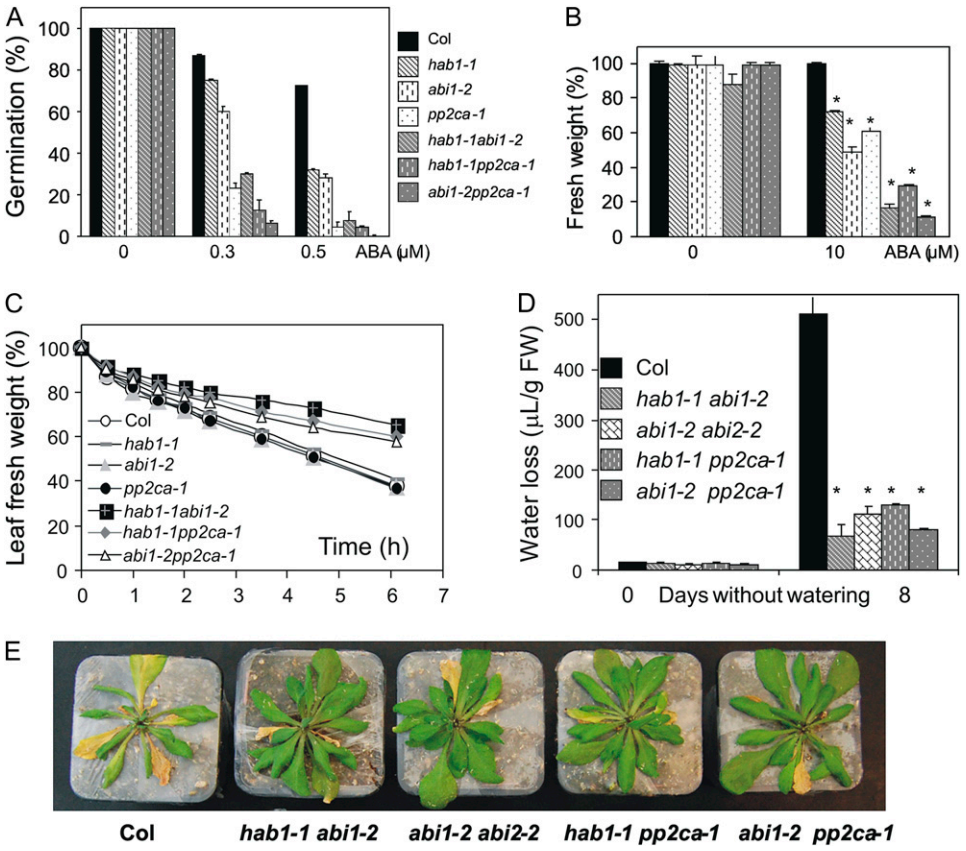

Figure 1. ABA-hypersensitive germination and growth inhibition of *hab1-1*, *abi1-2*, *pp2ca-1*, and double *hab1-1abi1-2*, *hab1-1pp2ca-1*, *abi1-2pp2ca-1* mutants compared to wild type. A, Percentage of seeds that germinated and developed green cotyledons in the presence of the indicated concentrations of ABA. Approximately 200 seeds of each genotype were sowed on each plate and scored 10 d later. The assays were done in the period encompassed between 1 to 2 months after seed harvesting. Values are averages ± SD for three independent experiments (not indicated in the figure); $P < 0.05$ (Student's *t* test) when comparing data from each genotype and wild type in ABA medium. B, Percentage of fresh weight from the different mutants as compared to wild type. The percentage was calculated with respect to the fresh weight of wild type in Murashige and Skoog medium either lacking or containing 10 μM ABA. Fresh weight of wild type was reduced by 39% in plates supplemented with ABA as compared to medium lacking ABA. Values are averages from three independent experiments ± SD ($n = 15$). * indicates $P < 0.01$ (Student's *t* test) when comparing data from each genotype and wild type in the same assay conditions. C, Reduced water loss measured in detached leaves of double *hab1-1abi1-2*, *hab1-1pp2ca-1*, *abi1-2pp2ca-1* mutants as compared to wild type or single parental mutants. Values are averages from two independent experiments ($n = 5$). SD values were lower than 8%. D, Quantification of water loss in 30-d-old plants after 8 d without watering. Values are averages from two independent experiments ± SD ($n = 10$). * as in B. FW, Fresh weight. E, Enhanced drought resistance of the indicated double mutants as compared to wild type. Photograph was taken 10 d after withholding water. Shoot was cut to better show the effect of drought on rosette leaves.

abi1-2pp2ca-1 mutants showed enhanced sensitivity to ABA-mediated inhibition of seed germination and growth inhibition than single parental mutants (Fig. 1, A and B). Likewise, short-term water loss assays showed that combined inactivation of either *PP2CA* and *HAB1* or *PP2CA* and *ABI1* resulted in a phenotype of reduced water loss compared to Columbia wild type (Col) or single parental mutants (Fig. 1C). Additionally, water-loss data were obtained under greenhouse conditions after exposing 21-d-old plants to drought stress by stopping irrigation and minimizing soil evaporation. Figure 1D shows that, after 8 d, the double mutants showed reduced water loss as compared to wild type. In agreement with these data, the double mutants did not show symptoms of wilting and they had turgid green rosette leaves, whereas wild-type plants wilted and rosette leaves yellowed (Fig. 1E).

Generation and Analysis of Double *abi1-2abi2-2* Mutants

A T-DNA mutant of *ABI2* was identified in the Salk collection, corresponding to donor stock number SALK_015166, and it was named *abi2-2*. Sequencing of the T-DNA flanking region in *abi2-2* showed that the insertion was localized 19 nucleotides upstream of the TGA stop codon. The T-DNA insertion severely impaired *ABI2* expression, based on quantitative real-time (qRT)-PCR analyses (see Fig. 3); however, the

abi2-2 allele showed wild-type responses in ABA-mediated inhibition of seed germination and growth assays, as well as ABA-induced stomatal closing (Fig. 2). In contrast, the double *abi1-2abi2-2* mutant showed higher sensitivity to ABA-mediated inhibition of germination and growth by ABA than single parental mutants (Fig. 2, A and B). Additionally, it showed enhanced ABA-induced stomatal closing (Fig. 2C), reduced water loss (Fig. 1D), and enhanced resistance to drought stress (Fig. 1E) as compared to wild-type plants.

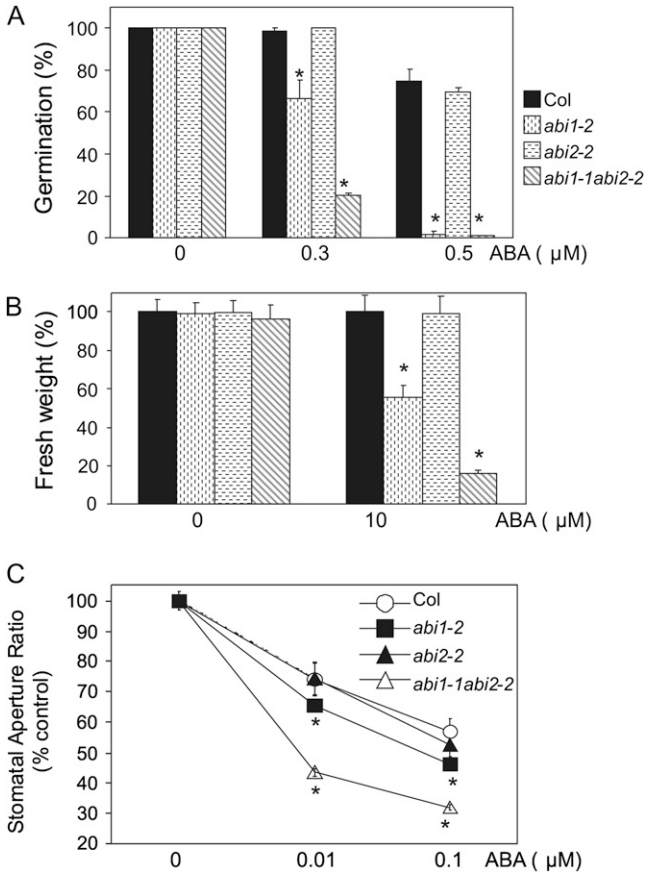

Figure 2. ABA-hypersensitive response of double *abi1-2abi2-2* mutant compared to wild type and single parental mutants. A, Percentage of seeds that germinated and developed green cotyledons in the presence of the indicated concentrations of ABA. Approximately 200 seeds of each genotype were sowed on each plate and scored 10 d later. The assays were done in the period encompassed between 1 to 2 months after seed harvesting. Values are averages ± SD for three independent experiments; *, $P < 0.05$ (Student's *t* test) when comparing data from each genotype and wild type in the same assay conditions. B, Percentage of fresh weight from the different mutants as compared to wild type. The percentage was calculated with respect to the fresh weight of wild type in Murashige and Skoog medium either lacking or containing 10 μM ABA. Fresh weight of wild type was reduced by 22% in plates supplemented with ABA as compared to medium lacking ABA. Values are averages from three independent experiments ± SD ($n = 15$); *, $P < 0.01$ (Student's *t* test) when comparing data from each genotype and wild type in the same assay conditions. C, ABA-hypersensitive stomatal closing in double *abi1-2abi2-2* mutant. Stomatal apertures were measured 2 h and 30 min after addition of 0.01 or 0.1 μM ABA. Data represent average aperture ratio (width/length) of three independent experiments ± SE (n = 30–40 stomata per experiment); *, $P < 0.01$ (Student's *t* test) when comparing data from the mutant and wild type in the same assay conditions.

Generation and Analysis of ABA Response in Triple *hab1-1abi1-2pp2ca-1* and *hab1-1abi1-2abi2-2* Mutants

To further study the effect on ABA signaling of the combined inactivation of negative regulators of the pathway, we generated two triple mutants impaired in three of the above-described PP2Cs. Thus, *hab1-1abi1-2pp2ca-1* and *hab1-1abi1-2abi2-2* triple mutants were generated and qRT-PCR analyses confirmed that expression of *HAB1*, *ABI1*, and either *PP2CA* or *ABI2*, respectively, was severely impaired (Fig. 3A). The phenotype of the triple mutants in the absence and presence of exogenous ABA was analyzed. Interestingly, both mutants showed impaired growth in the absence of exogenous ABA, either under in vitro conditions or when plants were grown in soil under greenhouse conditions (Fig. 3B). The impaired growth of these mutants suggests that combined inactivation of three negative regulators of ABA signaling might lead to a partial constitutive response to endogenous ABA levels. For instance, roots of these mutants were shorter than those of wild type and root growth was extremely hypersensitive to ABA-mediated inhibition of growth (Fig. 3C). Indeed, root growth of the triple mutants in the absence of exogenous ABA was approximately 60% to 65% of wild type. This decrease was very similar to that obtained in wild-type seedlings grown in 10 μM ABA (Fig. 3C). Additionally, a comparison of root growth for different ABA-hypersensitive mutants reveals a progressive increase in their sensitivity to ABA-mediated inhibition of growth (Fig. 3C), which suggests that PP2Cs act in concert to modulate root growth sensitivity to ABA.

Germination was slower in triple mutants than in wild type (Fig. 4A) and, indeed, the germination percentage of triple *hab1-1abi1-2pp2ca-1* mutants was 40% of wild type 80 h after sowing (Fig. 4A). Seeds of the triple mutants were hypersensitive to very low concentrations (nM) of ABA in germination assays (Fig. 4B). Thus, the inhibitory ABA concentration to achieve 50% inhibition (ABA-IC_{50}) of seed germination was 10 and 40 nM for the triple *hab1-1abi1-2pp2ca-1* and *hab1-1abi1-2abi2-2* mutants, respectively, whereas it was 380 nM for wild-type seeds. Interestingly, the germination percentage of the triple *hab1-1abi1-2pp2ca-1* mutant in the absence of exogenous ABA was similar to that found in wild-type seeds germinated in 300 nM ABA. Double mutants that contain the *pp2ca-1* allele were more sensitive to ABA-mediated inhibition of germination than *hab1-1abi1-2*. Thus, the double *hab1-1abi1-2* mutant had an ABA-IC_{50} of 120 nM, whereas the ABA-IC_{50} for the double *hab1-1pp2ca-1* and *abi1-2pp2ca-1* mutants was 50 and 70 nM, respectively. Early seedling growth was more inhibited by ABA in the triple mutants than in the double mutants, and double

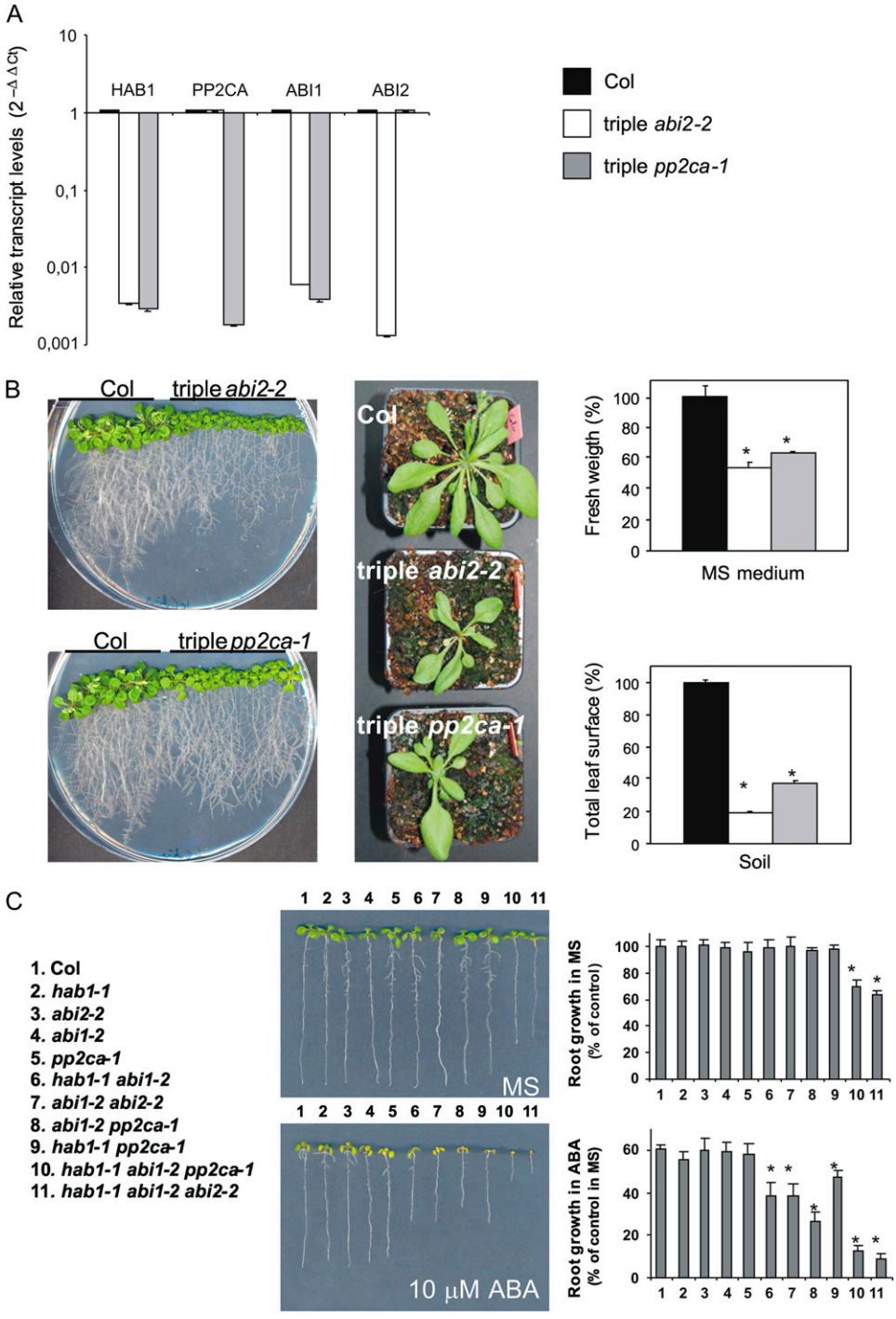

Figure 3. Growth impairment and ABA-hypersensitive growth inhibition of triple *hab1-1abi1-2abi2-2* (triple *abi2-2*) and *hab1-1abi1-2pp2ca-1* (triple *pp2ca-1*) mutants as compared to wild type. A, qRT-PCR analysis of *HAB1*, *PP2CA*, *ABI1*, and *ABI2* transcript levels in wild type and triple *hab1-1abi1-2abi2-2* and *hab1-1abi1-2pp2ca-1* mutants. Gene expression was analyzed in mRNAs extracted from 14-d-old seedlings treated with 10 μM ABA for 3 h. Data are averages ± SD from three independent experiments. B, Reduced growth in both Murashige and Skoog (MS) medium and soil of triple mutants compared to wild type. The photographs were taken after 3 weeks of plant growth in Murashige and Skoog medium or soil. The graphics show the quantification of fresh weight (n = 30) and leaf area (n = 10) in triple mutants with respect to wild type. Data are averages ± SD from two independent experiments. *, $P < 0.01$ (Student's t test) when comparing data from the mutant and wild type in the same assay conditions. C, ABA-hypersensitive root growth inhibition of double and triple mutants compared to wild type and single parental mutants. Representative seedlings were selected and a photograph was taken after 7 d of growth in medium lacking or supplemented with 10 μM ABA. Data are averages ± SD from three independent experiments (n = 15). *, $P < 0.01$ (Student's t test) when comparing data from the mutant and wild type in the same assay conditions. [See online article for color version of this figure.]

mutants containing the *pp2ca-1* allele were more ABA-hypersensitive than *hab1-1abi1-2* or single *pp2ca-1* mutants (Fig. 4C; Supplemental Fig. S1). Therefore, taking into account the sensitivity to ABA-mediated inhibition of germination and a dose-response (25–500 nM ABA) analysis of ABA-mediated inhibition of early growth (Supplemental Fig. S1), the following hierarchy of ABA hypersensitivity can be established: *hab1-1abi1-2pp2ca-1* > *hab1-1abi1-2abi2-2* > *hab1-1pp2ca-1* > *abi1-2pp2ca-1* > *pp2ca-1* ~ *hab1-1abi1-2*.

Finally, measurements of both stomatal aperture and aperture ratio (width/length) reveal that stomata of the triple *hab1-1abi1-2abi2-2* mutant were more closed than wild type in the absence of exogenous ABA (Fig. 5, A and B). Stomatal aperture measurements also showed that triple *hab1-1abi1-2abi2-2* mutant stomata were extremely hypersensitive to ABA-induced stomatal closing in the range of 10 to 100 nM ABA (Fig. 5). Interestingly, stomatal apertures in the triple *hab1-1abi1-2abi2-2* mutant in the absence of

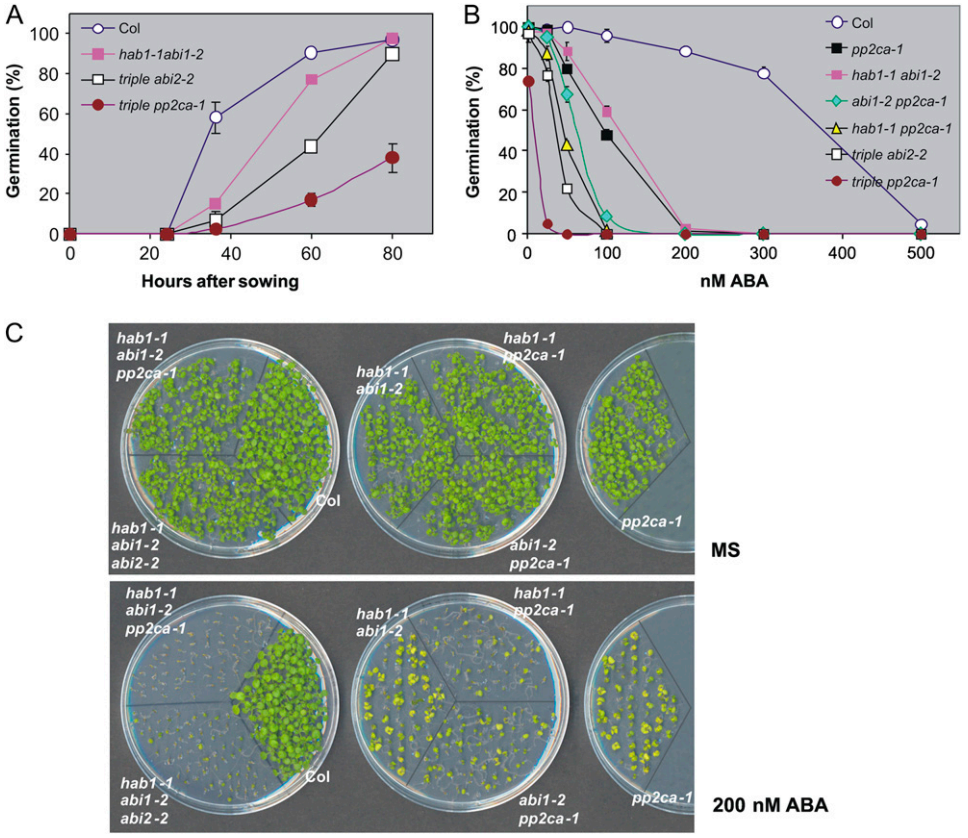

Figure 4. ABA-hypersensitive germination and early seedling growth inhibition of different mutants compared to wild type. A, Seed germination time course of triple *hab1-1abi1-2abi2-2* (triple *abi2-2*) and *hab1-1abi1-2pp2ca-1* (triple *pp2ca-1*) mutants compared to wild type. Data are averages ± SD from three independent experiments. B, Percentage of seeds that germinated and developed green cotyledons in the presence of the indicated concentrations of ABA. Approximately 200 seeds of each genotype were sowed on each plate and scored 5 d later. The assays were done in the period encompassed between 1 to 2 months after seed harvesting. Data are averages ± SD from three independent experiments. C, ABA-hypersensitive early seedling growth inhibition of different mutants compared to wild type. Approximately 80 seeds of each genotype were sowed on each plate and the photograph was taken 12 d later. MS, Murashige and Skoog medium.

exogenous ABA were similar to those found in wild type treated with 10 nM ABA. Additionally, we measured stomatal conductance in the absence of exogenous ABA for both triple mutants. As a result, we found decreased stomatal conductance for water vapor (g_s) and decreased transpiration (E) in both triple mutants as compared to wild type (Fig. 5, C and D). This result indicates that, in the absence of exogenous ABA, stomata of triple mutants have a lower aperture than wild type.

Transcriptomic Profiling Suggests That Triple Mutants Show a Constitutive Response to Endogenous ABA

The above data suggested that the phenotype of triple *hab1-1abi1-2pp2ca-1* and *hab1-1abi1-2abi2-2* mutants might reflect a partial constitutive response to endogenous ABA levels. To challenge this hypothesis, we have compared transcriptomic profiles of wild type and triple mutants using whole-genome long-oligonucleotide microarrays. First, we have identified the total number of genes up-regulated (>2-fold) and down-regulated (<0.5) in wild type by treatment with 10 μM ABA for 3 h (false discovery rate $P <$ 0.05), which qualify as ABA-responsive genes according to the ratio of expression Col + 10 μM ABA/Col no exogenous ABA (Fig. 6A). In addition, we have compared whole-genome expression of triple mutants with respect to wild type, in the absence of exogenous ABA (ratio of expression for triple no exogenous ABA/Col no exogenous ABA; Fig. 6A). The number of genes that (1) showed enhanced or diminished expression in the triple mutants with respect to the wild type, in the absence of exogenous ABA and (2) were ABA-responsive are indicated in Figure 6A and a complete list of these genes is provided as Supplemental Table S1. Thus, 194 or 266 ABA-responsive genes were up-regulated in the triple *abi2-2* or *pp2ca-1* mutants, respectively, in the absence of exogenous ABA. Conversely, 247 and 188 ABA-responsive genes were down-regulated in the triple *abi2-2* or *pp2ca-1* mutants, respectively, in the absence of exogenous ABA. Interestingly, 133 or 140 ABA-responsive genes were up-regulated or down-regulated, respectively, both in triple *abi2-2* and *pp2ca-1* mutants in the absence of exogenous ABA with respect to wild type. Independent confirmation of these results was obtained by qRT-PCR for two genes, *RAB18* and *RD29B*, which are up-regulated by ABA treatment (Fig. 6B). In the absence of exogenous ABA, these gene markers showed a 5- to 7-fold higher expression in the triple mutants than in wild type. Interestingly, expression of these genes in the triple mutants (in the absence of exogenous ABA) was approximately 50% to 60% of that found in wild type treated with 10 μM ABA. Finally, both triple mutants showed enhanced up-regulation by ABA of *RAB18* and *RD29B*, as their expression level upon ABA treatment was between 2- and 8-fold higher than in wild type (Fig. 6B).

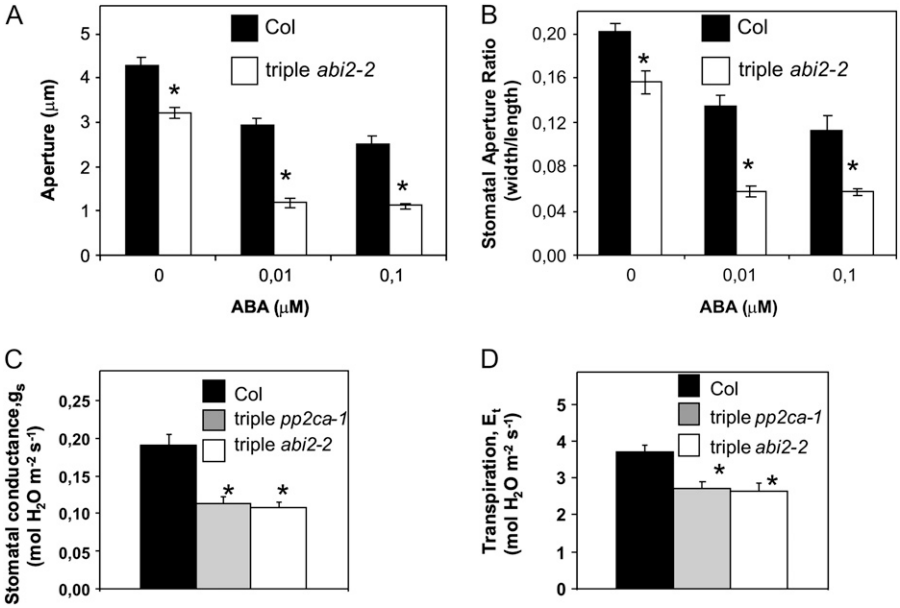

Figure 5. Reduced stomatal aperture in the absence of exogenous ABA for triple *hab1-1abi1-2abi2-2* (triple *abi2-2*) and *hab1-1abi1-2pp2ca-1* (triple *pp2ca-1*) mutants. A and B, ABA-hypersensitive stomatal closing in triple *hab1-1abi1-2abi2-2* mutant as compared to wild type. Plants were kept overnight in high humidity and then leaves were preincubated for 2 h in opening solution. Stomatal apertures were measured 2 h and 30 min after addition of 0, 0.01, or 0.1 μM ABA. Data represent the average of three independent experiments ± SEM (n = 30–40 stomata per experiment). C and D, Leaf gas-exchange measurements reveal both reduced stomatal conductance and transpiration in triple *pp2ca-1* and triple *abi2-2* mutants as compared to wild type. Data represent the average of two independent experiments ± SEM (n = 10 plants/experiment). *, $P < 0.01$ (Student's t test) when comparing data from each genotype and Col in the same assay conditions.

In summary, a partial constitutive up-regulation and down-regulation of ABA-responsive genes was found in both triple mutants in the absence of exogenous ABA (Supplemental Table S1). Furthermore, gene ontology (GO) analysis performed at The Arabidopsis Information Resource (TAIR; http://www.Arabidopsis.org/tools/bulk/go/index.jsp) reveals that these genes belong to the same categories that are overrepresented in ABA-treated wild-type seedlings, as, for instance, genes related to plant response to abiotic/biotic stimulus and stress response (oxidative stress, osmotic, salt, heat shock, and cold; Fig. 6C). Additionally, we identified ABA-responsive genes that were differentially up-regulated/down-regulated (>2-fold) between both triple mutants when compared with each other (Supplemental Table S2). For instance, genes encoding ABA-responsive seed storage proteins and AAA-type ATPases showed enhanced up-regulation in triple *abi2-2*, whereas genes encoding defensin-like proteins showed enhanced down-regulation compared to triple *pp2ca-1* (Supplemental Table S2). Interestingly, some genes encoding late embryogenesis-abundant proteins showed enhanced up-regulation in triple *pp2ca-1* compared to triple *abi2-2* (Supplemental Table S2).

Finally, to clarify whether the phenotypes described for the triple mutants reflect either enhanced synthesis of ABA or enhanced signaling, we have measured endogenous ABA levels in 12-d-old seedlings (Supplemental Fig. S2). Interestingly, endogenous ABA levels did not significantly differ when compared with data measured in triple mutants with respect to wild type (Supplemental Fig. S2). Moreover, after osmotic stress treatment (350 mM mannitol), both triple mutants did not produce more ABA than wild type (Supplemental Fig. S2). Therefore, these results indicate that the phenotypes observed in both triple mutants reflect a genuine effect of enhanced ABA signaling.

DISCUSSION

In this article, we show that different degrees of ABA hypersensitivity, ranging from mild to extreme, can be engineered through progressive inactivation of PP2Cs from group A. Thus, a fine tuning of ABA signaling can be accomplished in Arabidopsis through genetic inactivation of these genes, which might be of biotechnological interest once the corresponding orthologous genes are identified in crops. For instance, from this article and previous results of Saez et al. (2006), we conclude that inactivation of two major PP2Cs (HAB1/ABI1, ABI1/ABI2, ABI1/PP2CA, HAB1/PP2CA) is enough in Arabidopsis to generate drought-avoidant plants. Taking into account the important advance in RNA-mediated gene-silencing

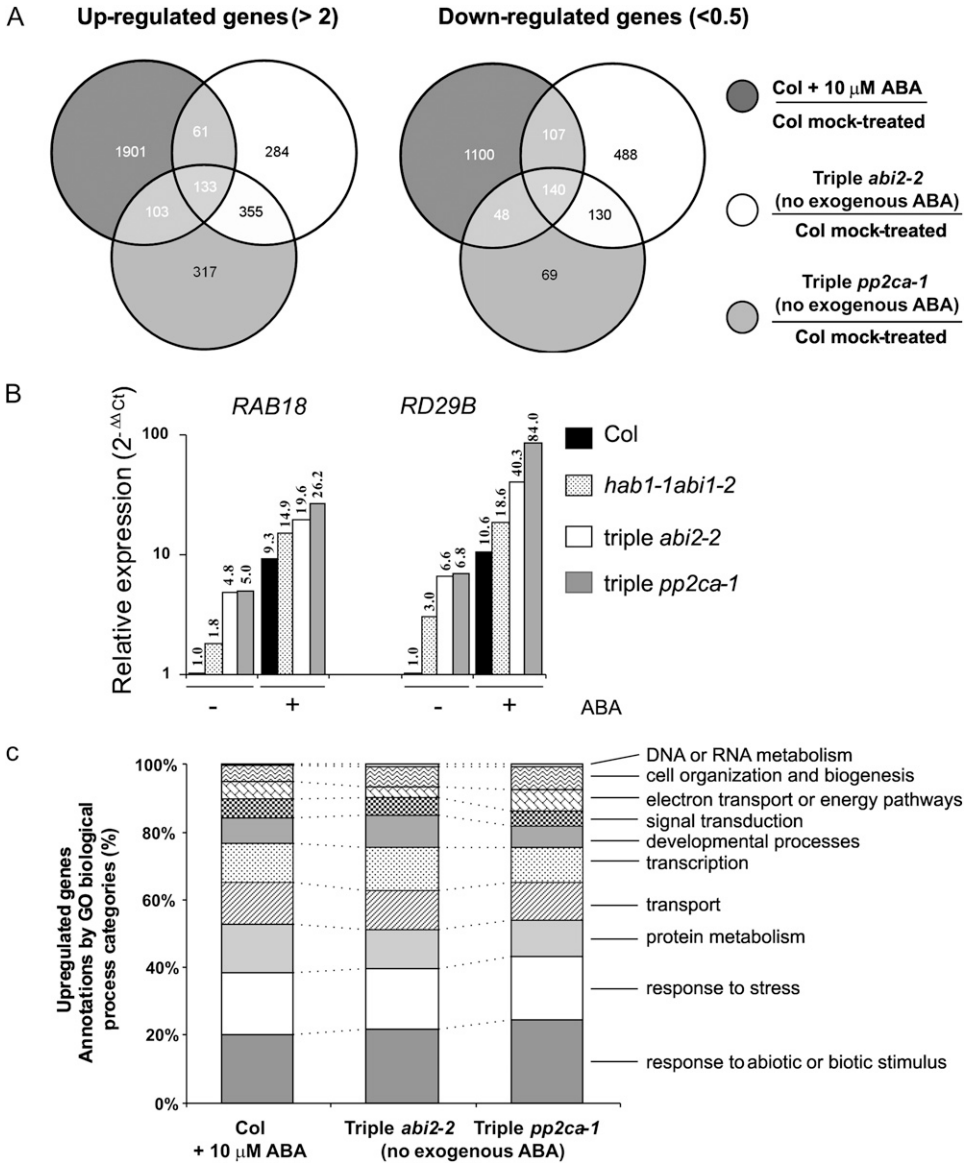

Figure 6. Triple *hab1-1abi1-2abi2-2* (triple *abi2-2*) and *hab1-1abi1-2pp2ca-1* (triple *pp2ca-1*) mutants show partial constitutive up-regulation and down-regulation of ABA-responsive genes in the absence of exogenous ABA. A, Venn diagrams. Number of ABA-responsive genes that show up-regulation and down-regulation in triple mutants in the absence of exogenous ABA. B, Expression of *RAB18* and *RD29B* in triple mutants with respect to wild type. qRT-PCR analyses were made in triplicate on RNA samples of 2-week-old seedlings that were either mock or 10 μM ABA-treated for 3 h. Numbers indicate the expression level of the *RAB18* and *RD29B* genes under mock (−) or ABA treatment (+) in each mutant genotype with respect to the wild type (value 1). C, Comparison of GO categories for ABA-responsive genes in ABA-treated wild type versus triple mutants in the absence of exogenous ABA.

technology, either through the use of hairpin RNAi or artificial microRNAs (Ossowski et al., 2008), it is reasonable to suggest that combined inactivation of these PP2Cs might be attained in crop plants where genome information is available. However, this work also shows that overactivation of ABA signaling is detrimental for plant growth in the absence of water stress because triple mutants that show a partial constitutive response to ABA are impaired in growth. Therefore, the multiplicity of PP2Cs that regulate ABA signaling appears to be a versatile mechanism to adequately control ABA response both in the absence or presence or stress.

The analysis of combined mutations in PP2Cs from group A has revealed a genetic interaction between the ABI1/HAB1 and PP2CA branches, which extends our knowledge on these PP2Cs. For instance, although it was known that PP2CA plays a major role in regulating ABA signaling in seeds, the phenotypes of double mutants that combine the *pp2ca-1* allele with either *hab1-1* or *abi1-2* reveal that PP2CA also has a key role in regulating water loss in vegetative tissue. This evidence could not be obtained from the analysis of single *pp2ca* mutants, which show similar water loss than wild type due to a slight ABA-hypersensitive response, as discussed by Kuhn et al. (2006). Additionally, enhanced sensitivity to ABA-mediated inhibition of seed germination was found in the double *hab1-1pp2ca-1* and *abi1-2pp2ca-1* mutants, whose ABA-IC_{50} was 50 and 70 nM, respectively, compared to *pp2ca-1* (ABA-IC_{50} = 110 nM). These double mutants were also more sensitive to ABA-mediated inhibition of root growth than *pp2ca-1*. Therefore, taken together, these results confirm that PP2CA, as shown previously for

HAB1 and ABI1, is a key regulator of ABA signaling both in seeds and vegetative tissues (Kuhn et al., 2006). This conclusion is consistent with gene expression data found at public databases (Supplemental Fig. S3). In addition, the phenotypes of the different mutants hereby reported suggest that PP2Cs provide a threshold of negative regulation required for a normal response to ABA. If ABI1/HAB1 and PP2CA branches regulate independently ABA signaling, the loss of one branch would be enough to overcome this threshold and, therefore, lead to ABA hypersensitivity. For instance, even though HAB1 and ABI1 are expressed in *pp2ca-1*, this mutant shows a strong ABA-hypersensitive phenotype in seeds. Alternatively, it is possible that ABA signaling is negatively regulated through a mechanism that involves an accurate PP2C dosage effect. For instance, such a mechanism is supported for the progressive increase in root growth sensitivity to ABA observed in single, double, and triple *pp2c* mutants (Fig. 4C).

Finally, we show that triple *pp2c* mutants are extremely sensitive to exogenous ABA. Particularly noticeable is the nanomolar sensitivity of the triple mutants for ABA-mediated inhibition of seed germination, which is particularly appealing, taking into account that other PP2Cs that regulate ABA signaling in seed are still active in such mutants. For instance, the triple *hab1-1abi1-2abi2-2* mutant shows an ABA-IC_{50} of 40 nM, even though two PP2Cs (PP2CA/AHG3 and AHG1) that are essential components of ABA signaling in seed are active. This fact suggests the existence of at least two branches of PP2Cs to negatively regulate ABA signaling in seed. Additionally, triple mutants show a partial constitutive response to ABA in different assays. For instance, root growth of triple mutants in the absence of exogenous ABA was very similar to wild type in the presence of 10 μM ABA. Partial stomatal closure was found in triple mutants as compared to wild type and, interestingly, in the absence of exogenous ABA the triple mutants showed a stomatal aperture only slightly higher than wild type treated with 10 nM ABA. This partial constitutive response to ABA was also found by gene expression analysis. Thus, expression of *RAB18* and *RD29B* in triple mutants, in the absence of exogenous ABA, was only 50% to 60% of that found in wild type treated with 10 μM ABA. Whole transcriptome analysis reveals that approximately 15% or approximately 25% of genes up-regulated or down-regulated by ABA in wild type, respectively, were constitutively up-regulated or down-regulated in the triple mutants in the absence of exogenous ABA. Even though this effect represents only a fraction of the whole set of ABA-responsive genes, it leads to impaired plant growth. This phenotype is reminiscent of that found in plants that overexpress DRE-binding protein DREB1A under the control of a 35S promoter, which show constitutive expression of many stress tolerance genes, but also show severe growth retardation under normal growing conditions (Kasuga et al., 1999). Similarly, constitutive expression of ABRE-binding factors ABF3 and ABF4 also led to constitutive expression of ABA-responsive genes and growth retardation (Kang et al., 2002). Therefore, PP2Cs play a major role in regulating ABA signaling both under stress as well as normal growth conditions. Additionally, the phenotype of triple *pp2c* mutants serves to illustrate the importance of ABA in stress response as well as growth regulation.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) plants were routinely grown under greenhouse conditions in pots containing a 1:3 vermiculite:soil mixture. For in vitro culture, seeds were surface sterilized by treatment with 70% ethanol for 20 min, followed by commercial bleach (2.5% sodium hypochlorite) containing 0.05% Triton X-100 for 10 min and, finally, four washes with sterile distilled water. Stratification of the seeds was conducted in the dark at 4°C for 3 d. Then seeds were sowed on plates composed of Murashige and Skoog basal salts, 0.1% MES acid, 1% agar, and 1% Suc. The pH was adjusted to 5.7 with KOH before autoclaving. Plates were sealed and incubated in a controlled-environment growth chamber at 22°C under a 16-h-light/8-h-dark photoperiod at 80 to 100 $\mu E\ m^{-2}\ s^{-1}$.

Mutant Identification by PCR Screening

The *hab1-1*, *abi1-2*, and *pp2ca-1* alleles have been described previously (Leonhardt et al., 2004; Saez et al., 2004, 2006; Kuhn et al., 2006). A line containing a single T-DNA insertion in *ABI2* was identified in the SALK T-DNA collection (Alonso et al., 2003), SALK_15166, and obtained from the Nottingham Arabidopsis Stock Centre (NASC; http://nasc.nott.ac.uk). To identify individuals homozygous for the T-DNA insertion, genomic DNA was obtained from kanamycin-resistant seedlings and submitted to PCR genotyping using the following *ABI2* primers: forward 5′-AGTGACTTCAGTGCGGCGAGT and reverse 5′-CCTTCTTTTTCAATTCAAGGAT. As a T-DNA left-border primer of the pROK2 vector, we used LBpROK2: 5′-GCCGATTTCGGAACCACCATC.

To generate the *abi1-2abi2-2*, *abi1-2pp2ca-1*, and *hab1-1pp2ca-1* double mutants, we transferred pollen of either *abi2-2* or *pp2ca-1* to the stigmas of emasculated flowers of *abi1-2* and *hab1-1*. To generate the triple *hab1-1abi1-2abi2-2* and *hab1-1abi1-2pp2ca-1* mutants, we crossed either *abi1-2abi2-2* or *abi1-2pp2ca-1* double mutants with *hab1-1abi1-2* (Saez et al., 2006; Rodrigues et al., 2009), respectively. The resulting F2 individuals were genotyped by PCR for the presence of the double or triple mutants.

Germination, Growth, and Stomatal Aperture Assays

To measure sensitivity to ABA-mediated inhibition of germination, seeds (approximately 200 seeds/experiment) were plated on solid medium composed of Murashige and Skoog basal salts, 1% Suc, and increasing concentrations of ABA. To score seed germination, either radicle emergence or the percentage of seeds that had germinated and developed fully green expanded cotyledons was determined. The ABA-resistant growth was scored as described by Saez et al. (2006). Data were obtained for three independent experiments, each done with 15 plants. To quantify root growth inhibition, 5-d-old seedlings grown vertically onto Murashige and Skoog plates were transferred to either Murashige and Skoog plates or Murashige and Skoog plates supplemented with 10 μM ABA. After 7 d, the plates were scanned on a flatbed scanner to produce image files suitable for quantitative analysis using National Institutes of Health Image software (ImageJ version 1.37). Data were obtained for three independent experiments, each done with 15 plants. Assays of ABA-induced stomatal closing were performed as described by Saez et al. (2006). Data were expressed as the average of three experiments where 30 to 40 stomata were measured for each one.

Leaf Gas-Exchange Measurements

For leaf gas-exchange measurements, plants were grown in hydroponics (Hoagland solution at 50% in 250-mL nontransparent pots) inside a growth

chamber (12 h at 150 μmol photons m^{-2} s^{-1}, approximately 60% humidity, and 26°C day/20°C night air temperature). Nutrition solution was changed every 2 to 3 d to prevent nutrition and water limitations. From preliminary light response curves, photosynthesis was proved to be saturating at photon flux densities above 500 μmol photons m^{-2} s^{-1}. Light-saturating net photosynthesis (A_N), leaf conductance for water vapor (g_s), transpiration rate (E), and substomatal CO_2 concentration (C_i) were therefore measured under steady-state conditions at 800 μmol photons m^{-2} s^{-1} with an open gas-exchange infrared gas analyzer (Li-6400; LI-COR) on the youngest fully expanded leaf of 38- to 45-d-old plants for Col and triple mutants. CO_2 concentration in the leaf chamber was maintained at 400 μmol CO_2 mol^{-1} air, whereas humidity and vapor pressure deficit were kept around 45% and 2 kPa, respectively.

Drought Stress and Water Loss Assays

Two different water-loss assays were performed. Short-term assays were performed basically as described by Saez et al. (2006) in detached leaves at the same developmental stage and size from 21-d-old plants. Four samples of three leaves per genotype were excised and fresh weight was determined by submitting the leaves to the drying atmosphere of a flow laminar hood for 6 h. Data are averages ± SE from three independent experiments (n = 5). Long-term assays were performed after withholding water in plants maintained under greenhouse conditions basically as described by Saez et al. (2006). Eight plants of each genotype (two independent experiments) were grown under normal watering conditions for 21 d and then subjected to drought stress for 8 d by completely terminating irrigation and minimizing soil evaporation by covering pots with plastic wrap film. Eight leaves from each plant were removed, weighed, incubated in demineralized water for 3 h, and weighed again. The difference in weight was considered as water loss.

RNA Analyses

These assays were performed as described by Saez et al. (2006). Briefly, plants were grown on Murashige and Skoog plates supplemented with 1% Suc. After 10 d, approximately 30 to 40 seedlings were either mock or 10 μM ABA treated. After 3 h, plant material was collected and frozen in liquid nitrogen. qRT-PCR amplifications and measurements were performed using an ABI PRISM 7000 sequence detection system (Perkin-Elmer Applied Biosystems). The sequences of the primers used for PCR amplifications were the following ones: for *HAB1* (At1g72770), forward 5′-AACTGCTGTTGTTGCCTTG and reverse 5′-GGTTCTGGTCTTGAACTTTCT; for *ABI1* (At4g26080), forward 5′-ATGATCAGCAGAACAGAGAGT and reverse 5′-TCAGTTCAAGGGTTTGCT; for *ABI2* (At5g57050), forward 5′-AGTGACTTCAGTGCGGCGAGT and reverse 5′-CCTTCTTTTTCAATTCAAGGAT; for *PP2CA* (At3g11410), forward 5′-CTTTGTCGTAACGGTGTAGC and reverse 5′-TTGCTCTAGACATGGCAAGA; for *RAB18* (At5g66400), forward 5′-ATGGCGTCTTACCAGAACCGT and reverse 5′-CCAGATCCGGAGCGGTGAAGC; for *RD29B* (At5g52300), forward 5′-ATGGAGTCACAGTTGACACGTCC and reverse 5′-GAGATAGTCATCTTCACCACCAGG; and for *β-actin-8* (At1g49420), forward 5′-AGTGGTCGTACAACCGGTATTGT and reverse 5′-GAGGATAGCATGTGGAAGTGAGAA.

qRT-PCR amplifications were monitored using Eva-Green fluorescent stain (Biotium). Relative quantification of gene expression data was carried out using the $2^{-\Delta\Delta C}{}_T$ or comparative C_T method (Livak and Schmittgen, 2001). Expression levels were normalized using the C_T values obtained for the *β-actin-8* gene. The presence of a single PCR product was further verified by dissociation analysis in all amplifications. All quantifications were made in triplicate on RNA samples obtained from three independent biological replicas.

RNA Amplification, Labeling, and Microarray Hybridization

These assays were performed as described by Rodrigues et al. (2009), according to MIAME guidelines (Brazma et al., 2001).

Identification of Differentially Expressed Genes and GO Analysis

Significance analysis of microarrays (Tusher et al., 2001) was performed on the three normalized datasets to identify differentially expressed genes. The parameters for significance analysis of microarrays were adjusted so that the false discovery rate probability for every experiment was below 0.05. A 2-fold expression cutoff was considered. A functional category analysis of the genes simultaneously up-regulated or down-regulated in the three genotypes (Col, triple *abi2-2*, and triple *pp2ca-1*) was carried out by the Munich Information Center for Protein Sequences (MIPS; http://mips.gsf.de/proj/funcatDB/search_main_frame.html). Only overrepresented categories with a P value smaller than 0.05 were further considered. Functional categorization of up-regulated genes in each of the genotypes, based on the high-level terms in the GO hierarchy, was conducted using TAIR (http://www.Arabidopsis.org/tools/bulk/go/index.jsp). Venn diagrams were generated to illustrate differences in expression.

ABA Content Measurement

Three independent biological samples of 12-d-old seedlings grown in Murashige and Skoog plates were used for ABA content determination. Seedlings were either mock- or 350 mM mannitol treated for 8 h. After measuring fresh weight, plants were ground in liquid nitrogen and then extracted with 1 mL extraction buffer (80% methanol, 100 mg/L butylated hydroxytoluene, and 0.5 g/L citric acid monohydrate) overnight at 4°C (Xiong et al., 2001). After centrifugation at 1,000g for 20 min at 4°C, supernatants were collected and dried. Samples were resuspended in buffer containing 10% methanol, 25 mM Tris, pH 7.5, 100 mM NaCl, and 1 mM $MgCl_2$, and they were subjected to ABA measurement using the Phytodetek ABA test kit (Agdia).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. ABA-hypersensitive early seedling growth inhibition of different mutants compared to wild type at different concentrations of ABA ranging from 25 to 500 nM ABA.

Supplemental Figure S2. Comparison of the endogenous ABA levels in wild type and triple mutants under nonstressed conditions or after treatment with 350 mM mannitol for 8 h.

Supplemental Figure S3. Cladogram and gene expression data from clade A PP2Cs.

Supplemental Table S1. ABA-responsive genes constitutively up-regulated (threshold of 2-fold, ratio >2) or down-regulated (threshold of 2-fold, ratio <0.5) in the triple mutants in the absence of exogenous ABA.

Supplemental Table S2. Differential expression of ABA-responsive genes constitutively up-regulated or down-regulated in the triple *abi2-2* and *pp2ca-1* mutants compared each other (genes selected previously from Supplemental Table S1).

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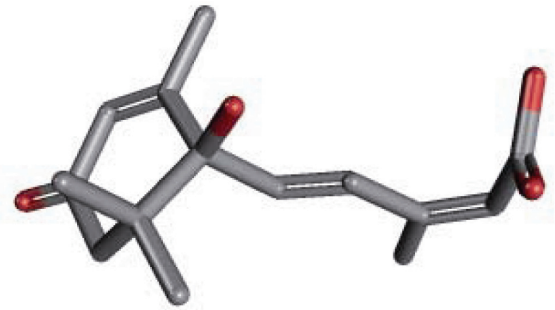


Discusión

El ácido abscísico es una hormona vegetal que se encuentra en todas las plantas superiores con un papel fundamental tanto en el desarrollo y crecimiento de la planta, como en la respuesta de la planta frente al estrés [79, 253], teniendo un notable papel en la tolerancia a sequía según se refleja en los experimentos incluidos en esta memoria. El ABA actúa regulando procesos importantes del desarrollo de la semilla como son la tolerancia a la desecación, la síntesis y el almacenamiento tanto de lípidos como de proteínas e inicia dormición. En las respuestas de la planta frente al estrés el ABA participa en situaciones de estrés hídrico promoviendo tolerancia a salinidad y sequía. En estas condiciones se sintetiza ABA de *novo* en diferentes partes de la planta lo que resulta en un rápido incremento de hasta 40 veces los niveles normales de ABA en hojas, mientras que la cantidad de ABA en raíces se incrementa progresivamente a medida que se produce pérdida de agua en tejidos [27]. El ABA sintetizado participa en diferentes respuestas adaptativas para la supervivencia de la planta como el mantenimiento de la elongación de la raíz [225], el cierre de los estomas y la expresión de genes de respuesta [49, 168], como se comprueba en este trabajo.

El principal objetivo que nos planteamos al comienzo de esta tesis doctoral fue profundizar en el conocimiento de la ruta de transducción de señal del ABA, el estudio detallado de PP2Cs de relevante importancia en la ruta como HAB1 que no había sido caracterizado, funciones, e interacción con otros posibles componentes clave, que no estaban descritos al comienzo de este trabajo para explicar el mecanismo y modo de acción de esta hormona. Algunos años antes de iniciarse esta memoria, se habían realizado estudios de clonaje molecular que revelaban la estructura primaria de proteínas fosfatasas

de tipo Ser/Thr en plantas. Se distinguían dos familias estructuralmente distintas que se denominaron PP1/PP2A y PP2C presentes en animales y plantas. Estudios de genética molecular y bioquímica realizados en *Arabidopsis thaliana* identificaban a las PP2C como proteínas clave en los procesos de transducción de señal en plantas. Las PP2Cs ABI1 y ABI2 se perfilaban como proteínas fundamentales en la ruta de transducción de señal de ABA a partir del descubrimiento de los mutantes *abi1-1D* y *abi2-1D*.

Otra PP2C denominada como KAPP (kinase associated protein phosphatase) destacaba como un elemento importante en las rutas de señalización donde estaban implicadas proteínas RLK (receptor like kinase). Además resaltaba la PP2C de alfalfa que actúa como regulador negativo en las rutas donde participan quinasas MAPK en plantas [203].

HAB 1 se identifica como una nueva PP2C a partir de la clonación tanto del cDNA como de su secuencia genómica por homología con *ABI1* y *ABI2* respectivamente. El cDNA de *AtP2C-HA/HAB1* contiene una pauta de lectura abierta de 1536 pb y codifica un proteína de 511 aminoácidos con un peso molecular de 55,7 KDa. La proteína está compuesta por un dominio C-terminal donde reside la actividad fosfatasas de y un dominio N-terminal de 180 aminoácidos. HAB1 posee una homología de un 55% y de un 54% con ABI1 y ABI2, siendo su expresión inducible por ABA [204].

En la ruta de señalización por ABA se han identificado reguladores negativos como la proteína fosfatasa 2C (PP2C) del grupo A HAB1 que caracterizamos en este trabajo como un regulador negativo de la ruta de señalización por ABA, así como reguladores positivos de la señalización por ABA como las proteínas SnRK2 [31, 78, 248, 254, 255] y los recientemente

caracterizados receptores acoplados a proteínas G (GPCRs) GTG1 y GTG2 localizados en la membrana plasmática [183] además de los receptores de ABA tipo PYL/PYR/RCAR que regulan las PP2Cs en respuesta a ABA [139, 184].

Los eventos de fosforilación y desfosforilación de proteínas son los eventos principales de señalización inducidos por estrés osmótico en plantas superiores, en ellos participan los miembros de la familia de quinasas del tipo SNF1 (sucrose non fermenting 1), quinasa que desempeña un papel fundamental en respuesta al estrés osmótico en Arabidopsis, maíz y arroz [141].

Las fosfatasas de tipo 2C son enzimas monoméricas presentes tanto en procariotas como en eucariotas. Implicadas en distintas vías de señalización, una muestra de la importancia de las PP2Cs de tipo Ser/Thr en plantas es su elevado número de genes, al menos hay 76 miembros según un análisis de bases de datos en Arabidopsis [220]. Un elevado número si comparamos las 6 encontradas en el genoma de levaduras, las 8 encontradas en gusano, las 2 en mosca y las 15 en el genoma de humanos [32]. Aunque siendo menos numerosas en humanos también se les atribuyen funciones importantes como a la PP2Cα implicada en señalización de apoptosis, en la regulación de genes supresores de tumores como p53 y, en la regulación de quinasas dependientes de ciclinas [123]

Las fosfatasas PP2C participan en la regulación de distintas rutas de plantas, ejemplos descritos en la literatura son los reguladores negativos KAPP y POLTERGEIST de la ruta CLAVATA, en la cuál se regulan el tamaño de los brotes y de los meristemos florales [263, 277]. Otro ejemplo es el canal de K^+

AKT2 regulado por la fosfatasa PP2CA que controla el transporte de K^+ y la polarización de la membrana [37] .

En la ruta de transducción de señal por ABA las PP2Cs juegan un papel fundamental. Existen evidencias genéticas apoyadas en el estudio de mutantes como en el caso de los mutantes *abi1* y *abi2*, en los que el análisis de revertientes intragénicos y, más recientemente el análisis de mutantes de pérdida de función que realizamos en este trabajo, novedoso en el campo de la señalización por ABA ya que con anterioridad a esta tesis no se habían analizado mutantes de pérdida de función en PP2Cs de plantas, confirman la función de las PP2Cs como reguladores negativos de la señalización por ABA [68, 150, 207, 208, 227, 238]. La sobre expresión transitoria de *ABI1* y *PP2CA* en protoplastos de maíz bloqueaba la expresión de genes delatores que portaban promotores inducibles por ABA [227]. La identificación y caracterización fisiológica de los mutantes revertientes *abi1-R1* a *abi1R7*, en un fondo genético Landsberg *erecta*,como mutantes de pérdida de función fue crucial para asignar la función de regulador negativo a ABI1 en la señalización por ABA [68]. Posteriormente se caracterizaron mutantes revertientes para *ABI2 abi2-R1* y los dobles mutantes *abi1-R4abi2-R1* y *abi1R5-abi2R1* cuyo análisis en ensayos de respuesta a ABA confirmaron la función de regulador negativo de ABI2 [150]. Al comienzo de este trabajo de investigación existía cierta controversia sobre el papel de ABI1 como regulador negativo de la señalización por ABA [266] basada en que la sobre expresión de ABI1 en Arabidopsis no afecta a la señalización por ABA, a pesar de que si ABI1 actuase como regulador positivo la sobre expresión llevaría a un reforzamiento de la señalización por ABA, algo que no se describía en el trabajo citado. El

hecho de que ABI1 sea un regulador negativo de la señalización por ABA está avalado por la sobre expresión de esta proteína en protoplastos de maíz donde se comprueba que se reprime la expresión de genes inducibles por ABA [227].

Los análisis de actividad PP2C de ABI1 y ABI2 llevados a cabo en los mutantes *abi1-1R5* y *abi2-1-R1* revelan que estas dos proteínas contribuyen en un 50% de la actividad PP2C inducida por ABA [150]. Ello indica que otras PP2Cs deben participar en la ruta de señalización por ABA. Además experimentos de inhibición de PP2CA por la expresión de un ARN antisentido llevan a un incremento de la sensibilidad a ABA durante el desarrollo de la tolerancia al frío y la germinación de la semilla [238]. Adicionalmente en esta memoria se muestra como HAB1 clonado en base a su homología con ABI1 y ABI2, podía ser un candidato ideal para participar en ese otro 50% de actividad PP2C inducida por ABA, como finalmente comprobamos que ocurre.

Para caracterizar la función de la PP2C HAB1 en la ruta de señalización de ABA realizamos un análisis de pérdida y de ganancia de función con el aislamiento y posterior análisis del mutante *hab1-1* y la generación y análisis de plantas transgénicas *35S:HAB1*. Hasta la realización de esta tesis no se habían caracterizado mutantes de inserción de T-DNA en genes de *PP2Cs* involucrados en la señalización por ABA. Los ensayos dosis-respuesta indican que *hab1-1* es hipersensible a la inhibición de la germinación por ABA en comparación con el fenotipo silvestre. En los ensayos en presencia de paclobutrazol, un inhibidor de giberelinas, observamos cómo *hab1-1* requiere un incremento mayor de giberelinas para germinar con respecto al silvestre, por lo que podemos deducir que HAB1 podría tener un importante papel como promotor de la germinación, lo que muestra que la germinación podría estar

promovida no sólo por giberelinas si no también por la regulación negativa de ABA a través de la acción de HAB1. Sin embargo los valores de pérdida de agua por transpiración son similares al silvestre, lo que podría ser debido a la redundancia funcional entre PP2Cs. La expresión sostenida de *HAB1* en las líneas *35S:HAB1* produce a un fenotipo de insensibilidad a ABA [208]. Analizamos también las respuestas mediadas por ABA tanto en semillas como en tejido vegetativo obteniendo que la expresión sostenida de *HAB1* lleva a una reducida sensibilidad a ABA. Obteniendo los mismo resultados en ensayos de crecimiento de raíz, donde las plantas *35S:HAB1* muestran una reducida sensibilidad a la inhibición del crecimiento por ABA en comparación con el fenotipo silvestre. El análisis de las líneas *35S:HAB1* en ensayos de pérdida de agua por transpiración muestran insensibilidad a ABA en cierre de estomas, siendo el valor de la pérdida de agua dos veces mayor respecto al fenotipo silvestre, por lo que hay un incremento en la sensibilidad al estrés por sequía.

El efecto inhibitorio del crecimiento de raíz a elevadas concentraciones de ABA ha sido atribuido a la activación de la ruta de señalización por etileno [6, 62, 208]. Además se podría establecer un vínculo entre el control del crecimiento por ABA y el control de ciclo celular, en el cuál participa la quinasa ICK1 (protein kinase inhibitor 1) inducible por ABA [258]. En la caracterización de las plantas *35S:HAB1* hemos observado resistencia al efecto inhibitorio del ABA a 30 μM tanto en crecimiento de raíz como del meristemo apical, ya que estas plantas son capaces en estas condiciones de pasar del estado vegetativo al reproductivo y florecer, algo que no ocurre en el silvestre. Esto nos lleva a plantear que *HAB1* podría desempeñar un papel en la parada de ciclo celular, ya que la expresión mantenida de *HAB1* atenúa la inhibición del crecimiento

por ABA, la regulación de *HAB1* por ABA podría contribuir a la regulación de la parada de ciclo celular inducida por ABA en tejidos meristemáticos. En la líneas *35S:HAB1* también hemos analizado los genes de respuesta a ABA *RAB18*, *RD29A* y *P5CS1* en respuesta al tratamiento con ABA exógeno, comprobando que sus correspondientes mRNAS se reducen en comparación con el fenotipo silvestre.

En este trabajo se caracteriza el papel de la fosfatasa 2C HAB1 en la ruta de transducción de señal del ABA como regulador negativo de la señalización por ABA mediante un abordaje genético de pérdida y de ganancia de función

Un objetivo actual de los estudios que se están realizando para conocer en mayor detalle la ruta de señalización por ABA es determinar proteínas que interaccionen con los distintos componentes descritos de la ruta, especialmente aquellos que regulan procesos clave de la misma. Este ha sido uno de los principales objetivos de esta tesis y por este motivo hemos realizado una búsqueda de proteínas que pudieran ser dianas de interacción de la PP2C HAB1. En estos ensayos hemos empleado el sistema de búsqueda por doble híbrido en levaduras, utilizando como cebo un fragmento de *HAB1* que comprendía únicamente la región catalítica para que junto con la unión al DNA no se produjera transactivación del sistema. En estos estudios se empleó una librería de cADN de Arabidopsis. Uno de los clones positivos que mayor frecuencia fue encontrado correspondía al cDNA completo de la proteína SWI3B, un componente de un complejo remodelador de cromatina Swi/Snf [215] descrito inicialmente en levaduras, sugiriendo que AtSWI3B es una de las dianas prevalentes de interacción con HAB1. Posteriormente se caracterizó la interacción de estas proteínas por un mapeo de la interacción mediante

delecciones seriadas, observándose que era imprescindible el dominio catalítico intacto de HAB1 para que se produjera la interacción, además de ser necesarios los 220 primeros residuos de SWI3B. Hay que resaltar que interacción entre HAB1 y SWI3B es específica ya que HAB1 no interacciona, al menos en ensayos de doble híbrido, con otros miembros de la familia SWI como SWI3A, SWI3C, o SWI3D, resultado que concuerda con la diversificación de funciones que ha sido descrita para estos factores [85, 214]. Posteriormente se realizaron varios ensayos para corroborar que esta interacción se produce *in planta*. En primer lugar se determinó la localización subcelular de ambas proteínas mediante experimentos de expresión de proteínas unidas a GFP junto con ensayos de fraccionamiento subcelular y análisis posterior por western blot mostrando que la localización de SWI3B era nuclear y que HAB1 se encontraba tanto en el núcleo como en el citoplasma sugiriendo que ambas proteínas podían encontrarse ya que compartían localización. Además se emplearon diferentes ensayos para determinar si estas proteínas interaccionaban *in vivo*, concretamente, ensayos de complementación bimolecular de la fluorescencia (BiFC) basados en la reconstrucción de la proteína fluorescente amarilla (YFP) y la consiguiente emisión de luz, si existe interacción entre dos proteínas recombinantes formadas por un fragmento de la YFP fusionado a las proteínas de interés, en este caso HAB1 y SWI3B. El ensayo de BiFC dio como resultado la interacción de HAB1 y SWI3B en hojas de tabaco infiltradas con *Agrobacterium*. Adicionalmente realizamos experimentos de coinmunoprecipitación y análisis por western blot que mostraban, por ensayos de biología molecular, los mismos resultados obtenidos por BiFC, poniéndose de manifiesto, mediante ensayos en levaduras

y en plantas, que estas proteínas interaccionan. La siguiente pregunta que pretendíamos contestar era si esta interacción tenía consecuencias funcionales, en este sentido se ha descrito que mutantes homocigotos para componentes de la maquinaria de remodelado de cromatina presentan efectos fenotípicos en otras especies [44] siendo los mutantes *swi3b-1* y *swi3b-2* letales en el embrión de Arabidopsis [214] . Por este motivo se analizó la respuesta a ABA, en crecimiento y germinación, en mutantes de Arabidopsis *swi3b +/-* observándose una reducción de la sensibilidad a ABA y sugiriendo que SWI3B es un regulador positivo de la señalización por ABA. Este efecto se confirmó con los mutantes de TILLING (targeting induced local lesions in genomes) *swi3b-3* y *swi3b-4* que presentaban sustituciones amino-acídicas en el domino SANT de la proteína afectando su función como revelaba un estudio *in silico* [170]. Finalmente observamos que el mutante *swi3b-3* no solo tenía afectada la sensibilidad a ABA en el tejido vegetativo y la semilla sino que estos efectos iban acompañados de alteraciones en la expresión de genes de respuesta a ABA como son *RD29B* y *RAB18,* poniendo de manifiesto que SWI3B está implicada en la expresión génica mediada por ABA. Por otro lado el estudio de la inhibición de la germinación y el establecimiento de plántula inducidos por ABA en un doble mutante *hab1-1 swi3b-3* nos permitió establecer que HAB1 realiza su función aguas arriba de SWI3B en la cascada de señalización por ABA, ya que el doble mutante presenta un fenotipo insensible a ABA al contrario del mutante *hab1-1*. HAB1 modulaba la respuesta a ABA a través de un supuesto complejo remodelador de cromatina SWI/SNF. Para analizar la presencia de HAB1 en la cromatina de la planta y la posible influencia de la regulación por ABA realizamos experimentos de

inmunoprecipitación de cromatina (ChIP), partiendo de la generación de las líneas transgénicas *hab1-1::ProHAB1-HAB1-dHA* que sobre expresan HAB1 marcado con un doble epítopo HA (hemaglutinina) en un fondo genético *hab1-1*. En estos estudios se observó que la presencia de HAB1 era destacada en regiones de los promotores de *RD29B* y *RAB18* cercanas a los elementos de respuesta a ABA como se demostró por RT-PCR cuantitativa a partir de extractos de plantas HAB1-dHA y, que la unión era significativamente mayor que el observado en precipitados del control negativo *hab1-1*. Es interesante resaltar que el tratamiento con ABA eliminaba la presencia de HAB1 en los alrededores de los promotores de *RAB18* y *RD29B*. En esta línea cabe destacar que plantas que sobre expresaban *HAB1* bajo el control de un promotor 35S presentaban un expresión reducida de genes inducibles por ABA de forma contraria al mutante de pérdida de función *hab1-1* que mostraba el efecto a la inversa como habíamos descrito con anterioridad. Por lo tanto HAB1 podría reprimir la transcripción inducida por ABA a través de la interacción directa con la cromatina, inhibición que al parecer desaparece en presencia de ABA (Figura 7).

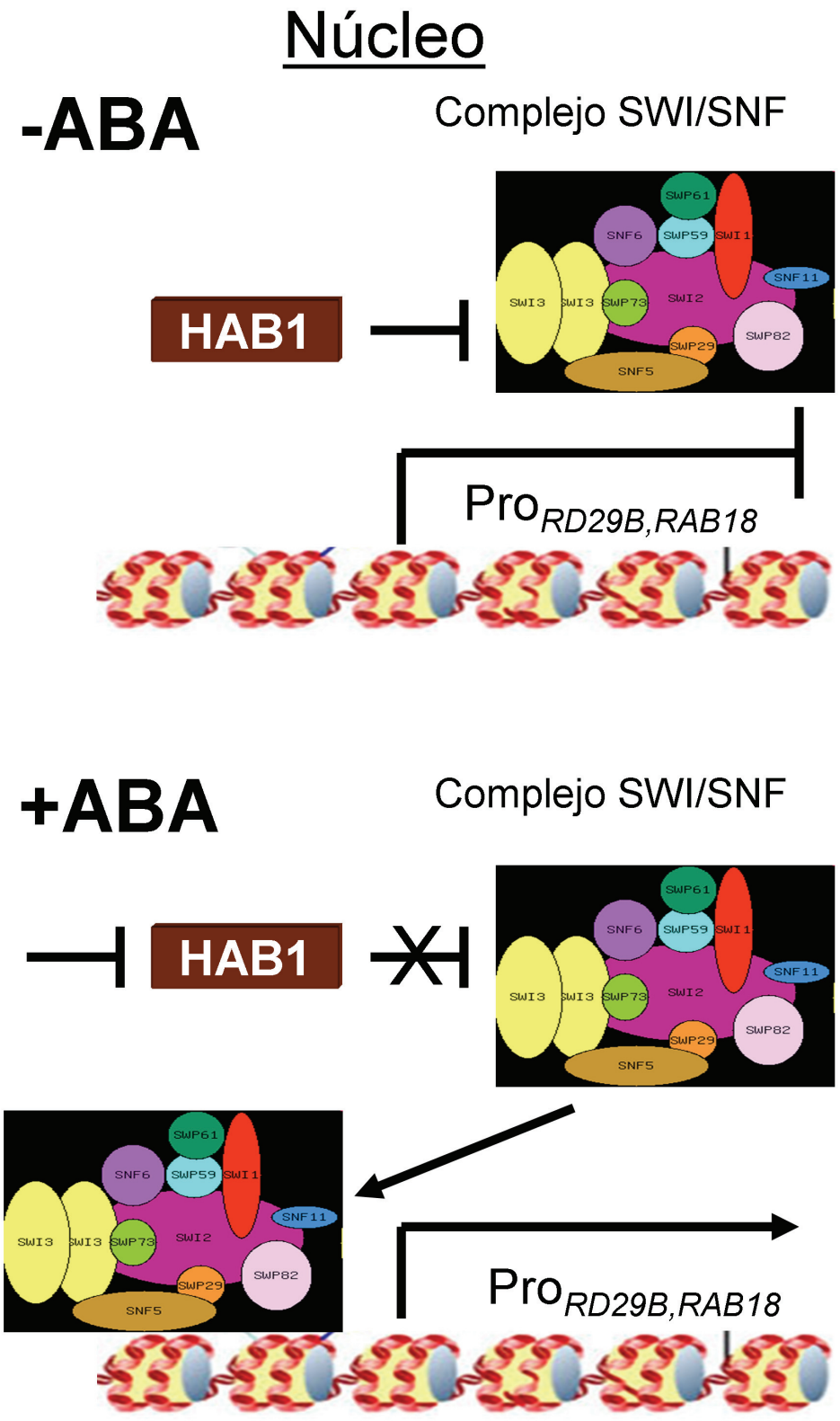


Figura 7. Modelo de la implicación de HAB1, SWI3B y un supuesto complejo SWI/SNF de plantas en la regulación transcripcional en respuesta a ABA sobre el molde de cromatina. Adaptado de Sáez y cols. The Plant Cell. Vol 20. 2972-2988. 2008.

Con estos ensayos hemos profundizado en el conocimiento de los mecanismos moleculares que regulan la señalización por ABA y además hemos descrito una función de una PP2C en el núcleo, hecho que no había sido descrito con anterioridad [37, 71, 153, 178, 272, 275] falta por determinar si otros componentes del complejo SWI/SNF también existen en plantas y son importante en la señalización por ABA.

En este trabajo de tesis se ha descubierto un nuevo interactor de HAB1 que es SWI3B, un supuesto componente del complejo remodelador de la cromatina SWI/SNF, que proporciona un eslabón de unión entre la señalización por ABA y la regulación de la respuesta transcripcional de la planta que tiene lugar en el molde de cromatina [210]

La cromatina permite empaquetar una cantidad de ADN celular muy grande en poco espacio, por medio de la repetición de una unidad, el nucleosoma [95]. El nucleosoma no solo tiene una función estructural, sino que permite la regulación de la transcripción génica. Así pues, del concepto inicial de arquitectura estática se ha pasado a un concepto de alto dinamismo en la estructura de los nucleosomas. Este sistema tan dinámico tiene dos reguladores principales, los remodeladores de cromatina que utilizan la hidrólisis del ATP para cambiar los contactos entre el DNA y las histonas y, las enzimas modificadoras de nucleosomas que añaden o eliminan modificaciones covalentes a determinados residuos de las histonas o del DNA [22, 95, 230]. A estos reguladores hay que añadir proteínas “pasivas” con un papel en la regulación de la transcripción génica [95]. Entre las enzimas modificadoras se incluyen complejos proteicos que acetilan o deacetilan los residuos de Lys presentes en el extremo N-terminal de histonas, histona acetiltransferasas e

histona deacetilasas [95]. Por otro lado, los remodeladores de cromatina dependientes de ATP usan la energía derivada de la hidrólisis del ATP para alterar la accesibilidad al núcleo del ADN del nucleosoma induciendo cambios conformacionales en la interacción entre el octámero de histonas y el ADN, por el desplazamiento temporal del octámero de histonas del ADN, o por cambios en el tipo de histonas que componen el nucleosoma [9, 95]. Por lo tanto, el elemento que es regulado es el ADN que se encuentra alrededor del octámero de histonas en el nucleosoma y que deja de ser accesible a factores de transcripción.

Los complejos remodeladores de cromatina dependientes de ATP se clasifican como SWI2/SNF2 (SWITCH2/SUCROSE NONFERMENTING); ISWI (IMITATION SWITCH), Mi-2/CHD (Mi-2/Chromodomain-Helicase-DNA binding protein), y las subfamilias INO80 [7, 17].

El complejo SWI/SNF de levaduras fue el primero en ser descrito [187]. De manera similar al complejo Swi2/Snf2 de levaduras, el complejo SWI/SNF contiene un núcleo central compuesto por tres polipéptidos, Swi3, Snf5 y Swp73, que son requeridos para el ensamblaje y actividad del complejo [230, 271] (Figura 8).

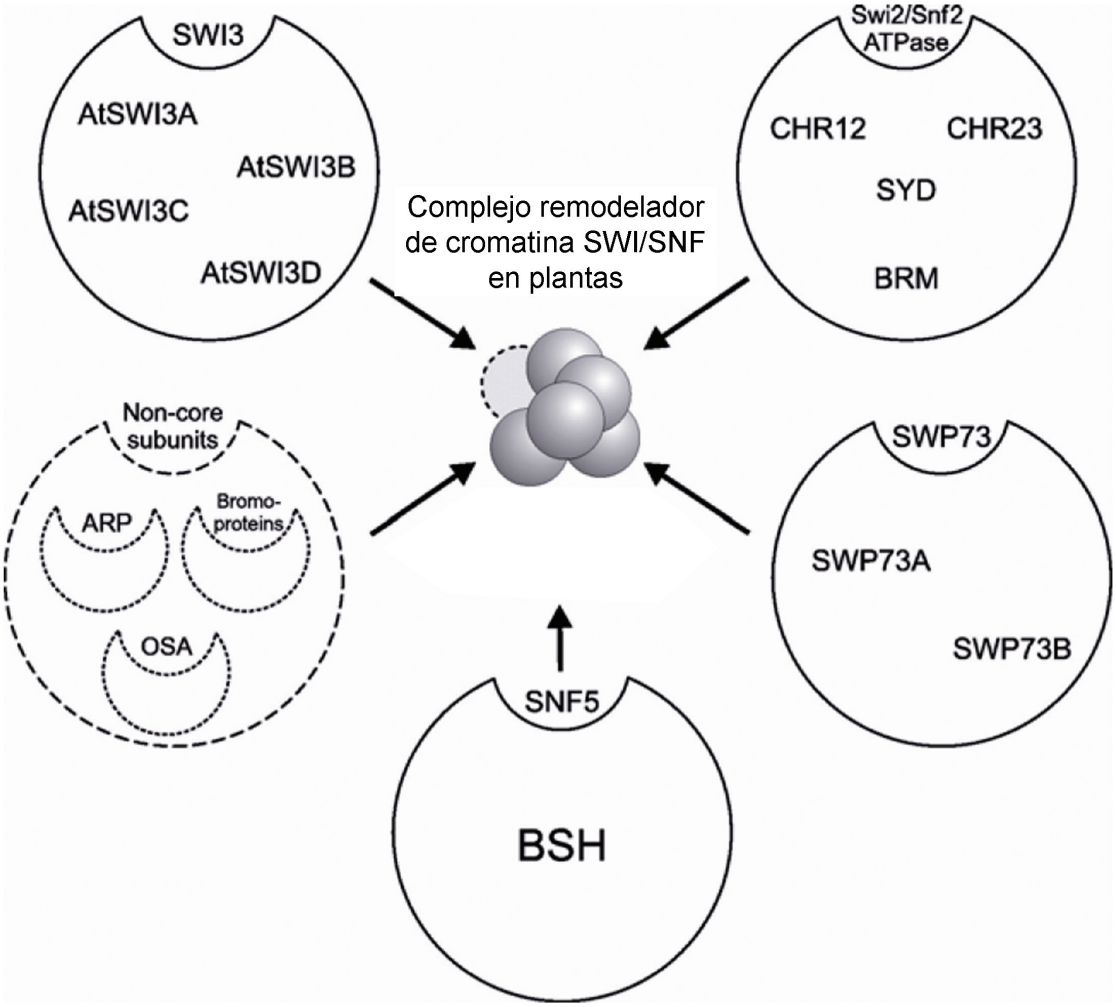


Figura 8. Complejos SWI/SNF en plantas. Los complejos SWI/SNF en Arabidopsis pueden estar formados por una combinación de proteínas identificadas que constituyen el núcleo (círculos con líneas continúas) y un número desconocido de subunidades candidatas auxiliares (círculos con líneas discontinúas). Adaptado de Jerzmanowski. Biochimica et Biophysica Acta. 1769. 330-345. 2007.

En plantas hay un gran número de genes que potencialmente pueden codificar subunidades remodeladoras de cromatina dependientes de ATP [16, 48, 198, 235, 251, 256]. Por ejemplo, en *Arabidopsis thaliana* hay 42 ATPasas similares a SNF2 [108] (Figura 8), que pueden ser clasificadas en diferentes subfamilias y grupos de subfamilias [53]. De estas, 4 pertenecen a la subfamilia canónica SWI2/SNF2 [251]. Estudios genéticos sugieren que la familia de

genes de Arabidopsis SNF2 contiene tanto reguladores negativos como positivos de la transcripción génica [214].

Arabidopsis posee 4 proteínas que pertenecen a la subfamilia Snf2 (Figura 9), BRM (BRAHMA) y SYD (SPLAYED) que son proteínas grandes y CHR12 (CHROMATIN REMODELLING 12) y CHR23 (CHROMATIN REMODELLING 23) que son más pequeñas. Todas ellas presentan los mismos dominios en la zona N-terminal incluyendo un dominio HSA que media interacciones requeridas para la activación transcripcional dependiente de SWI/SNF [247]. Solo BRM tiene un bromodominio en la zona C-terminal que en levaduras une complejos de remodelado a cromatina hiperacetilada [102]. La zona C-terminal de BRM tiene un módulo que interacciona con el ADN y con histonas [48]. No se conoce el papel de la zona C-terminal de SYD pensándose que tiene un efecto negativo en la acumulación de SYD durante el desarrollo [235]. Aunque ni el mutante nulo para *brm* [48, 85] ni el de *syd* [121, 122, 257] son letales en el embrión, el desarrollo del embrión en el doble mutante *brm/syd* se detiene en el estadío de corazón [9] indicando funciones redundantes entre los dos genes en el desarrollo temprano. La diferencia morfológica entre las plantas *brm* y *syd* sugiere que durante las fases finales del desarrollo, BRM y SYD, en general controlan diferentes eventos moleculares. Los mutantes *brm* y *syd* presentan un perfil de expresión de genes solo un 1% diferente con el fenotipo silvestre [9]. Sin embargo hay un número significativo de genes que están afectados de forma coordinada en ambos mutantes sugiriendo que tanto BRM como SYD son necesarios para la correcta expresión de estos genes [9]. Los mutantes de Arabidopsis deficientes en el gen AtSWI3C son prácticamente indistinguibles del mutante *brm* [85]

[214, 243] con la única diferencia del que *brm* es estéril y *atswi3c* no. Además el doble mutante *brm*/*atswi3c* parece y se comporta exactamente como los mutantes *brm* [108] sugiriendo que BRM y AtSWI3C actúan en un único complejo que es el responsable de las mayoría de las funciones biológicas de BRM durante el desarrollo postembrionario [95].

Los mutantes deficientes en CHR12 y CHR23, no presentan defectos fenotípicos visibles [108], aunque la sobre expresión de CHR12 incrementa la detención del crecimiento frente al estrés medioambiental [160]. Teniendo en cuenta todos estas observaciones, BRM y SYD son parcialmente redundantes y pueden compensarse una a la otra, sobre todo en embriogénesis temprana, sin embargo no hay compensación entre las Snf2s CHR12 y CHR23 [108].

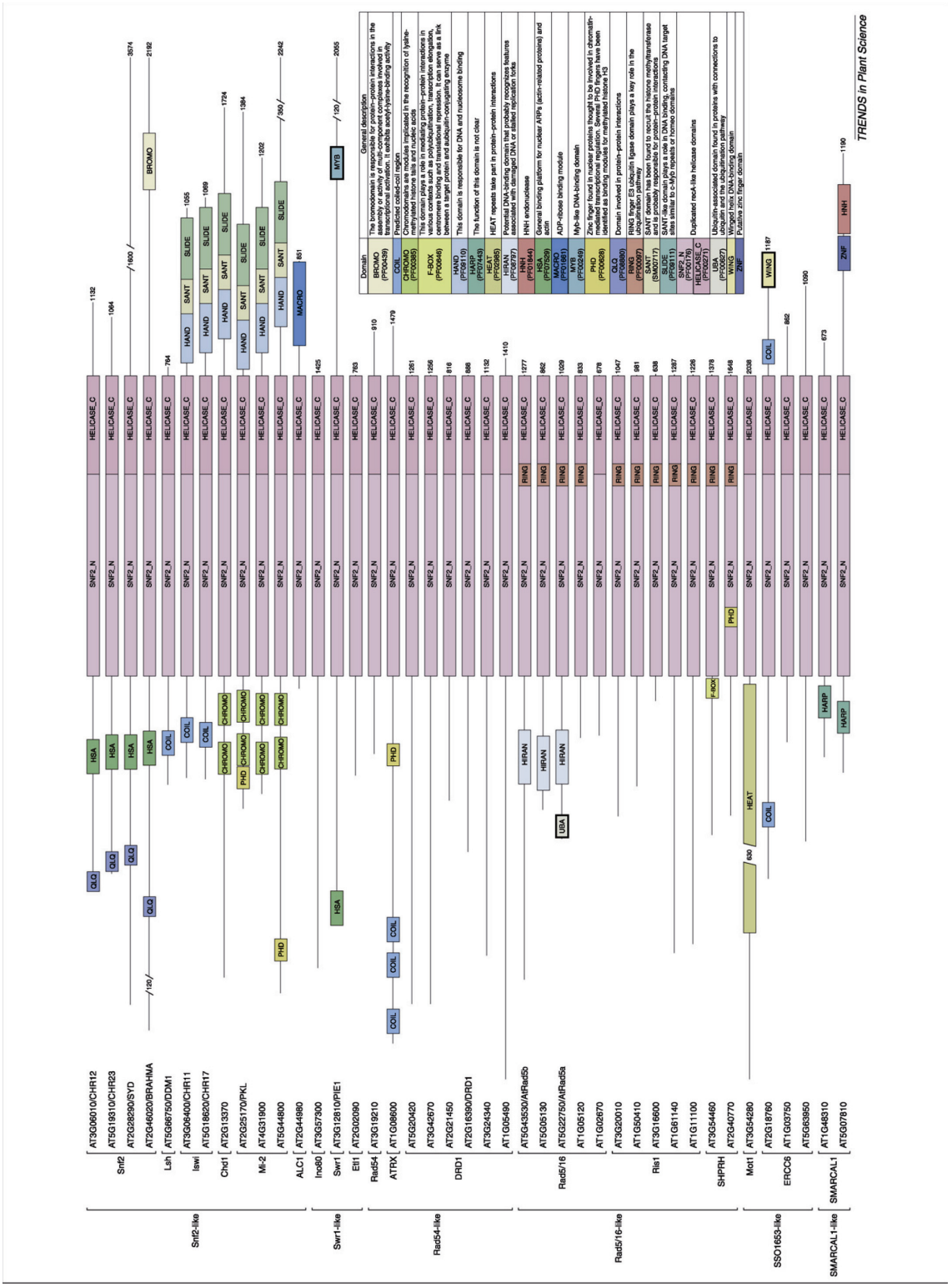


Figura 9. Arquitectura de los dominios de la familia de proteínas de Arabidopsis Snf2. Las proteínas son nombradas de acuerdo a su identificador TAIR (http:ttwww.arabidopsis.org/) /nombres comunes y agrupadas en subfamilias y grupos de acuerdo con la clasificación de miembros de la familia Snf2. Los dominios están dibujados como rectángulos con diferentes colores y las zonas intermedias como líneas, mostrando la longitud de la secuencia total al final del dibujo. Los colores rosa y rojo se han empleado para indicar los dominios con actividad enzimática y los otros colores muestran dominios implicados en unión. Los dominios se identificaron por análisis informático. Knizewski y cols. Trends in Plant Science. (13)10 557-565:2008.

Arabidopsis contiene 4 genes que codifican para homólogos de SWI3 [215] denominados, *AtSWI3A*, *AtSWI3B*, *AtSWI3C* y *AtSWI3D* y que tienen en común con su homólogo en levaduras y ortólogos en Drosophila (Moira), ratón (Srg3) y humano (BAF170 y BAF155) dominios SWIRM, SANT y un dominio de cremallera de leucinas. El dominio SWIRM confiere a la proteína la capacidad de interaccionar con el ADN y los nucleosomas [42] mientras que el dominio SANT (denominado así por presentarlo las proteínas SWI3, ADA2, N-Cor y TFIIIB) confiere a la proteína la capacidad de interaccionar con la cola de histonas no acetiladas [15, 277] y finalmente el dominio rico en cremallera de leucinas que permite a la proteína de oligomerizar o dimerizar con otras proteínas que contengan cremalleras de leucina como muestra la capacidad de homodimerización de las subunidades de SWI3 [29]. Las variantes de SWI3 en Arabidopsis no tienen una gran identidad de secuencia entre ellas sugiriendo que presentan funciones no redundantes o parcialmente solapantes. En este sentido, el análisis de la familia AtSWI3 ha puesto de manifiesto un diversificación funcional de las cuatro variantes muy remarcable [214]. Mientras AtSWI3A y AtSWI3B forman homo y heterodímeros e interaccionan con BSH (BUSHY GROWTH) (el homólogo en Arabidopsis, de SNF5) las proteínas AtSWI3C y AtSWI3D solo pueden unir a AtSWI3B en ensayos de doble híbrido en levadura. Las mutaciones en los genes que codifican para AtSWI3A y AtSWI3B paran el desarrollo del embrión en el estado globular. Por un posible efecto de impronta genética, las mutaciones atswi3b producen la letalidad de la mitad de las macro y microesporas. La mutación del gen que codifica atswi3c provoca una estatura semi-enana, inhibición de la elongación de la raíz, rizado de la hoja, desarrollo aberrante del estambre y fertilidad reducida. Las plantas

que llevan mutaciones atswi3d presentan un enanismo severo, alteraciones en el número y desarrollo de los órganos florales y esterilidad completa tanto femenina como masculina [193]. SNF5 tiene un papel crítico en la organización y función de los complejos SWI/SNF, en levadura son esenciales para el ensamblaje y la correcta unión al promotor de los complejos [61]. La región C-terminal de estas proteínas presenta una región conservada de 200 aminoácidos que media la interacción entre el complejo remodelador y numerosos factores celulares como la ciclina E/CDK2 [286] y represores transcripcionales [142]. En Arabidopsis, solo BSH (At3g17590) presenta una similitud significativa a SNF5. BSH complementa la mutación snf5 en levaduras [18]. La supresión parcial de la expresión de BSH mediante estrategias de RNA antisentido produce un fenotipo pleiotrópico que implica dominancia apical reducida e infertilidad [18] indicando que BSH tiene numerosas funciones fisiológicas. Además no se han descrito o registrado colecciones de mutantes por inserción de T-DNA de BSH lo que podría estar indicando que la haploinsuficiencia de BSH podría alterar completamente la transmisión gametofítica de un alelo inactivado.

Ya que las proteínas BSH y ATSWI3 pueden formar complejos SWI/SNF con diferentes subunidades SWI/SNF2, se predice que las mutaciones que afecten a *BSH* y *SWI3* causarán defectos más severos que las de los genes de las subunidades SWI2/SNF2. De hecho, el silenciamiento parcial de BSH en plántula produce defectos pleiotrópicos que incluyen infertilidad y reducción de la dominancia apical [18] mientras que el silenciamiento similar de *ATSWI3B/CHB2* produce enanismo, retraso en la floración, alteraciones en la plántula y el desarrollo de la hoja [284].

Varios trabajos muestran que hay factores, que modifican la cromatina, implicados en la señalización por ABA, [231, 232] sin embargo teniendo en cuenta el profundo impacto del ABA en la regulación de la expresión génica y la gran cantidad de factores de transcripción implicados en este proceso se puede predecir que todavía hay por descubrir muchos factores que modifican la cromatina y que están implicados en la señalización por ABA.

Los datos aportados en este trabajo muestran los pasos finales de la cascada de señalización por ABA, esquema que se completa con los resultados recientemente publicados que muestran datos sobre la parte inicial de la cascada de señalización por ABA incluyendo el descubrimiento y la caracterización de un receptor de ABA denominado PYR/PYL/RCAR, que inhiben la actividad de las enzimas fosfatasas (PP2C) asociadas a la respuesta del ABA [31, 54, 72, 105, 149, 159, 171, 172, 184, 213, 237, 273].

En la tesis que presentamos se aportan experimentos que ayudan a resolver el controvertido papel de ABI1 en la señalización por ABA. Para ello realizamos la identificación y caracterización de dos nuevos alelos recesivos de ABI1, *abi1-2* y *abi1-3* y de ABI2, *abi2-2*, además de la generación posterior de dobles mutantes *hab1-1abi1-2* y *hab1-1abi1-3*, *abi1-2abi2-2*. Los mutantes sencillos *abi1-2 y abi1-3* muestran mayor sensibilidad a ABA en ensayos de crecimiento y germinación en comparación con el fenotipo silvestre Columbia, lo que se corresponde con lo descrito en la literatura para los mutantes revertientes a*bi1-R1* a *abi1-R7.* Además los mutantes *abi1-2 y abi1-3* presentan hipersensibilidad en el cierre de estomas inducido por ABA, en este caso, al contrario de lo que ocurría en el alelo recesivo *abi1-1R4* que mostraba un fenotipo igual al de la planta silvestre en respuesta a concentraciones de ABA

de 100nM [150]. Esta última observación puede ser debida a las diferencias de fondo genético de cada mutante o bien a que *abi-1R4* no es un alelo nulo, algo que no ocurre en el caso de los mutantes de pérdida de función por inserción de T-DNA que hemos caracterizado en esta memoria. En ensayos de pérdida de agua en hojas cortadas en los mutantes *abi1-2* y *abi1-3* no encontramos diferencias significativas con el fenotipo silvestre. Esto puede ser debido a que en los ensayos de pérdida de agua en hojas cortadas reflejan distintos grados de diferencias en la apertura de estomas entre los mutantes y el silvestre, al principio de los experimentos de sequía más que, más tarde en los eventos de señalización inducida por marchitamiento [115]. Sin embargo en experimentos de sequía realizado en plantas intactas, *abi1-2* y *abi1-3* mostraban una reducción en la pérdida de agua en comparación con el fenotipo silvestre. Del mismo modo, cuando estudiamos la expresión de genes de respuesta a ABA había una inducción mayor aunque modesta, de 1.5 a 3 veces respecto al fenotipo silvestre. También hemos identificado y caracterizado un mutante de T-DNA de la colección Salk para *ABI2* al que denominamos *abi2-2*, que mostraba respuestas similares al fenotipo silvestre en ensayos de inhibición de la germinación de la semilla y crecimiento así como en el cierre de estomas. Generamos un doble mutante *abi1-2 abi2-2* y encontramos que presenta una sensibilidad mayor a ABA en inhibición de la germinación y crecimiento que ambos mutantes sencillos parentales.

En la señalización por ABA juegan un papel fundamental las proteínas HAB1, ABI1, ABI2 y PP2CA como reguladores negativos en respuestas de la planta tanto en semillas como en tejido vegetativo y en respuestas frente al estrés. Para desentrañar posibles interacciones genéticas y redundancia

funcional entre ellas, se identifica y se caracteriza en diferentes tipos de ensayos, un colección de mutantes sencillos, dobles triples (Tabla 1). Esta serie de mutantes obtenidos por inactivación progresiva de los genes correspondientes a las PP2Cs, nos muestran diferentes grados crecientes de hipersensibilidad a ABA. Esta metodología de modulación de la respuesta a ABA podría utilizarse para modular la respuesta a ABA en cultivos de interés agrícola con la inactivación de genes ortólogos. En esta memoria se demuestra además que podemos conferir mayor tolerancia a sequía inactivando dos genes PP2C que participen en la ruta de señalización por ABA. Otra de las tecnologías es el silenciamiento en plantas mediante la utilización de pequeños RNAs y micro RNAs [180]. No obstante hay que tener en cuenta que la inactivación combinada de tres PP2Cs en los mutantes triples resultan en una respuesta parcial constitutiva a ABA aún en ausencia de estrés, ya que presentan penalización en el crecimiento.

Estudiando las diferentes combinaciones de pérdida de función en PP2Cs hemos comprobado que existe interacción genética entre las dos ramas del grupo A de las PP2Cs, la rama representada por ABI1/HAB1 y la representada por PP2CA/AHG3, que es la respuesta a una de las preguntas que nos planteábamos al realizar este abordaje. Hemos comprobado cómo a pesar de que PP2Ca estaba caracterizada como el principal regulador de la señalización por ABA en semillas, también desempeña una función en la regulación de la pérdida de agua en tejido vegetativo. Algo que ocurre en los mutantes dobles con pp2ca y no se había determinado en el mutante sencillo [115].. Del mismo modo que se observa mayor sensibilidad a la inhibición de la germinación y a la inhibición del crecimiento de raíz en los mutantes dobles

hab1-1pp2c-1a y *abi1-2pp2ca-1* que en el sencillo *pp2ca-1.* De esta forma podemos confirmar que PP2CA es un regulador negativo de la señalización por ABA tanto en semillas como en tejido vegetativo [115]. Los fenotipos de los diferentes mutantes con los que hemos trabajado sugieren que PP2C proporciona un umbral de regulación negativa que se requiere para una normal respuesta de ABA. Si ABI1/HAB1 y PP2CA/AHG3 se regularan de forma independiente en la señalización por ABA, la pérdida de de una de estas dos ramas originaría un desequilibrio llevando a una hipersensibilidad por ABA. También es posible que la señalización por ABA esté regulada de forma precisa a través de un mecanismo que involucre un efecto dependiente de la dosis de PP2C, como observamos en el progresivo incremento en la sensibilidad a ABA en el crecimiento de raíz que ocurre en el mutante sencillo, doble y triple en *pp2c.*

También constatamos que deben existir dos ramas que regulan negativamente la señalización por ABA en semillas, ya que el mutante *hab1-1abi1-2abi2-2* muestra una sensibilidad en ensayos de inhibición de la germinación del orden de nanomolar, algo que llama la atención si pensamos que en ese mutante aún siguen funcionales las PP2Cs PP2CA/AHG3 y AHG1.

Los mutantes triples muestran una respuesta parcial constitutiva a ABA en los distintos ensayos que hemos realizado, como son crecimiento de raíz y apertura de estomas y en expresión de genes inducibles por ABA en ausencia de ABA en todos los casos. En un análisis del transcriptoma completo observamos que en el genotipo silvestre el 15% de los genes están inducidos y el 25% están reprimidos por ABA, mientras que en los triples mutantes están

constitutivamente inducidos o reprimidos en ausencia de ABA. De esta forma se podría explicar las alteraciones en crecimiento que tienen estos mutantes.

En este trabajo caracterizamos los dobles mutantes *hab1-1abi1-2* y *hab1-1abi1-3* a partir de los mutantes parentales sencillos. No existen estudios previos a este trabajo que caractericen dobles mutantes de pérdida de función en PP2Cs por inserción de T-DNA. Si se había descrito un doble *abi1-R4abi2-R1* que presenta una mayor respuesta a ABA que los mutantes parentales sencillos [150]. La inactivación combinada de *HAB1* y de *ABI1* en los dobles mutantes *hab1-1abi1-2* y *hab1-1abi1-3* lleva a una mayor hipersensibilidad a ABA respecto a los mutantes parentales sencillos. Presentan mayor inhibición de la germinación en presencia de ABA y mayor inhibición en el establecimiento de plántula en condiciones de estrés osmótico. El ABA tiene un papel clave en la coordinación de diferentes aspectos de la respuesta por estrés hídrico mediando en la supervivencia de la planta, en semillas y en plántulas el ABA actúa previniendo la germinación y detiene crecimiento. En los dobles mutantes que generamos lo que esperábamos es eliminar el freno que suponen HAB1 y ABI1 para las respuestas mediadas por ABA para hacer frente al estrés, eliminando la cooperación negativa que ejercen estas proteínas en los estadíos del desarrollo de semilla y plántulas.

En distintos ensayos realizados en los dobles mutantes *hab1-1abi1-2* y *hab1-1abi1-3* observamos un reforzamiento mediado por ABA de la inhibición de la germinación de la semilla y las respuestas que se daban en tejido vegetativo mediadas por ABA estaban más inducidas en los mutantes dobles que en los sencillos. Por otra parte, la inhibición del crecimiento en presencia de ABA era dramática en los mutantes dobles, además de que la pérdida de

agua por transpiración también está significativamente reducida en los mutantes dobles con respecto a los sencillos, tanto en ensayos con hojas cortadas como en los ensayos de sequía sin riego en plantas. En cuanto a la expresión de genes de respuesta a ABA y particularmente en los genes de respuesta a estrés como *RAB18*, *RD29B* y *P5CS1,* en los dobles mutantes había una inducción mayor de estos genes que en los mutantes parentales sencillos. Todos estos resultados sugieren que las funciones de HAB1 y ABI1 como reguladores negativos de la señalización por ABA se solapan parcialmente y, revelan una modulación de la respuesta a ABA a partir de la inactivación combinada de estos genes.

La ruta de biosíntesis y de señalización de ABA están consideradas como rutas compuestas por potenciales dianas para la mejora de plantas en condiciones de estrés. Está descrito que plantas transgénicas con mayores niveles de ABA tienen una mejor respuesta en condiciones de estrés [90, 191], al igual que el uso de compuestos que aceleran la acumulación de ABA [94]. Por otro lado, a pesar de que se han descrito muchos mutantes hipersensibles a ABA, no se ha relacionado bien la hipersensibilidad a ABA con la tolerancia a la sequía [50]. Se han identificado mutantes con una respuesta mejorada en ABA que si mostraban una reducción en el consumo de agua, como el caso de *era1, abh1, gcr1* [84, 182, 185] y mutantes en los que aunque había una hipersensibilidad a ABA no mostraban una reducción en el consumo de agua [267, 268]. Los mutantes hipersensibles a ABA que comparten un reducción en el consumo de agua se caracterizan por tener reforzada la respuesta a ABA en estomas y una reducción de la pérdida de agua, es el caso de *hab1-1abi1-2* y *hab1-1abi1-3* y de *era1, abh1, gcr1* (Tabla 1).

Basándonos en los resultados presentados, sugerimos que el silenciamiento en especies de cultivo de genes de PP2Cs con funciones similares a ABI1 y HAB1 podría ser una herramienta biotecnológica para mejorar los mecanismos de tolerancia a sequía.

Mutante	**Fenotipo**	**Gen**
abh1 (r)	(H) Semilla y cierre estomatal	Nuclear cap-binding protein
abi1-1 (d)	(I) Semilla, cierre estomatal y tejido vegetativo.	PP2C Gly180Asp
abi1-1R1-7 intragenic revertant (r)	(H) Semilla y crecimiento	PP2C Gly180Asp + segunda mutación
abi1-2 (r)	(H) Semilla, crecimiento y cierre estomatal	PP2C
abi1-2 pp2ca-1 (r)	(H) Semilla, crecimiento y cierre estomatal	PP2C
abi1-2abi2-2 (r)	(H) Semilla, crecimiento y cierre estomatal	PP2C
abi1-3 (r)	(H) Semilla, crecimiento y cierre estomatal	PP2C
abi2-1 (d)	(I) Semilla y tejido vegetativo	PP2C Gly168Asp
abi2-1R1 revertiente intragénico (r)	(H) en el doble mutante *abi1-1R4 abi2-1R1*	PP2C Gly168Asp + Glu186Lys
abi2-2	Fenotipo silvestre	PP2C
abi3 (r)	(I) Semilla	TF B3 domain
abi4 (r)	(I) Semilla	TF AP2 domain
abi5 (r)	(I) Semilla	TF bZIP domain
abi8 (r)	(I) Semilla y tejido vegetativo	Función desconocida
ahg1 (r)	(H) Semilla	PP2C
era1 (r)	(H) Semilla y cierre estomatal	Farnesil transferasa
fry1 (r)	(H) *RD29A:LUC* Superinducción en semilla	Inositol polyphosphate 1-phosphatasa
gcr1 (r)	(H) en crecimiento, cierre estomatal y expresión génica	Receptor acoplado a proteínas G
gpa1 (r)	(H) Semilla, inhibición del cierre estomatal	Subunidad α de una proteína unida a GTP heterotrimérica
gtg1gtg2 (r)	(I) Semilla, crecimiento, cierre estomatal	GPCR-type G proteins
hab1 (r)	(H) Semilla, crecimiento y cierre estomatal	PP2C
hab1-1 abi1-2 abi2-2 (r)	(H) Semilla, crecimiento y cierre estomatal	PP2C
hab1-1 abi1-2 pp2ca-1 (r)	(H) Semilla, crecimiento y cierre estomatal	PP2C
hab1-1 abi1-2(r)	(H) Semilla, crecimiento y cierre estomatal	PP2C
hab1-1 pp2ca-1 (r)	(H) Semilla, crecimiento y cierre estomatal	PP2C
hab1-1 swi3b-3	(I) Semilla, crecimiento y expresión génica	PP2C, SWI
hyl1 (r)	(H) Semilla y crecimiento	Nuclear dsRNA binding protein implicada en procesamiento de mRNA.
ost (r)*1*	(I) Cierre estomatal	PK (SnRK2)

pldα1 (r)	(I) cierre estomatal, inhibición de apertura estomatal	Fosfolipasa Dα1
pp2ac-2 (r)	(H) Semilla y tejido vegetativo	subunidad catalítica de PP2A
pp2ca/ahg3 (r)	(H) Semilla, crecimiento y cierre estomatal	PP2C
pp2ca-1 (r)	(H) Semilla, crecimiento y cierre estomatal	PP2C
rboh D/F (r)	(I) Semilla, crecimiento y cierre estomatal	NADPH oxidasa (producción de ROS)
rcn1 (r)	(I) Semilla y cierre estomatal	subunidad reguladora de PP2A
rop10 (r)	(H) Semilla, crecimientoy cierre estomatal	GTPasa pequeña de membrana plasmática
rpk1 (r)	(I) Semilla, crecimiento, cierre estomatal y expresión génica	leucine-rich receptor-like kinase
sad1 (r)	*(H)RD29A:LUC* Superinducción. Semilla y crecimiento. Defectivo en biosíntesis e inducción de ABA	Sm-like snRNP proteins (mRNA processing)
snrk2.2 snrk2.3 (r)	(I) Semilla, germinación, inhibición crecimiento de la raiz	PK (SnRK2)
snrk2.2 snrk2.3 snrk2.6 (r)	(I) Semilla, crecimiento y expresión génica	PK (SnRK2)
swi3b-1 +/-	(I) Semilla, crecimiento y expresión génica	SWI
swi3b-2 +/-	(I) Semilla, crecimiento y expresión génica	SWI
swi3b-3	(I) Semilla, crecimiento y expresión génica	SWI Asp-245-Asn
swi3b-4	(I) Semilla, crecimiento y expresión génica	SWI Ser-264-Phe

Tabla 1. Mutantes de *Arabidopsis thaliana* que presentan alteraciones en la respuesta a ácido abscisico. (d) dominante. (r) recesivo. (I) insensible a ABA (H) hipersensible a ABA. Sombreado: mutantes generados y caracterizados en esta tesis.

En el Informe sobre el Desarrollo Mundial de 2008 del Banco Mundial se muestra claramente que la agricultura puede contribuir considerablemente al desarrollo económico y la reducción de la pobreza en los países menos adelantados. Aunque esta función se reduce considerablemente en los países de ingresos medios, en ellos la agricultura sigue desempeñando un papel importante en relación con la reducción de la pobreza, que sigue siendo desproporcionadamente rural, a pesar de la cada vez menor importancia relativa de la agricultura en las economías nacionales. El problema de la inversión insuficiente en agricultura se complica en las épocas de agitación económica, como la actual crisis económica mundial.

El cambio climático y el continuo deterioro de las tierras cultivables junto con el incremento de la población mundial, representan una seria amenaza para la producción mundial agrícola que debería garantizar el alimento a una población mundial que según la Organización de las Naciones Unidas para la Agricultura y la Alimentación (FAO), se estima alcanzará los 9100 millones de personas en el 2050. La producción agrícola y alimentaria de muchos países en desarrollo puede verse afectada negativamente, en especial en los países de bajos ingresos y un índice elevado de hambre y pobreza y que son ya muy vulnerables a la sequía, las inundaciones y los ciclones.

La sequía está clasificada como la causa más común de la grave escasez de alimentos en los países en desarrollo. África tiene la mayor tasa de prevalencia de hambre, considerándose el continente más seco después de Oceanía. Sólo en Etiopía, más de 57 millones de personas se han visto afectadas por la sequía en los últimos 30 años. Los pequeños campesinos necesitan acceso a semillas de alta calidad, fertilizantes, abonos y tecnologías

para poder impulsar la producción y la productividad. En esta línea de trabajo está la mejora de la tolerancia de las principales especies de cultivo al estrés abiótico, como por ejemplo el arroz *(Oryza sativa L.)*. El desarrollo de nuevos cultivos con mejor tolerancia al estrés podría tener sin duda un importante efecto sobre la producción global mundial. Los abordajes de ingeniería genética ofrecen una atractiva alternativa a las técnicas convencional de mejora (Figura 10).

Por ello en esta memoria mostramos que la inactivación combinada de dos fosfatasas con relevancia en la ruta de transducción de ABA como son las combinaciones de HAB1/ABI1, ABI1/ABI2, ABI1/PP2CA y HAB1/PP2CA, genera tolerancia a sequía en la especie modelo *Arabidopsis thaliana*. Esta aportación biotecnológica podría ser aplicable en genes ortólogos o de funciones similares para la mejora de los mecanismos de respuesta a sequía en especies de cultivo complementando los métodos tradicionales de mejora y la variedad genética.

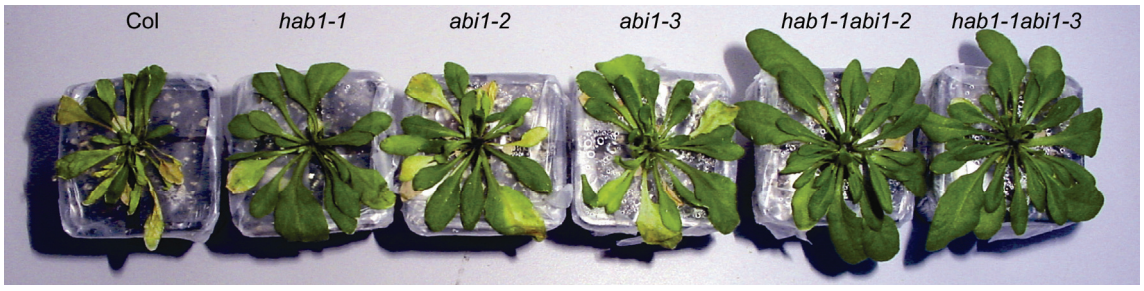
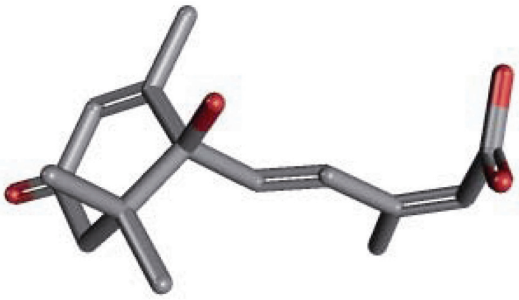


Figura 10. Mejora de la tolerancia a sequía en los dobles mutantes *hab1-1 abi1-2* y *hab1-1 abi1-3* con respecto al silvestre (Col) y a los mutantes parentales sencillos *hab1-1*, *abi1-2* y *abi1-3*.



Conclusiones

La PP2C HAB1 es un regulador negativo de la señalización por ABA, esto se demuestra con el fenotipo de hipersensibilidad a ABA en semillas del mutante recesivo de inserción de T-DNA *hab1-1* junto con la reducida sensibilidad a ABA de las plantas 3*5S:HAB1.*

HAB1 interacciona con SWI3B una proteína de *Arabidopsis thaliana* homóloga a la subunidad SWI3B del complejo remodelador de cromatina SWI/SNF de levaduras.

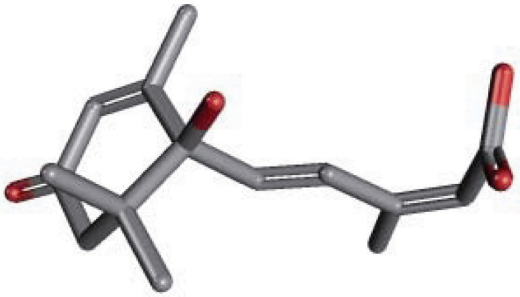
SWI3B es un nuevo regulador positivo de la señalización por ABA.

HAB1 modula la respuesta a ABA a través de la regulación de un supuesto complejo remodelador de cromatina SWI/SNF.

La inactivación combinada de las PP2Cs del grupo A HAB1 y ABI1 refuerza la sensibilidad a ABA y provoca la reducción del consumo de agua en *Arabidopsis thaliana*.

La inactivación progresiva de las PP2Cs del grupo A HAB1, ABI1, ABI2 y PP2CA/AHG3 provoca una modulación en la hipersensibilidad a ABA. La respuesta a ABA tanto en condiciones de estrés como en el crecimiento normal de la planta está regulada de forma sutil por la acción integrada de estos genes.

La modulación de la sensibilidad a ABA obtenida en *Arabidopsis thaliana* a través de la inactivación genética de los genes de PP2Cs del grupo A podría ser una aplicación biotecnológica, una vez se identifiquen los genes ortólogos en especies de interés agronómico.



Abreviaturas

a
AAOS. Aldehído abscísico oxidasa.
AAPK. ABA ACTIVATED SERIN-THREONINE PROTEIN KINASE.
ABA. Ácido Abscísico.
ABAP1 ARMADILLO BTB ARABIDOPSIS PROTEIN 1.
ABF2. ABSCISIC ACID RESPONSIVE ELEMENT-BINDING FACTOR 2.
ABH1. ABA HYPERSENSITIVE 1.
ABI1 y 2. ABA-INSENSITIVE 1 y 2.
ABI3/VP1. ABA INSENSITIVE 3/VIVIPAROUS1.
ABO1/ELO2. ABA-OVERLY SENSITIVE 1/ Fatty acyl-CoA elongase.
ABREs. ABA Response Elements. Elementos de respuesta a ABA.
ADN Acido desoxirribonucleico.
AHG1 y 2. ABA-HYPERSENSITIVE AT GERMINATION 1 y 2.
AKT1. ARABIDOPSIS K TRANSPORTER 1.
AREB. ABA-RESPONSIVE ELEMENT BINDING PROTEIN.
ARFs. Auxin response factors. Factores de respuesta a auxinas.
ARN. Ácido ribonucleico.

b
BiFC. Complementación bimolecular de la fluorescencia.
bHLH. basic helix-loop-helix family.
BRM. BRAHMA.
BUSHY/BSH. BUSHY GROWTH.
BY2. Bright Yellow 2
bZIP. cremallera básica de leucinas.

c
CBL1. CALCINEURIN B-LIKE PROTEIN 1.
CEs. Coupling Elements.
ChlH/ABAR. Mg Chelatase subunit/ ABA-RESPONSIVE PROTEIN.
ChIP. Coinmunoprecipitación de cromatina.
CHR12. CHROMATIN REMODELLING 12.
CHR23. CHROMATIN REMODELLING 23.
CIPK15/PKS3/ SnRK3.1. CBL-INTERACTING PROTEIN KINASE.
CPL3. C-TERMINAL DOMAIN PHOSPHATASE-LIKE 3.

d
DMAPP. Dimeltilalil Pirofosfato.
DRE. Drought Response Element. Elemento de respuesta a sequía.

e
f
FAD. dinucleótido de flavina-adenina.
FCA1. putative cytosine deaminase.

g
GFP. GREEN FLUORESCENT PROTEIN
GPA.1 GTP-BINDING ALPHA SUBUNIT GCR1.
GPCR. G PROTEIN COUPLED RECEPTOR.
GPR98/GPHR. G protein-coupled receptor 89b

GTG1 y GTG2. GPCR-TYPE G PROTEIN 1 and 2.
.

h
HAB1. HYPERSENSITIVE TO ABA1/HOMOLOGY TO ABI1.
HYL1. HYPONASTIC LEAVES 1.

i
IPP. Isopentenil pirofosfato.
ISWI: IMITATION SWITCH.

j
k
KAPP KINASE ASSOCIATED PROTEIN PHOSPHATASE.
KEG. RING E3 LIGASE KEEP ON GOING.

l
LanC. lantibiotic synthetase component C.
LCYB. ciclasa β de licopeno.
LEA. DEHYDRIN LEA. LATE EMBRYOGENESIS ABUNDANT.

m
MAPK6 Mitogen-activated protein kinase.
MEP. metileritritol fosfato.
Mi-2/CHD. Mi-2/Chromodomain-Helicase-DNA binding protein.
MoCo. cofactor de molibdeno.

n
NAP1. NUCLEOSOME ASSEMBLY PROTEIN 1.
NCED. 9-cis-epoxicarotenoide dioxigenasa.
NSY. neoxantina sintasa.

o
OST1/SnRK2.6. OPEN STOMATA 1.

p
PDR12. Pleiotropic drug resistant transporter.
PHK. phosphorylase kinase fosforilasa kinasa.
PLD. phospholipase D alpha.
PP2A. PROTEIN PHOSPHATASE 2 A.
PP2C. PROTEÍNAS FOSFATASAS DE TIPO 2C.
PP2B. PROTEIN PHOSPHATASE 2 B.
PYL. PYRABACTIN RESISTANCE 1-LIKE.
PYR. PYRABACTIN RESISTANCE 1.

q
r
RCAR. REGULATORY COMPONENT OF ABA RECEPTOR 1.
ROS. Especies reactivas de oxígeno.
RPK1. RECEPTOR-LIKE PROTEIN KINASE 1.

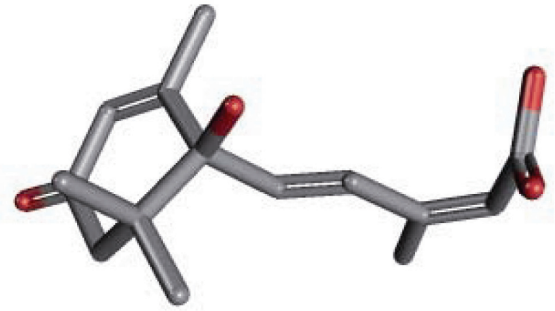
RCN1. ROOTS CURL IN NPA.

s
SAD2. SUPERSENSITIVE TO ABA AND DROUGHT 2.
SAL1/FIERY1 3'(2'),5'-bisphosphate nucleotidase/ inositol or phosphatidylinositol phosphatase.
SANT dominio proteico (denominado así por presentarlo las proteínas SWI3, ADA2, N-Cor y TFIIIB).
SIZ1. SUMO E3 ligase.
SnRK2. SNF1-RELATED PROTEIN KINASE 2.
snRNP. U1 small nuclear ribonucleoprotein. pequeña ribonucleoproteina nuclear.
SOS2/SnRK3.11. SALT OVERLY SENSITIVE.
SYD. SPLAYED.
STRS1 y 2. STRESS RESPONSE SUPPRESSOR 1 y 2.
SWI/SNF. SWITCH2/SUCROSE NONFERMENTING.

t
u
UV. Ultravioleta

v
w
x
y
z
ZEP. zeaxantina epoxidasa.

.



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