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La cisteína y su contribución a diferentes procesos de señalización en *Arabidopsis thaliana*

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Índice

ABREVIATURAS

ABA	Ácido Abscísico
ADP	Adenosín-5'-difosfato
AMP	Adenosin-5'-monofosfato
APS	Adenosin-5'-fosfosulfato
ATP	Adenosin-5'-trifosfato
BR	Brasinosteroides
CAS	β-Cianoalanina sintasa
CC	*Coiled coil*
CK	Citoquininas
CoA	Coenzima A
Cys	Cisteína
DES	L-Cys desulfhidrasa
EDS1	*Enhanced Disease Susceptibility 1*
ET	Etileno
ETI	*Effector-Triggered Immunity*
ETRs	Receptores de etileno asociados al retículo endoplasmático
Fd_{ox}	Ferredoxina oxidada
Fd_{red}	Ferredoxina reducida
GA	Giberelina
γ-EC	γ-Glutamilcisteína
γ-ECS	γ-Glutamilcisteinil sintetasa
GGT4	γ-glutamil transpeptidasa 4
GS	Glutatión Sintetasa
GSH	Glutatión reducido
GSSG	Glutatión oxidado
GST	Glutatión S-Transferasa
HR	Respuesta Hipersensible
JA	Ácido jasmónico
LRR	Repeticiones ricas en leucina
MoCo	Cofactor de molibdeno
NADH	Nicotinamida-adenina-dinucleótido-fosfato
NDR1	*Non-race-specific Disease Resistance 1*
NPR1	*Nonexpressor of Pathogenesis Genes 1*
OAS	O-Acetilserina
OASTL	O-Acetilserina(tiol)liasa
PAD4	*Phytoalexin Deficient 4*
PAMPs	*Pathogen-Associated Molecular Patterns*
PAPS	3'-Fosfoadenosín-5'-fosfosulfato

PC	Fitoquelatina
PCS	Fitoquelatina Sintasa
PE	Fosfatidiletanolamina
PI3K	Fosfatidilinositol 3-quinasa
PI3P	Fosfatidilinositol 3-fosfato
PLP	Piridoxal-5´-fosfato
PPi	Pirofosfato inorgánico
PTI	*PAMP-Triggered Immunity*
PR	*Pathogenesis-Related*
PRRs	*Pattern Recognition Receptors*
Rboh	*Respiratory Burst Oxidase Homolog*
RCBs	*Rubisco-Containing Bodies*
ROS	Especies reactivas del oxígeno
SA	Ácido salicílico
SABP2	*SA-Binding Proteína 2*
SAGs	Genes Asociados a la Senescencia
SAR	Resistencia Sistémica Adquirida
SAT	Serina acetiltransferasa
SAV	Vacuolas Asociadas a la Senescencia
SCF	*Skp-Cullin-F-box*
SDCs	*Sulfur-containing Defence Compounds*
SED	*Sulfur-Enhanced Defense*
SIR	*Sulfur-Induced Resistance*
SURE	*Sulfur-Responsive Element*
TIR	*Toll/ Interleucina-1*
TMV	Virus del Mosaico del Tabaco
TOR	*Target of Rapamycin*

Resumen

La cisteína es la primera molécula sintetizada en plantas que contiene azufre reducido y ocupa una posición central en el metabolismo debido a sus funciones bioquímicas. Las células de *Arabidopsis thaliana* contienen diferentes enzimas O-Acetilserina(tiol)liasas (OASTL) que catalizan la biosíntesis de cisteína y están localizadas en el citosol, plastidios y mitocondrias, para dar lugar a múltiples conjuntos subcelulares de cisteína. Se ha avanzado mucho en el estudio de las OASTLs más abundantes, sin embargo, hasta ahora, la información sobre las menos abundantes es escasa. En el primer capítulo de esta tesis se establece la reacción enzimática catalizada por la isoforma minoritaria citosólica OASTL CS-LIKE (AT5G28030). Para ello, expresamos esta enzima en bacterias y caracterizamos la proteína recombinante purificada. Nuestros resultados demuestran que CS-LIKE cataliza la desulfuración de L-cisteína a sulfuro, amonio y piruvato. Por tanto, CS-LIKE es una nueva L-Cisteína desulfhidrasa localizada en el citoplasma y hemos propuesto que se designe DES1. El impacto y la funcionalidad de DES1 en el metabolismo de la cisteína se pone de manifiesto por el fenotipo de los mutantes insercionales de T-DNA *des1-1* y *des1-2*. Los resultados obtenidos nos permiten proponer que DES1 es una L-Cisteína desulfhidrasa involucrada en mantener la homeostasis de cisteína en el citosol, principalmente durante los estadíos más tardíos del desarrollo o bajo perturbaciones ambientales.

En el segundo capítulo de esta tesis se describe ampliamente el fenotipo de senescencia prematura de los mutantes *des1*, observándose un incremento de la expresión de los genes asociados a la senescencia, de otros marcadores como vacuolas asociadas a la senescencia (SAV) y una alteración del perfil transcripcional. Además se ha observado en ambos mutantes una clara inducción del proceso de autofagia mediante la acumulación de la proteína ATG8 y fundamentalmente de su forma conjugada. Puesto que la ausencia de DES1 en estos mutantes provoca una reducción en los niveles endógenos de sulfuro, hemos observado que tratamientos con H_2S revierten la presencia de vacuolas asociadas a la senescencia, así como la inducción de la autofagia. Esta reversión de la autofagia por sulfuro se observa también en plantas sometidas a limitación por carbono. A nivel transcripcional también observamos que el sulfuro reduce considerablemente la alteración transcripcional de genes que muestra el mutante *des1-1* en estadíos de crecimiento tardíos. Por tanto, proponemos que el H_2S actúa como una molécula señalizadora que regula negativamente la autofagia y modula el perfil transcripcional en *Arabidopsis*.

En *A. thaliana*, la mayoría de la cisteína es sintetizada en el citosol por la acción de la isoforma mayoritaria citosólica OAS-A1. Por otra parte, la única cisteína desulfhidrasa citosólica descrita hasta la fecha es DES1. De esta manera DES1 y OASA1 llevan a cabo funciones opuestas para mantener la homeostasis de cisteína en

el citosol. En el tercer capítulo de esta tesis se describe el papel de la cisteína en la respuesta inmune de las plantas. Partiendo de la base de que los transcriptomas de los mutantes deficientes en OAS-A1 y DES1 presentaban una alta correlación con la respuesta a distintos estreses bióticos, se estudió la respuesta de ambos mutantes a la infección por distintos patógenos de plantas. Los mutantes *des1* son resistentes a patógenos necrótrofos y biotrófos y acumulan ácido salicílico, es decir, se comportan como mutantes SAR (resistencia sistémica adquirida). En cambio, los mutantes *oas-a1* son más sensibles a ambos tipos de patógenos y carecen de respuesta hipersensible durante la resistencia inducida por efector (ETI: *effector-triggered immunity*). Estos resultados muestran que la cisteína podría ser un metabolito crucial durante la interacción planta-patógeno, ya que su desequilibrio en el citosol tiene claras consecuencias en la susceptibilidad de las plantas a patógenos.

Puesto que la isoforma citosólica OAS-A1 es la principal enzima OASTL de *Arabidopsis*, un control eficiente de su actividad es crucial para mantener la homeostasis de cisteína en condiciones de estrés. Tanto el estrés abiótico como biótico conducen a la producción de especies reactivas del oxígeno y nitrógeno, que conducen a la producción de peroxinitrito, un potente agente nitrante que tiene la capacidad de nitrar de forma no estocástica los residuos de tirosina de las proteínas. En el cuarto capítulo de esta tesis hemos demostrado que OAS-A1 sufre un proceso de inactivación producido por la modificación específica del residuo de tirosina 302 mediante nitración, inhibiendo la actividad enzimática de OAS-A1. Esta modificación post-traduccional de OAS-A1 mediante nitración puede representar un rápido y eficiente mecanismo regulatorio para controlar la biosíntesis de cisteína y glutatión en respuesta a distintos factores de estrés.

Introducción

1. EL AZUFRE EN LAS PLANTAS

El azufre es un macronutriente esencial presente en la naturaleza tanto en formas orgánicas como inorgánicas. En sus diferentes estados de oxidación, representa uno de los elementos más versátiles y la forma oxidada más común es el sulfato. Las plantas, microorganismos y hongos, a diferencia de los animales, son capaces de reducir el sulfato a sulfuro e incorporarlo a los metabolitos orgánicos mediante un proceso dependiente de energía, dando lugar a una amplia variedad de compuestos orgánicos esenciales para el desarrollo y crecimiento de las plantas. Además, las plantas son la fuente más importante de aminoácidos azufrados esenciales para humanos y animales, como son la cisteína y la metionina (Wirtz y Droux, 2005). El azufre se encuentra en vitaminas como la biotina y la tiamina, cofactores como coenzima A, cofactor de molibdeno (MoCo), ácido lipoico y agrupaciones sulfoférricas que participan en el transporte de electrones. Los glucosinolatos, fitoalexinas, tioninas y defensinas son compuestos azufrados que intervienen en la defensa de la planta frente a patógenos (Rausch y Wachter, 2005). El azufre también está presente en los sulfolípidos que son esenciales para la formación de las membranas tilacoidales de los cloroplastos. Algunas hormonas de plantas son inactivadas por sulfatación, como los brasinoesteroides y el ácido jasmónico; por el contrario, la sulfatación es esencial para la función de hormonas peptídicas que estimulan el crecimiento celular (Hirai y Saito, 2008).

La cisteína es el primer compuesto orgánico con azufre reducido sintetizado en la planta a partir de la asimilación fotosintética del sulfato. La cisteína es indispensable no solo como aminoácido, formando parte de las proteínas, sino también para la estabilización de las estructuras terciaria y cuaternaria de las proteínas debido a su participación en la formación de los puentes disulfuro y por su papel regulador en los sitios activos de muchas enzimas. La cisteína ocupa una posición central en el metabolismo primario y secundario de la planta debido a sus funciones bioquímicas. Es el metabolito precursor del antioxidante glutatión, del aminoácido metionina y de gran parte de las biomoléculas esenciales y compuestos de defensa mencionados anteriormente, donde el mecanismo catalítico de muchas de estas biomoléculas está basado en la reactividad del grupo tiol (Droux, 2004). La conversión entre grupos tiólicos libres y la formación de puentes disulfuro y viceversa, constituye un sistema dinámico que es la base para las modificaciones redox que sufren las proteínas. Estas modificaciones están involucradas en la regulación de la actividad de determinadas enzimas y a su vez en la regulación del metabolismo de las células en repuesta a señales ambientales (Wirtz y Droux, 2005).

2. ASIMILACIÓN FOTOSINTÉTICA DE SULFATO EN PLANTAS

La utilización de mutantes de bacterias y levaduras, así como la secuenciación completa del genoma de *Arabidopsis thaliana*, ha dado lugar a un gran avance en el conocimiento del proceso de la asimilación de sulfato en plantas (Figura 1) (Barroso *et al.*, 1997; Hell, 1997; Leustek *et al.*, 2000; Saito, 2004; Noji *et al.*, 2006; Takahashi *et al.*, 2011).

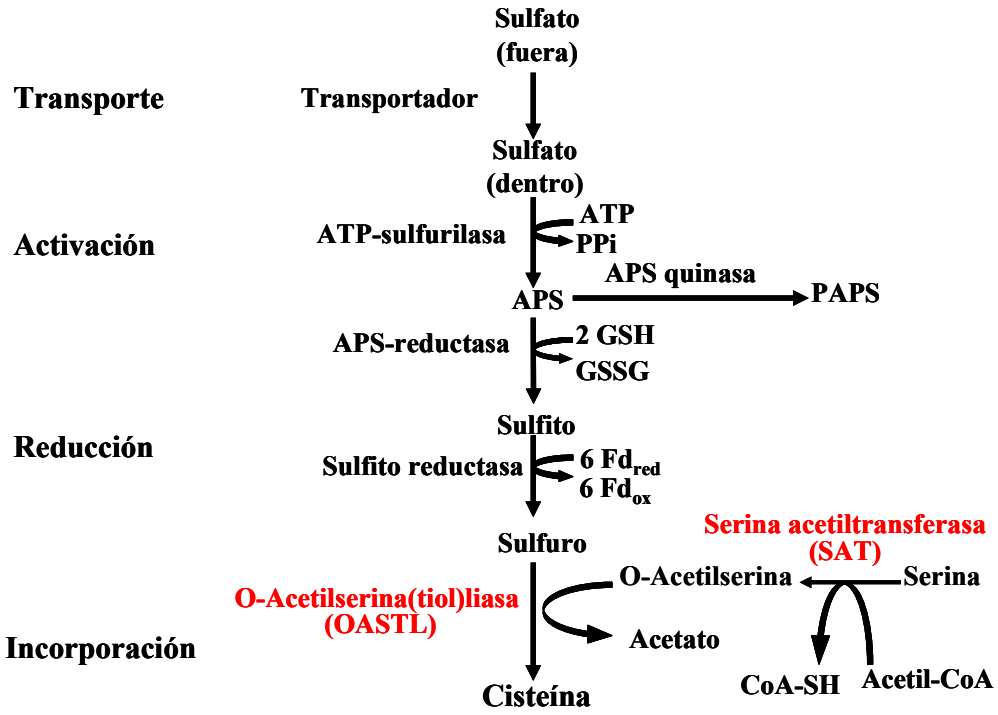

Figura 1. Ruta de asimilación de sulfato en plantas. En rojo aparecen las enzimas implicadas en la biosíntesis de cisteína.

La biosíntesis de cisteína constituye la última etapa de la asimilación fotosintética de sulfato. Las cuatro etapas fundamentales de este proceso son: transporte de sulfato al interior celular, activación del sulfato, reducción del sulfato activado a sulfuro e incorporación del azufre reducido a esqueletos carbonados (Figura 1).

2.1.Transporte de sulfato

Las plantas toman el sulfato del suelo y lo distribuyen a todas las partes de la planta. Se conocen bien los mecanismos para el transporte del sulfato a través de la membrana plasmática a nivel de las raíces, donde se utiliza un gradiente electroquímico establecido por la ATPasa de protones, de forma que los transportadores de sulfato transportan al interior de la célula una molécula de sulfato por cada tres protones. Esta

etapa es considerada el paso más regulado de la ruta de asimilación de azufre en la planta (Vauclare *et al.*, 2002).

Todos los transportadores conservan en la zona central de la cadena polipeptídica 12 dominios transmembrana. Las divergencias en las secuencias de aminoácidos de los extremos amino y carboxi terminal determinan las diferencias en la afinidad por el sulfato y las funciones de los mismos (Vidmar *et al.*, 2000). Además, contienen en el extremo carboxi terminal un dominio denominado STAS (*sulfate transporters and antisigma factor antagonists*) que puede tener un papel regulador en el control de la actividad y localización de los transportadores de membrana (Shibagaki y Grossman, 2004; Rouached *et al.*, 2005). En *A.thaliana* se han identificado 14 genes que forman una familia de transportadores de sulfato (Howarth *et al.*, 2003b). Según sus secuencias, estos genes se pueden dividir en 5 grupos (*SULTR1*-*SULTR5*), dependiendo tanto de la afinidad al sustrato como de la función que ejercen (Hawkesford y De Kok, 2006).

Los miembros del grupo *SULTR1* representan a los transportadores de alta afinidad y la mayor parte son regulados transcripcionalmente en respuesta a la disponibilidad de sulfato. *SULTR1;1* y *SULTR1;2* se expresan mayoritariamente en raíces, facilitando el transporte de sulfato al interior de las raíces en condiciones de limitación de azufre. *SULTR1;2* es el más abundante y *SULTR1;1* tiene un valor de K_m más bajo y está más fuertemente inducido en condiciones de limitación de azufre (Takahashi *et al.*, 2000; Yoshimoto *et al.*, 2002). *SULTR1;3* se expresa específicamente en el floema y es el responsable del transporte del sulfato desde las zonas fuentes hasta los sumideros (Yoshimoto *et al.*, 2003).

Los transportadores del grupo *SULTR2* son de baja afinidad por sulfato, se localizan en tejidos vasculares y están involucrados en el transporte a larga distancia. Las dos isoformas de este grupo, *SULTR2;1* y *SULTR2;2,* se expresan en raíces y hojas. *SULTR2;1* se induce considerablemente en condiciones de limitación de azufre en raíces (Takahashi *et al.*, 2000; Takahashi, 2010).

El grupo *SULTR3* es el más desconocido y ninguno de los miembros de este grupo parece estar regulado por la cantidad de azufre en la planta. La expresión de *SULTR3;1, 3;2* y *3;3* está restringida a hojas (Buchner *et al.*, 2004). Recientemente se ha demostrado que varios miembros de este grupo controlan la translocación del sulfato en diferentes etapas del desarrollo de semillas (Zuber *et al.*, 2010).

Los grupos *SULTR4* y *SULTR5* se localizan en el tonoplasto (Kataoka *et al*, 2004). El grupo *SULTR4* interviene en el transporte de sulfato desde la vacuola al citosol. Además, su expresión se induce en condiciones de estrés por azufre (Hawkesford y De Kok, 2006). Recientemente, se ha demostrado que uno de los dos genes pertenecientes al grupo *SULTR5* (*SULTR5;2*) codifica un transportador de

molibdato de alta afinidad y se ha renombrado como *MOT1 (*Tomatsu *et al.,* 2007*;* Baxter *et al.,* 2008).

No está claro qué tipo de transportadores interviene en el transporte de sulfato al cloroplasto en plantas. Se ha descrito que la entrada de sulfato compite con la translocación de fosfato y triosa-fosfato (Gross *et al.*, 1990).

2.2. Activación de sulfato

Una vez el sulfato ha entrado en la planta, puede ser almacenado en raíces u hojas en el interior de las vacuolas o ser utilizado en el proceso de asimilación, siendo las concentraciones de cisteína o glutatión las que regulan esta distribución (Bolchi *et al.*, 1999).

El sulfato tiene que ser reducido hasta sulfito, pero el potencial de oxido-reducción del par redox sulfato/sulfito a pH 7,0 es extraordinariamente negativo, $E'_o[SO_4^{2-}/ SO_3^{2-}]$= -516 mV. Al no existir donadores fisiológicos de electrones con potenciales redox tan negativos, la reducción directa del sulfato no es posible, por lo que el sulfato inorgánico tiene que ser previamnete activado.

Las ATP sulfurilasas (EC 2.7.7.4) catalizan la activación del sulfato hidrolizanando la unión entre los fosfatos alfa y beta del ATP y añadiendo sulfato al fosfato alfa. La energía almacenada en el enlace anhidro fosfato-sulfato del producto de la reacción, adenosin 5´-fosfosulfato (APS), permite la reducción posterior del sulfato. Existen 4 genes que codifican distintas isoenzimas ATP sulfurilasas en *Arabidopsis*, de las cuales tres están localizadas en plastidios y una en el citosol (Hatzfeld *et al.*, 2000a; Mugford *et al.*, 2009). Las ATP sulfurilasas se expresan tanto en raíces como en hojas, y en *Arabidopsis,* la actividad en raíces es el 30% de la actividad en hojas (Lee y Leustek, 1999). Esto indica que ambos órganos juegan un papel importante en la asimilación de sulfato.

La reacción de adenilación del sulfato está desplazada hacia la formación de ATP, por ello los productos de la reacción, APS y pirofosfato (PPi), deben ser mantenidos a baja concentración por las enzimas pirofosfatasas, APS reductasas y APS quinasas. Estas últimas metabolizan el APS, catalizando la fosforilación dependiente de ATP del APS en la posición 3´ dando lugar a 3´-fosfo adenosina-5´-fosfosulfato (PAPS). PAPS es la forma activa del sulfato usada para la síntesis de compuestos secundarios sulfatados en plantas. La presencia de actividad ATP sulfurilasa y APS quinasa en el citosol está ligada a la síntesis de PAPS, que es sustrato de sulfotranferasas que catalizan sulfataciones, donando grupos sulfatos a otros metabolitos como sulfolípidos y glucosinolatos. Todas esta enzimas sulfotransferasas que catalizan estas reacciones se encuentran en el citosol (Rotte y Leustek, 2000; Wittstock y Halkier, 2002; Mugford *et al.*, 2011).

2.3. Reducción del sulfato activado a sulfuro

La reducción del sulfato es llevada a cabo por las enzimas APS reductasas (EC 1.8.99.2) y sulfito reductasas (EC 1.8.7.1). Ambas se localizan en los plastidios de hojas y raíces. El residuo de sulfato del APS es reducido a sulfito por la APS reductasa. *Arabidopsis* contiene tres genes que codifican APS reductasas. Estas enzimas son polipéptidos de aproximadamente 50 kDa, con un dominio catalítico amino terminal, homólogo a las APS y PAPS reductasas de bacterias (Bick *et al.*, 2000) y un dominio carboxilo terminal, que interacciona con el reductor, homólogo a las tiorredoxinas y glutarredoxinas (Gutierrez-Marcos *et al.*, 1996; Setya *et al.*, 1996). Las APS reductasas usan eficientemente el glutatión reducido como donador de electrones. El mecanismo de reacción de las APS reductasas se divide en 2 etapas. En primer lugar, un residuo de cisteína reducido reacciona con APS para formar el complejo intermediario "enzima-Cys-S-SO_3^{-2}" y AMP. En segundo lugar, el extremo carboxilo terminal media la interacción con el glutatión, reduciéndose el grupo unido a la enzima y obteniéndose al final sulfito libre y "enzima-Cys-S-SG". Posteriormente la enzima activa se regenera con una segunda molécula de glutatión (Weber *et al.*, 2000).

El sulfito producido en el cloroplasto es reducido a sulfuro mediante la transferencia de seis electrones en una reacción catalizada por la enzima sulfito reductasa, que es dependiente de ferredoxina en plantas y NADPH en células no fotosintéticas (Yonekura-Sakakibara *et al.*, 2000). Las sulfito reductasas son hemoproteínas homo-oligoméricas compuestas de 2 a 4 subunidades idénticas. Cada subunidad contiene un sirohemo y una agrupación sulfo-férrica [4Fe-4S]. *Arabidopsis* tiene un único gen que codifica una sulfito reductasa y se ha caracterizado en detalle que la disminución de esta actividad produce una respuesta significativa en el metabolismo primario y secundario (Khan *et al.*, 2010).

2.4. Incorporación del azufre reducido a esqueletos carbonados

La síntesis de cisteína representa la última etapa de la asimilación de sulfato y consiste en la incorporación del sulfuro al esqueleto carbonado de la O-acetilserina (OAS) dando lugar a L-cisteína.

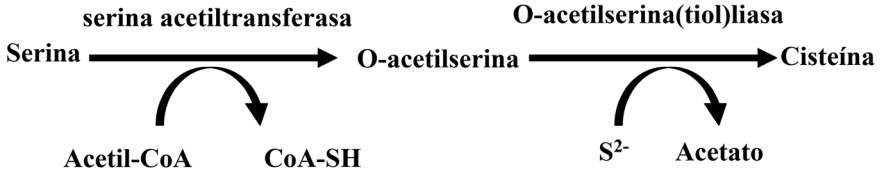

Figura 2. Reacciones catalizadas por la serina acetiltransferasa y O-acetilserina(tiol)liasa.

Este proceso tiene lugar mediante dos reacciones enzimáticas catalizadas por las enzimas serina acetiltranferasas que genera la OAS (SAT, EC 2.3.1.30) y O-acetilserina(tiol)liasas que incorpora el sulfuro al esqueleto carbonado de la OAS (Barroso *et al.,* 1997) (Figura 2).

En el genoma de *A. thaliana* se han identificado cinco genes *SAT* (Howarth *et al.*, 2003a) y ocho genes *OASTL* (Wirtz *et al.*, 2004) (Figura 3a). La presencia de múltiples cDNAs en las bases de datos sugiere que esta organización es similar en otras especies de plantas. Las diferentes isoformas se localizan en el citosol, los cloroplastos y las mitocondrias, resultando en una compleja variedad de isoformas y en diferentes conjuntos o "pools" subcelulares de cisteína (Figura 3b).

En base a las características cinéticas y abundancia de los transcritos en *Arabidopsis*, las isoformas mayoritarias en el cloroplasto de SAT y OASTL son las isoformas codificadas por los genes *SAT53* (At1g55920) y *OAS-B* (At2g43750). Las responsables mayoritarias de la síntesis de cisteína en el citosol están codificadas por *SAT52* (At5g56760) y *OAS-A1* (At4g14880); y *SAT1* (At3g13110) y *OAS-C* (At3g59760) codifican las mayoritarias en la mitocondria (Barroso *et al.*, 1995; Howarth *et al.*, 2003a; Haas *et al.*, 2008; Heeg *et al.*, 2008; Lopez-Martin *et al.*, 2008a; Watanabe *et al.*, 2008a; Watanabe *et al.*, 2008b; Krueger *et al.*, 2009).

Otra isoforma mayoritaria mitocondrial, denominada ATCYS-C1, participa en la ruta metabólica para la destoxificación de cianuro. Análisis bioquímicos han demostrado que esta enzima posee preferentemente actividad β-cianoalanina sintasa (CAS) y cataliza la formación de β-cianoalanina a partir de cisteína y cianuro (Hatzfeld *et al.*, 2000b; Meyer *et al.*, 2003). Recientemente un análisis detallado de mutantes insercionales de T-DNA deficientes en ATCYS-C1 ha demostrado que la actividad CAS mitocondrial es esencial para mantener bajos niveles de cianuro, permitiendo el correcto desarrollo de los pelos radicales. Este estudio propone a la molécula de cianuro como una nueva molécula señalizadora en plantas (Garcia *et al.*, 2010).

Las isoformas minoritarias citosólicas están codificadas por los genes *SATN* (At4g35640), *SAT106* (At2g17640), *ATCYS-D1* (at3g04940) y *ATCYS-D2* (At5g28020), y no han sido caracterizadas hasta la fecha.

Otra isoforma minoritaria denominada inicialmente CS-LIKE (At5g28030) ha sido objeto de estudio durante el desarrollo de esta tesis como se presenta a continuación. En este estudio hemos establecido inequívocamente que cataliza la desulfuración de L-Cys a sulfuro, amonio y piruvato, y por tanto es una nueva enzima L-Cys desulfhidrasa (EC 4.4.1.1) y se ha propuesto que se designe como DES1, que es la forma en la que se mostrará a partir de este momento (Alvarez *et al.*, 2010).

La isoforma minoritaria de localización cloroplástica CS26 (At3g03630) ha sido también recientemente caracterizada como una S-sulfocisteína sintasa, describiéndose

por primera vez en plantas. Además se ha revelado que esta actividad juega un papel esencial en la función del cloroplasto y su regulación redox (Bermudez *et al.*, 2010).

a) **b)**

Gen	Posición en el genoma
SAT 52	At5g56760
SAT 53	At1g55920
SAT 1	At3g13110
SAT 106	At2g17640
SAT N	At4g35640
OAS-A1	At4g14880
OAS-B	At2g43750
OAS-C	At3g59760
ATCYS-C1	At3g61440
ATCYS-D2	At5g28020
ATCYS-D1	At3g04940
DES1	At5g28030
CS26	At3g03630

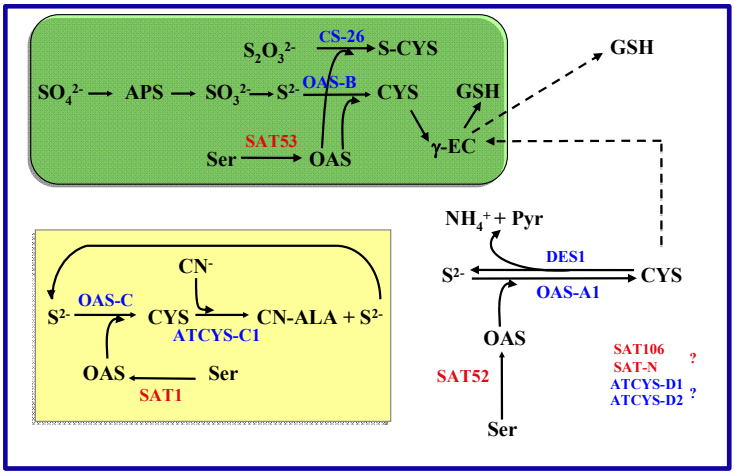

Figura 3. a) Genes *SAT* y *OASTL* presentes en el genoma de *A. thaliana.* b) Localización subcelular de las isoformas SAT y OASTL en *Arabidopsis thaliana*. Las cinco isoenzimas con actividad SAT se muestran en color rojo y las isoenzimas OASTL en azul.

Las enzimas SAT de plantas contienen 2 dominios muy conservados: el dominio N-terminal, donde predominan estructuras α-hélices, está involucrado en la interacción proteína-proteína entre las subunidades SAT, mientras que el dominio C-terminal esencial para la catálisis y la interacción de SAT con OASTL contiene una repetición en tándem de un hexapéptido muy conservado en la estructura de las acetiltransferasas y posee una estructura de hojas plegadas-β (Vaara, 1992; Bogdanova y Hell, 1997; Wirtz *et al.*, 2001).

La enzima OASTL es un homodímero de 60-70 kDa de peso molecular que contiene como grupo prostético dos moléculas de piridoxal-5´-fosfato (PLP) por dímero (Leon *et al.,* 1987). La comparación de las secuencias de aminoácidos muestra una alta homología entre las enzimas de plantas y de bacterias, conservándose en todas ellas la secuencia de aminoácidos del sitio activo TSGNT y el aminoácido lisina involucrado en la unión al cofactor (Figura 4). Concretamente, en la proteína OAS-A1 esta lisina ocupa la posición 46 y forma una base de Schiff con el PLP, que junto con otras interacciones, ancla el PLP al sitio activo. Distintos análisis cinéticos, mutagénesis dirigidas y ensayos de unión a ligandos han permitido elucidar el papel de los residuos clave en el sitio

activo, de modo que la Asn^{77} y la Gln^{147} de la proteína OAS-A1 son aminoácidos claves en la unión al sustrato OAS, mientras que la Thr^{74} y Ser^{75} están involucrados en la incorporación del sulfuro para formar cisteína. El sitio de interacción con SAT está muy conservado en plantas y bacterias, y lo constituye el loop β8A-β9A desde el aminoácido Lys ^{217} hasta Phe ^{230} (Figura 4) (Bonner *et al.*, 2005).

Las verdaderas OASTL se asocian con SAT formando el complejo cisteína sintasa, a diferencia de las isoformas ATCYS-C1, CS26 y DES1, que presentan actividades enzimáticas diferentes a OASTL y varios cambios de aminoácidos en el loop β8A- β9A, que es necesario para la interacción con SAT (Yamaguchi *et al.*, 2000; Hatzfeld *et al.*, 2000b; Bonner *et al.*, 2005; Francois *et al.*, 2006; Alvarez *et al.*, 2010; Bermudez *et al.*, 2010).

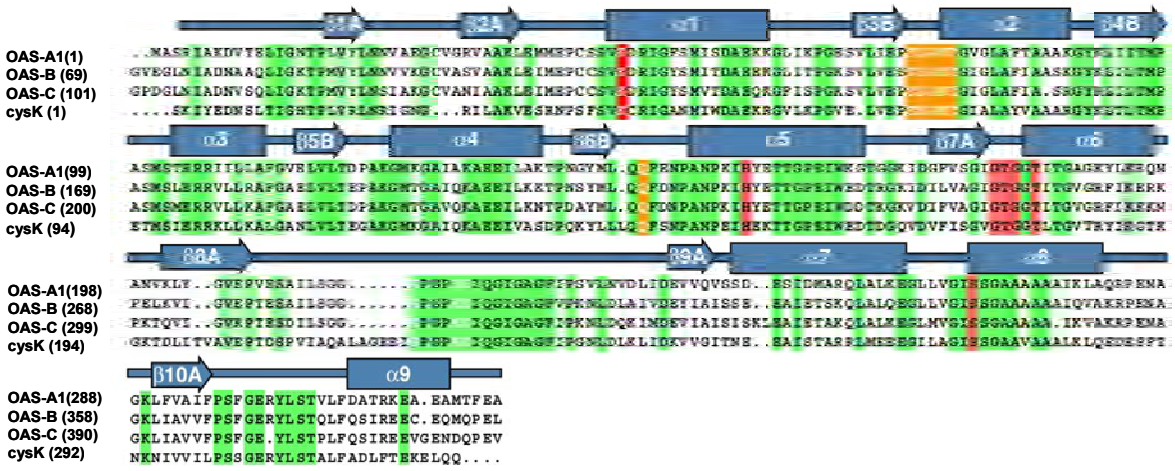

Figura 4. Comparación de las secuencias de aminoácidos y estructura secundaria de las isoformas OASTLs mayoritarias de plantas y de la OASTL de *Salmonella typhimurium* codificada por el gen cysK (Bonner *et al.*, 2005). Entre paréntesis se indica la posición del aminoácido al inicio del alineamiento de las secuencias. En rojo se resaltan los aminoácidos que intervienen en la interacción con el PLP y en naranja aquellos que forman parte del sitio activo. Los aminoácidos conservados entre las diferentes isoformas se muestran en verde.

Análisis bioquímicos realizados con enzimas recombinantes mostraron que el complejo cisteína sintasa, de un peso molecular aproximado de 300 kDa, podría estar constituido por dos homodímeros de OASTL y una molécula homotetrámera de SAT (Droux *et al.*, 1998). Más tarde, mediante análisis de las estructuras tridimensionales de SAT y OASTL de bacterias y plantas, se ha propuesto una estructura $\alpha_6\beta_4$ para las subunidades del complejo cisteína sintasa de plantas, constituido por un dímero de trímeros de SAT y dos homodímeros de OASTL (Wirtz y Hell, 2006). La formación del complejo cisteína sintasa parece ser un mecanismo regulador característico de la síntesis de cisteína. La enzima SAT es activa cuando forma parte del complejo, produciéndose un aumento de su V_{max} y su afinidad a los sustratos y por tanto obteniéndose un mayor

rendimiento en la producción de OAS. Por otro lado, la enzima OASTL está inactiva cuando forma parte del complejo (Hindson y Shaw, 2003), actuando como regulador de la actividad SAT, ya que modifica su estabilidad y propiedades catalíticas. La fracción OASTL libre es catalíticamente activa y por tanto responsable de la formación de cisteína (Droux *et al.*, 1998; Wirtz y Hell, 2006). Se ha demostrado que la actividad OASTL excede a la actividad SAT en plastidios 345 veces, 200 veces en el citosol y 10 veces en mitocondrias, lo que indica que la SAT está en su totalidad formando el complejo cisteína sintasa con una pequeña fracción del total de actividad OASTL, modificándose así las propiedades cinéticas de ambas (Heeg *et al.*, 2008).

Como se ha explicado anteriormente, la reducción asimilatoria de sulfato tiene lugar en los cloroplastos y por tanto se ha sugerido que las isoformas mayoritarias cloroplásticas podrían participar en la ruta de asimilación primaria de sulfato, y que las otras isoformas pueden tener funciones metabólicas y de señalización desconocidas hasta la fecha. De hecho, nuestro grupo de investigación está interesado en conocer si cada "pool" subcelular de cisteína está asociado a la biosíntesis de diferentes productos secundarios y por tanto si desempeña una función específica en el metabolismo de la planta.

Inicialmente, se propuso que las endomembranas podrían ser impermeables al transporte de cisteína debido a la reactividad del grupo tiol y que aquellos compartimentos con la capacidad de biosintetizar proteínas requerirían su propia producción de cisteína (Lunn *et al.*, 1990). El estudio detallado de mutantes insercionales de T-DNA de *Arabidopsis* deficientes en cada una de estas isoformas, la aplicación de las denominadas tecnologías "ómicas", así como la distribución subcelular de actividades enzimáticas y metabolitos, está permitiendo dilucidar las funciones específicas de las diferentes SAT y OASTL en la planta, y por tanto conocer la contribución de cada "pool" subcelular de cisteína a su fisiología.

Se ha comprobado que en el mutante de la isoforma cloroplástica OAS-B, la falta de síntesis de cisteína es compensada completamente por la de otros compartimentos, por lo que se requiere un transporte de sulfuro desde el cloroplasto a otros compartimentos celulares a través de las membranas. Se ha especulado que el sulfuro alcanza el citosol atravesando las membranas del cloroplasto mediante difusión. Sin embargo, el estroma del cloroplasto tiene un pH de 8,5 en condiciones de iluminación, donde el 95% del sulfuro estaría presente en la forma cargada HS^-. Por lo tanto, probablemente el sulfuro es transportado de forma controlada a través de la membrana del cloroplasto (Heldt *et al.*, 1973; Wu y Berkowitz, 1992; Wirtz y Hell, 2007).

Por otro lado, diferentes estudios en mutantes deficientes en las distintas isoformas SAT han demostrado que la mitocondria es el orgánulo más importante para la síntesis de OAS, ya que contiene el 80% de la actividad SAT total y la menor

actividad OASTL (Haas *et al*., 2008; Watanabe *et al.*, 2008b; Krueger *et al.,* 2009). A su vez, la mayor parte de la cisteína es sintetizada en el citosol por la isoforma OAS-A1, tal como ha sido demostrado en el análisis de mutantes deficientes en las OASTLs mayoritarias. OAS-A1 constituye el 23% del total de las proteínas OASTL y el 44% y 80% del total de la actividad OASTL en hojas y raíces respectivamente (Heeg *et al.*, 2008; Lopez-Martin *et al.*, 2008a; Watanabe *et al.*, 2008a). La concentración de cisteína en el citosol ha sido estimada en torno a 300 μM, mientras que en el resto de compartimentos celulares se encuentra por debajo de 10 μM (Krueger *et al.*, 2009).

En resumen, la utilización de mutantes insercionales deficientes en la síntesis de cisteína en mitocondrias, plastidios o citosol permitió entender que los compartimentos no afectados en cuestión propocionan cisteína a los otros compartimentos afectados de modo que la viabilidad de estos mutantes solo puede ser explicada por procesos de transportes eficientes entre el citosol y ambos orgánulos.

2.5. Regulación de la asimilación de sulfato

El transporte y asimilación de sulfato en plantas están fuertemente controlados por diversos mecanismos reguladores, fundamentalmente a nivel transcripcional (Davidian y Kopriva, 2010). La deficiencia en azufre induce el transporte de sulfato en las raíces tanto a nivel de los transportadores de alta como de baja afinidad, así como a nivel de los transportadores vacuolares SULTR4 (Kataoka *et al.*, 2004). Cuando se suministra sulfato u otras fuentes de azufre a las plantas, esta inducción desaparece rápidamente (Lappartient *et al.*, 1999). Los efectos de la limitación por sulfato son mimetizados por tratamiento con OAS, que se acumula durante estas condiciones, induciéndose los niveles de ARNm de los transportadores de sulfato, así como la capacidad de asimilación de sulfato (Smith *et al.*, 1997). Se han identificado en *Arabidopsis* elementos cis-reguladores involucrados en la regulación de la repuesta de la planta a la deficiencia de azufre (Maruyama-Nakashita *et al.*, 2005). El elemento mejor conocido tiene una secuencia de 16 pares de bases y se ha denominado elemento de respuesta al azufre (SURE: *sulphur-responsive element*). El elemento SURE está presente en los promotores de *SULTR1;1* y de otros genes de la ruta metabólica del azufre (Davidian y Kopriva, 2010).

La captación de sulfato está coordinada con la toma y asimilación de nitrógeno y carbono, de modo que en condiciones de limitación por nitrógeno los transcritos SULTR1;1 y 1;2 disminuyen y la captación de sulfato está fuertemente reducida. En cambio, la transcripción de los genes de transportadores de sulfato de alta afinidad se induce por sacarosa (Maruyama-Nakashita *et al.*, 2004). El producto de la reacción catalizada por el complejo cisteína sintasa, la OAS, es el punto de intersección entre las rutas de asimilación de nitrógeno, carbono y azufre, que necesitan una regulación muy

estricta para que la síntesis de los distintos aminoácidos se realice correctamente (Prosser *et al.*, 2001; Saito, 2004).

La asimilación de sulfato está también fuertemente regulada por la demanda de sulfuro, así como por las condiciones ambientales (Kopriva y Rennenberg, 2004; Koprivova *et al.*, 2008). Además del transporte de sulfato, se ha sugerido que el paso de la ruta más regulado es el llevado a cabo por las APS reductasas. Las APS reductasas son reguladas por el estado nutricional de azufre en las plantas, puesto que se inducen en condiciones de deficiencia de azufre y se inhiben por la presencia de azufre reducido, como H_2S, cisteína o glutatión (Kopriva y Koprivova, 2004).

Otra etapa de la ruta regulada en respuesta a diferentes procesos de estrés es la síntesis de cisteína. Mediante hibridación *in situ* se analizó la expresión de la isoforma mayoritaria citosólica OAS-A1 asociada a tejidos y se encontró una alta expresión en tricomas de hojas y tallos, revelando nuevos aspectos en la función biológica de los tricomas con el metabolismo del azufre y sugiriendo una función especializada de la isoforma OAS-A1 en los tricomas (Gotor *et al.*, 1997). Se había descrito que los tricomas podrían jugar un papel importante en la defensa frente a estreses bióticos o abióticos, y por ello se profundizó en el estudio de estas estructuras, demostrándose que la ruta de biosíntesis de glutatión es muy activa en este tipo de células y funciona eficientemente en los procesos de destoxificación (Gutierrez-Alcala *et al.*, 2000).

Estudios realizados en nuestro laboratorio indican que la isoforma mayoritaria citosólica OAS-A1 parece ser esencial en la respuesta de la planta frente a ciertos procesos de estrés abiótico como la salinidad (Barroso *et al.*, 1999). Además, la proteína OAS-A1 confiere tolerancia a salinidad en levaduras y por tanto juega un papel esencial en la protección frente a estrés salino (Barroso *et al.,*1999; Romero *et al.*, 2001). También se ha demostrado que la proteína OAS-A1 está implicada en la respuesta de la planta a metales pesados y la sobre-expresión de este gen en *Arabidopsis* produce plantas transgénicas muy resistentes a la presencia de metales pesados (Dominguez-Solis *et al.*, 2001). De esta forma, se demuestra que la disponibilidad de cisteína citosólica es una etapa limitante en la protección de la planta a estrés por metales (Dominguez-Solis *et al.*, 2004). La caracterización de mutantes nulos *oas-a1* parece sugerir que la proteína OAS-A1 juega un papel esencial en el control redox celular. En las plantas mutantes se observa no sólo una reducción del contenido total de cisteína y glutatión, sino además una alteración del estado redox que se manifiesta en un aumento del porcentaje de glutatión oxidado. Estos mutantes muestran una clara sensibilidad a la presencia de metales pesados, aunque los niveles de fitoquelatinas no difieren con respecto a las plantas silvestres, lo que sugiere que la hipersensibilidad a cadmio podría estar relacionada con una respuesta más general en la planta. Las plantas deficientes en OAS-A1 se encuentran oxidativamente estresadas en condiciones fisiológicas de crecimiento, probablemente como resultado de un desequilibrio entre la formación y la

eliminación de ROS. Por tanto, la contribución de la cisteína citosólica en la destoxificación de ROS y señalización redox sería una respuesta metabólica global de la planta a condiciones adversas (Lopez-Martin *et al.*, 2008a; Lopez-Martin *et al.*, 2008b).

Nuestro grupo de investigación también ha encontrado que los transcritos de todos los miembros de la familia génica SAT de *Arabidopsis* aumentan en respuesta a tratamientos con cadmio (Howarth *et al.*, 2003a). Estos resultados podrían sugerir que las isoformas SAT específicas junto con las OASTLs correspondientes podrían intervenir en los mecanismos de defensa de la planta frente a estrés por metal. Sin embargo, la sobre-expresión de SAT-52 y SAT-53, que codifican la isoformas mayoritarias citosólica y cloroplástica respectivamente, no mejoran la resistencia a metales pesados, a diferencia de lo que ocurre con la sobre-expresión de la isoforma OASTL citosólica. Estos resultados parecen contradictorios con el modelo actual en el que se asume que en la mayoría de los compartimentos celulares el nivel de enzimas OASTL se encuentra en exceso con respecto al nivel de enzimas SAT (Lopez-Martin *et al.,* 2005).

La coordinación adecuada del complejo cisteína sintasa es también un punto de control importante de la ruta de asimilación de sulfato. La formación del complejo es reversible y la interacción de las dos enzimas se ve fuertemente afectada por la concentración de OAS y sulfuro, de manera que el sulfuro induce la formación del complejo y por tanto la formación de OAS, mientras que la OAS favorece la disociación del complejo (Riemenschneider *et al.*, 2005a). Si se acumula OAS, el complejo se destabiliza, y por tanto se reduce la síntesis de OAS. El exceso de OAS reacciona con el sulfuro presente para formar cisteína gracias a la OASTL libre. La molécula de OAS regula además positivamente la expresión de los genes de la asimilación del sulfato (Hirai *et al.*, 2003). Por el contrario, el aumento de la síntesis de cisteína y glutatión reprime la expresión de la enzimas de la asimilación de sulfato. Este sistema sirve para coordinar la síntesis de OAS con las actividades de la ruta de asimilación de sulfato (Figura 5) (Hell *et al.*, 2002; Wirtz y Hell, 2006). Por tanto, la formación del complejo cisteína sintasa proporciona un mecanismo efectivo para controlar los cambios a nivel celular y molecular de la producción de cisteína, actuando como un sensor del estado intracelular del azufre en la planta (Yi *et al.*, 2010). La isoforma citosólica SAT, además, está regulada alostéricamente mediante retroinhibición por cisteína. Esta característica no se observa en las isoformas cloroplásticas y mitocondriales, que son insensibles a cisteína (Saito *et al.*, 1995; Noji *et al.*, 1998; Kumaran *et al.*, 2009).

A nivel post-traduccional, el procesamiento y modificación de las proteínas son procesos generalmente conocidos por su importancia en la actividad, estabilidad y función de las proteínas. Se ha descrito que las APS reductasas son reguladas por procesos redox (Bick *et al.*, 2001), de forma que cuando hay un exceso de tioles, el

dímero activo de APS reductasa se reduce a la forma monomérica inactiva (Kopriva y Koprivova, 2004).

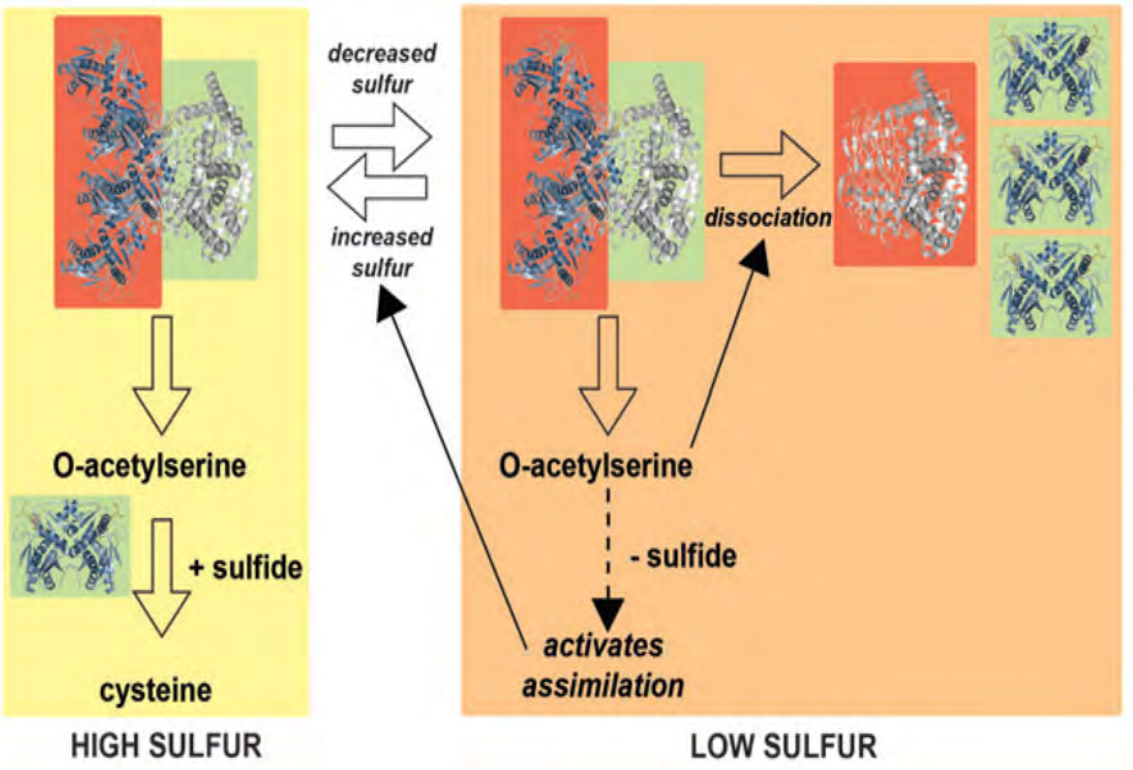

Figura 5. Modelo de regulación del complejo cisteína sintasa (Yi *et al.*, 2010).
Representación esquemática del modelo de formación del complejo cisteína sintasa. La estructura de las proteínas que forman el complejo se enmarca en recuadros de color verde cuando están activas, mientras que la forma inactiva se encuentra resaltada en color rojo. OASTL está representada como una estructura tridimensional en azul y SAT en gris.

Aunque a nivel transcripcional se ha visto que OAS-A1 es regulada en respuesta a diferentes procesos de estrés abiótico, poco se sabe de las modificaciones post-traduccionales que sufren las distintas OASTLs. Se ha descrito que las isoformas mayoritarias citosólicas y cloroplásticas de *Arabidopsis*, OAS-A1 y OAS-B, se pueden encontrar N-acetiladas. La actividad específica de las mismas no se ve alterada con dicha modificación (Wirtz *et al.*, 2010). Aunque la posible función biológica no está clara, se ha descrito que la acetilación N-terminal de estas isoformas citosólicas y cloroplásticas podría estar modificando la tasa de reciclaje de las mismas, posiblemente para generar señales de degradación (Hwang *et al.*, 2010).

Tanto el estrés abiótico como biótico conducen a la producción de especies reactivas del oxígeno y nitrógeno. La producción simultanea de óxido nítrico (NO) y radical superóxido en células de plantas sometidas a estrés se correlaciona con la

producción de un potente agente nitrante como es el peroxinitrito, que tiene la capacidad de nitrar de forma no estocástica los residuos de tirosina de las proteínas (Lozano-Juste *et al.*, 2011). En esta tesis hemos demostrado que OAS-A1 sufre un proceso de inactivación producido por la modificación específica del residuo de tirosina 302 mediante nitración, inhibiendo la actividad enzimática de OAS-A1 porque reduce drásticamente la capacidad de enlace del sustrato y la estabilización del cofactor PLP necesario para su actividad. La isoforma citosólica OAS-A1 es la principal enzima OASTL de *Arabidopsis*, de modo que un control eficiente de su actividad es crucial para mantener la homeostasis de cisteína bajo condiciones de estrés (Lopez-Martin *et al.*, 2008a). Esta modificación post-traduccional de OAS-A1 mediante nitración puede representar un rápido y eficiente mecanismo regulador para controlar la biosíntesis de cisteína y glutatión en respuesta a distintos factores de estrés (Alvarez *et al.*, 2011b).

2.6. Glutatión y fitoquelatinas

La cisteína es uno de los precursores de la síntesis del tripéptido γ-glutamilcisteinil-glicina, glutatión o GSH (Foyer *et al.*, 2001; Noctor *et al.*, 2002), que es el principal tiol de bajo peso molecular presente en las plantas. Este tripéptido, que se encuentra en equilibrio con su forma oxidada GSSG, actúa como antioxidante manteniendo el balance redox celular, es esencial en procesos de destoxificación de metales pesados y xenobióticos, actúa como cofactor en diversos procesos metabólicos, y como forma de almacén y de transporte del azufre reducido. Además, el glutatión participa en la regulación del crecimiento y desarrollo de las plantas (Droux, 2004; Meyer y Hell, 2005; Foyer y Noctor, 2011).

La sínteis de glutatión tiene lugar a través de dos etapas enzimáticas sucesivas dependientes de ATP, que son catalizadas por las enzimas γ-glutamil cisteína sintasa o γ-glutamil cisteína liasa (GSH1 o γ-ECS, EC 6.3.2.2) y glutatión sintetasa (GSH2, EC 6.3.2.3). La primera de estas enzimas cataliza la formación del pseudopéptido γ-glutamil-cisteína (γ-EC) a partir de L-glutamato y L-cisteína en presencia de ATP. En la mayoría de las plantas, GSH1 se localiza en los plastidios. En el segundo paso, la glutatión sintetasa une L-glicina al extremo carboxi-terminal del dipéptido γ-EC en una reacción dependiente de ATP. Esta segunda enzima se ha descrito en el citosol y en los plastidios, de manera que más del 90% está en el citosol y menos del 10% en los cloroplastos (Wachter *et al.*, 2005; Gromes *et al.*, 2008).

La sínteis de glutatión está limitada por la enzima GSH1, cuya actividad presenta retroinhibición por glutatión y se induce en distintas condiciones de estrés. En *Arabidopsis,* GSH1 es un monómero que contiene un puente disulfuro intramolecular cuya reducción inhibe fuertemente la actividad de la enzima debido a los cambios conformacionales del monómero. En otras plantas como *Brassica juncea*, intervienen

dos puentes disulfuro intramoleculares en la regulación de la actividad de la GSH1 (Jez *et al.*, 2004; Hothorn *et al.*, 2006; Hicks *et al.*, 2007; Gromes *et al.*, 2008). La enzima GSH2 tiene un *loop* rico en glicina que cubre el sitio activo de la enzima, pero no se ha descrito que esté sometido a regulación redox (Wang y Oliver, 1997).

El par redox GSH/GSSG protege al citosol y otros compartimentos celulares de las especies reactivas del oxígeno (ROS) como el radical superóxido, peróxido de hidrógeno o radicales libres, mediante el ciclo ascorbato-GSH (Mullineaux y Rausch, 2005; Rausch y Wachter, 2005). Las ROS se forman en la célula en numerosos procesos como el transporte electrónico durante la fotosíntesis y la respiración mitocondrial, y en otros compartimentos como los peroxisomas, implicados en procesos de óxido-reducción. La generación intracelular de ROS es una respuesta común a condiciones de estrés bióticos o abióticos tales como la infección por patógenos, la salinidad o los metales pesados, y en todas ellas el GSH juega un papel esencial en la protección de la planta.

Por otra parte, el glutatión forma conjugados (GS-X) por la acción de la enzima glutatión S-tranferasa (GST) con compuestos xenobióticos, como herbicidas y toxinas. Estos conjugados son transportados desde el citosol al interior de las vacuolas mediante transportadores de tipo ABC para su posterior degradación (Rea *et al.*, 1998; Dixon *et al.*, 2002).

El glutatión es además el precursor de las fitoquelatinas (PCs), que intervienen en la destoxificación de los metales pesados. La quelación de los metales en el citosol por ligandos de alta afinidad parece ser el mecanismo más importante que presentan las plantas en respuesta a metales pesados, de tal forma que este proceso reduce de 10 a 1000 veces la toxicidad de los metales pesados en el citosol (Sirko y Gotor, 2007).

Las fitoquelatinas se caracterizan por tener una estructura general del tipo $(\gamma\text{-Glu-Cys})_n\text{-Gly}$. El GSH es el precursor de las fitoquelatinas que son sintetizadas por la enzima fitoquelatina sintasa. Esta enzima es un polipéptido de 50 kDa que cataliza la transferencia de una unidad de γ-EC procedente de GSH a una molécula de GSH o de PC (Ducruix *et al.*, 2006). Metales tóxicos como el níquel, cadmio, mercurio o arsénico son eficientemente detoxificados via glutatión y PCs debido a la alta afinidad de estos polímeros por los metales pesados (Howe y Merchant, 1992; Cobbett y Goldsbrough, 2002).

El paso limitante para la biosíntesis de fitoquelatinas en respuesta a metales pesados parece ser la disponibilidad de sulfuro para la síntesis de cisteína (Goldsbrough, 2000). En condiciones normales, la cantidad de cisteína que se sintetiza es suficiente para la formación de los distintos compuestos azufrados necesarios para el funcionamiento de la planta. Sin embargo, en condiciones de estrés se tiene que inducir la expresión de distintos genes involucrados en la asimilación del sulfato para producir suficiente cantidad de cisteína, como ocurre en plantas de *A. thaliana* tratadas con

cadmio (Dominguez-Solis *et al.*, 2001; Howarth *et al.*, 2003a; Dominguez-Solis *et al.*, 2004). Del mismo modo, la expresión de los genes GSH1 y GSH2 se induce en respuesta a Cd para asegurar la síntesis de GSH en cantidad suficiente (Xiang y Oliver, 1998). El metal, una vez complejado con las fitoquelatinas, es transportado a las vacuolas para disminuir su toxicidad efectiva en el citosol. Existen transportadores dependientes de ATP que permiten la entrada del complejo PC-Cd formado en el citosol y de PC libre a la vacuola, para su posterior degradación (Van der Zaal *et al.*, 1999). En *Arabidopsis* se ha descrito que la enzima γ-glutamil transpeptidasa 4 (GGT4) interviene en la degradación de dichos conjugados en la parte interna del tonoplasto (Van der Zaal *et al.*, 1999; Grzam *et al.*, 2007).

2.7. Catabolismo de cisteína

En condiciones normales el contenido de cisteína en la planta se mantiene a niveles muy bajos debido a su toxicidad, por tanto la homeostasis de cisteína se regula de forma muy precisa en el citosol, compartimento responsable de la mayor tasa de síntesis de cisteína. Esta toxicidad se debe a que los tioles son susceptibles de ser oxidados dando lugar a especies con azufre en estado de oxidación más alto (especies reactivas del azufre). Estas especies son capaces de inhibir a enzimas y pueden ser consideradas otra clase de agentes productores de estrés oxidativo (Giles y Jacob, 2002). Además la cisteína libre, pero no el glutatión puede potenciar las propiedades pro-oxidantes del ión ferroso mediante la reacción de Fenton (Vanlerberghe *et al.*, 2002; Jacob *et al.*, 2003; Park y Imlay, 2003). Se ha calculado que los niveles de cisteína son al menos 10 veces más bajos que los de glutatión (Meyer *et al.*, 2001; Krueger *et al.*, 2009). Para mantener niveles no tóxicos de cisteína se han descrito varios tipos de enzimas responsables de la degradación de cisteína en diferentes especies de plantas (Papenbrock *et al.*, 2007).

Las L-Cys desulfhidrasas (DESs, EC 4.4.1.1) catalizan la formación de piruvato, amonio y sulfuro a partir de la cisteína y requieren PLP como cofactor. Esta actividad fue descrita por primera vez por Harrington y Smith (Harrington y Smith, 1980) que encontraron en células de tabaco que el sulfuro y el piruvato se producían en cantidades equimolares a partir de L-Cys. Posteriormente, la existencia de la actividad L-Cys desulfhidrasa fue observada en varios estudios usando diferentes especies de plantas (Rennenberg y Filner, 1983; Rennenberg *et al*, 1987). Sin embargo, los genes y las proteínas correspondientes a esta actividad no se llegaron a identificar (Schmidt, 2005). En esta tesis se ha llevado a cabo la purificación y caracterización de la proteína DES1, poniéndose de manifiesto que es la única L-Cys desulfhidrasa citosólica descrita hasta la fecha en *Arabidopsis thaliana* (Alvarez *et al.*, 2010).

Además de las enzimas L-Cys desulfhidrasas, las D-Cys desulfhidrasas (EC 4.4.1.15) usan específicamente D-Cys como sustrato para generar piruvato, amonio y sulfuro. La actividad D-Cys desulfhidrasa se ha descrito en diferentes organismos fotosintéticos como *Spinacia oleracea*, *Chlorella fusca*, *Cucurbita pepo, Cucumis sativus* y en cultivos de células en suspensión de *Nicotiana tabacum* (Rennenberg *et al*, 1987). Esta actividad presenta diferentes pH óptimos y diferente sensibilidad a inhibidores con respecto a la L-Cys desulfhidrasa (Rennenberg *et al*, 1987; Schmidt, 1982). En *A.thaliana,* se han identificado dos genes, At1g48420 y At3g26115, que codifican proteínas que presentan esta actividad dependiente de PLP (Riemenschneider *et al.*, 2005b). El papel fisiólogico de la D-Cys y de la actividad D-Cys desulfhidrasa están todavía por clarificar (Papenbrock *et al.*, 2007).

Otro tipo de enzimas que intervienen en la degradación de la cisteína son las L-Cys desulfurasas (EC 2.8.1.7) que liberan alanina y azufre elemental a partir de la cisteína y usan también PLP como cofactor. Estas proteínas son conocidas como NifS-like, ya que está actividad se describió por primera vez en la proteína NifS de *Azotobacter vinelandii*, que interviene en la biosíntesis de las agrupaciones sulfo-férricas de la nitrogenasa (Zheng *et al.*, 1993; Schmidt, 2005). Las plantas contienen tres proteínas NifS-like que están localizadas en la mitocondria (mtNifS/AtNFS1) (Kushnir *et al.*, 2001), en el cloroplasto (cpNifS/AtNFS2) (Leon *et al.*, 2002; Pilon-Smits *et al.*, 2002), y en el citosol (ABA3) (Heidenreich *et al.*, 2005). mtNifS proporciona azufre para la formación de biotina y el ensamblaje de las agrupaciones sulfo-férricas de las proteínas citosólicas y mitocondriales. cpNifS es necesaria para la biosíntesis de las agrupaciones sulfo-férricas en el cloroplasto y también interviene en la síntesis de tiamina. La tercera proteina NifS-like, ABA3, es citosólica e interviene en el último paso de la biosíntesis de ABA. No participa en la formación de las agrupaciones de Fe-S, pero es requerida para la sulfuración del cofactor de molibdeno (Van Hoewyk *et al*., 2008).

3. RESPUESTA INMUNE EN PLANTAS

Las plantas están expuestas al ataque de un amplio espectro de patógenos y herbívoros. De ellos, solo una pequeña proporción es capaz de invadir la planta y utilizarla como fuente de energía, ya que la planta posee mecanismos de defensa constitutivos e inducibles que impiden el desarrollo de los patógenos.

Algunos patógenos han evolucionado para evitar el reconocimiento por parte del hospedador, de forma que pueden retrasar las respuestas de defensas de la planta hasta que dejan de ser efectivas dando lugar a una interacción planta-patógeno compatible. Existen distintos tipos de interacción planta-patógeno en función de la estrategia de ataque y estilo de vida del patógeno: necrótrofas, biótrofas y hemibiótrofas (Tabla 1).

Los mecanismos de resistencia en plantas pueden ser divididos en dos categorías: resistencia basal o resistencia inducida por PAMPs y resistencia inducida por efector.

Tabla 1. Tipos de interacción planta-patógeno.

	NECRÓTROFA	BIÓTROFA	HEMIBIÓTROFA
Estrategias de ataque	Secretan enzimas degradadoras de pared. Toxinas.	Contacto íntimo intracelular con células de planta.	Fase inicial biótrofa y luego necrótrofa.
Características de la interacción	Matan los tejidos de la planta y luego los colonizan. Maceración de tejidos.	Las células de la planta quedan vivas. Daño mínimo.	Células vegetales vivas en la etapa inicial. Gran daño posterior.
Rango de huésped	Amplio	Restringido	Intermedio
Ejemplos	*Erwinia* (bacteria) *Botrytis* (hongo)	Nematodos endoparásitos Virus *Pseudomonas* (bacteria)	*Phytophtora infestans* (hongo) *Pseudomonas* (bacteria)

3.1. Resistencia basal o resistencia inducida por PAMPs

Las plantas pueden reconocer estructuras invariables o patrones moleculares asociados al patógeno (PAMPs: *pathogen-associated molecular patterns*) e inducir una respuesta que conduce a la resistencia inducida por PAMPs (PTI: *PAMP-triggered immunity*) (Nurnberger *et al.*, 2004; Ausubel, 2005; Zipfel y Felix, 2005; Chisholm *et al.*, 2006). Los receptores de reconocimiento de PAMPs están anclados a la membrana (PRRs: *pattern recognition receptors*) y controlan el espacio exterior mediante unión directa con los PAMPs o por asociación con proteínas de unión a PAMPs (Zipfel, 2008; Boller y Felix, 2009). Los receptores PRRs más caracterizados son los RLK, que son receptores quinasa que contienen un dominio con repeticiones ricas en leucina (LRR), como el receptor de la flagelina FLS2, que actúa junto con otro receptor RLK llamado BAK1, y el receptor del factor de elongación bacteriano EF-Tu llamado EFR (Gomez-Gomez y Boller, 2000; Kunze *et al.*, 2004; Chinchilla *et al.*, 2006; Zipfel *et al.*, 2006; Chinchilla *et al.*, 2007). *Arabidopsis* contiene más de 600 receptores RLKs. Otros PRRs son proteínas de membrana con dominios extracitoplasmáticos del tipo LysM, como CEBiP o CERK1 que se unen al PAMP fúngico quitina (Kaku *et al.*, 2006; Miya *et al.*, 2007). El reconocimiento de estos PAMPs por los receptores PRRs induce una vía de señalización intracelular dependiente de MAP quinasas (Asai *et al.*, 2002) (Figura 6). En esta cascada de señalización intervienen factores de transcripción específicos de plantas, como los pertenecientes a la superfamilia WRKY. Estos factores de

transcripción poseen motivos de dedos de zinc a través de los cuales interaccionan con las cajas W existentes en las regiones promotoras de muchos genes de defensa, incluidos los genes *PR (Pathogenesis-Related) 1* y *5*. Otra respuesta temprana inducida por PAMPs es la generación extracelular de especies reactivas del oxígeno por la NADPH oxidasa localizada en la membrana. Por otro lado, la síntesis y deposición del poliglucano callosa en el espacio extracelular a través de la callosa sintasa es una respuesta de defensa más tardía que permite el fortalecimiento de la pared celular.

Durante la evolución, los microorganismos han desarrollado mecanismos para evitar la respuesta de defensa de la planta introduciendo proteínas efectoras que son secretadas por los patógenos al interior de las células del hospedador para interferir en la señalización de la respuesta inmune suprimiendo la PTI y potenciando la infección. Los patógenos son por lo tanto virulentos y las proteínas efectoras como AvrPto, AvrPtoB y HopM1 son factores de virulencia necesarios para que se dé la enfermedad (Figura 6) (Nomura *et al.*, 2006).

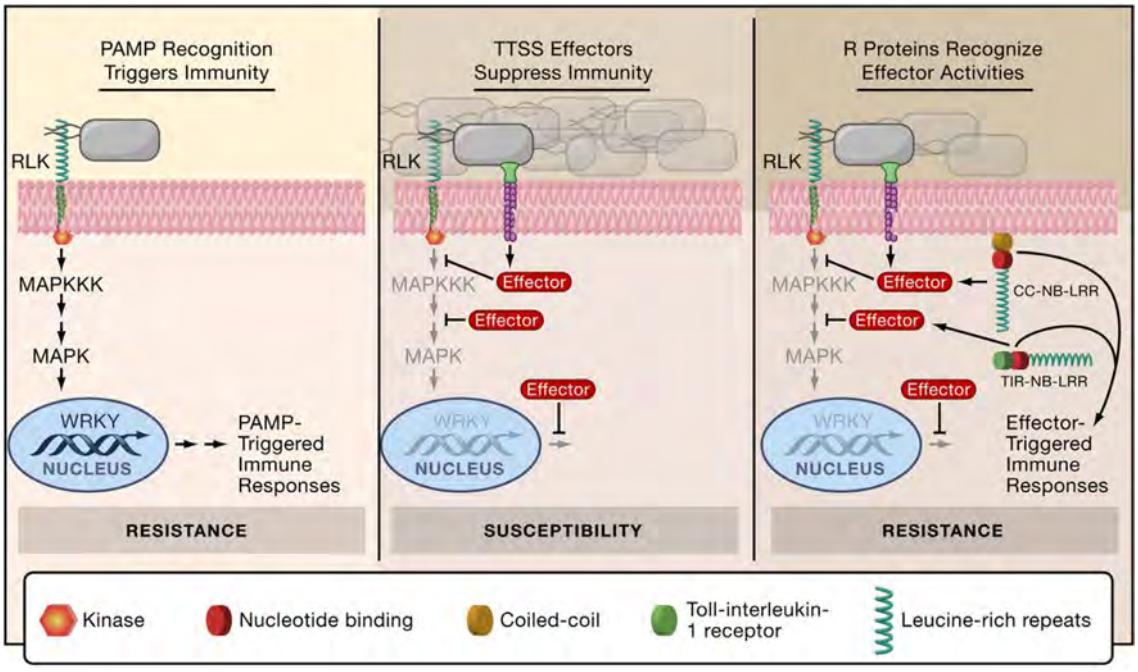

Figura 6. Co-evolución de los sistemas de establecimiento de la infección y tipos de reconocimiento (Chisholm *et al.*, 2006). En el primer caso, el reconocimiento de PAMPs por un receptor extracelular tipo RLK dispara la resistencia basal (PTI), y se pone en marcha la cascada de señalización dependiente de MAP quinasas. En segundo lugar, una bacteria patógena usa el sistema de secreción tipo III para introducir una proteína efectora que suprime la respuesta basal e induce enfermedad. En el tercer caso, las proteínas de resistencia (CC-NB-LRR y TIR-NB-LRR) de la planta reconocen a las proteínas efectoras correspondientes del patógeno y restaura la respuesta de resistencia inducida en este caso por efector (ETI).

3.2. Resistencia inducida por efector

Algunos cultivares de plantas han desarrollado mecanismos de resistencia específicos basados en la interacción gen a gen (Staskawicz *et al.*, 1995; Jones y Dangl, 2006). Durante la respuesta inducida por efector (ETI: *effector-triggered immunity*), una proteína efectora (AvrRpm1, AvrB, AvrRpt2, AvrPto, etc.) procedente del patógeno es reconocida directa o indirectamente por una proteína R de resistencia de la planta y esta interacción inicia el programa de defensa de la planta lo suficientemente rápido como para detener la propagación del patógeno (Figura 6) (Dangl y Jones, 2001; Chisholm *et al.*, 2006; Jones *et al.*, 2006). Las proteínas R han sido agrupadas en numerosas clases según la homología de secuencia (Figura 7). El grupo más amplio se denomina NB-LRR y contiene un dominio central de unión a nucleótidos (NB) más un dominio carboxilo terminal con repeticiones ricas en leucinas (LRR) (Fluhr, 2001; Belkhadir *et al.*, 2004). A este grupo pertenece al menos la mitad de las posibles proteínas R en *Arabidopsis.* Este grupo puede ser subdividido en dos, aquellos que poseen en el extremo amino terminal una región homóloga al receptor Toll/ Interleucina-1 (TIR) y un segundo subgrupo que posee un dominio *coiled coil* (CC) en el extremo amino (Meyers *et al.*, 1999; Pan *et al.*, 2000). La mayoría de las proteínas R intracelulares pertenecen a estos dos subgrupos, siendo el subgrupo de receptores TIR el más abundante en *Arabidopsis* (Meyers *et al.*, 2003; Takken y Tameling, 2009). Por otro lado, existen receptores intracelulares que son proteínas quinasas como PTO, cuya unión con el receptor intracelular tipo NB-LRR PRF es necesaria para que se dé resistencia (Ronald *et al.*, 1992; Martin *et al.*, 1993). Existen también receptores en la membrana plasmática que presentan dominios LRR, como CF-2 y XA21. XA21 presenta además un dominio intracelular serina/ treonina quinasa (Figura 7) (Kajava, 1998; Dangl y Jones, 2001).

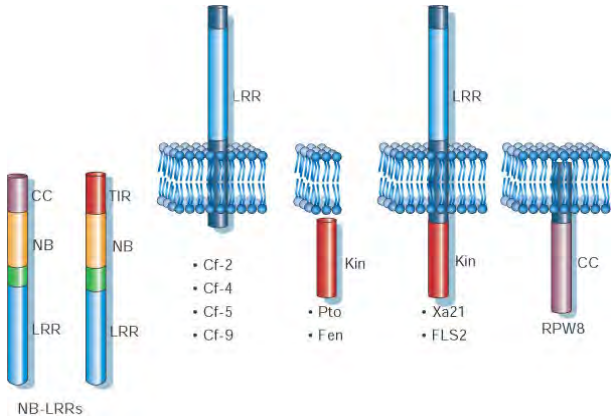

Figura 7. Representación de la localización y estructura de las principales clases de proteínas de resistencia de plantas (Dangl y Jones, 2001).

3.3. La explosión oxidativa y la respuesta hipersensible

Una de las primeras respuestas a la infección por patógenos es la producción de especies reactivas del oxígeno (Lamb y Dixon, 1997). Se han propuesto varias funciones de las ROS en la defensa de las plantas frente a los patógenos (Figura 8). En primer lugar, pueden afectar directamente a los microorganismos. El radical superóxido puede dismutar espontáneamente a peróxido de hidrógeno, que es relativamente estable y tóxico para los microbios al nivel en que es producido en plantas (Chen y Schopfer, 1999). En segundo lugar, las ROS unen covalentemente algunos elementos de la pared celular como glicoproteínas estructurales, y son indispensables para la polimerización de la lignina, de manera que permiten reforzar la pared celular como barrera de defensa (Huckelhoven, 2007). En tercer lugar, hay que destacar su función en señalización mediando la activación de genes de defensa, el control redox de factores de transcripción o la interacción con otros componentes de señalización como las cascadas de fosforilación. El peróxido de hidrógeno se considera la ROS más importante implicada en señalización. Algunas proteínas de defensa, como la fenilalanina amonio liasa (PAL) y la glutatión S-transferasa, así como la cascada de señalización de las MAP quinasas se activan tras la adición de peróxido de hidrógeno (Mou *et al.*, 2003; Yoshioka *et al.*, 2003).

La mayoría de las respuestas de defensa se activan en los dos mecanismos de resistencia, PTI y ETI, pero sus cinéticas son distintas (Tao *et al.*, 2003). Hay dos fases de producción de ROS: una primera fase transitoria e inespecífica que tiene lugar a los pocos minutos de la interacción con el patógeno y una segunda fase más intensa y prolongada en el tiempo que ocurre horas después del ataque del patógeno. La primera fase de explosión oxidativa se da tanto en la PTI como en la ETI, de forma que a los pocos minutos del ataque del patógeno, la producción de ROS es inducida a tan alto nivel que satura los mecanismos antioxidantes existentes en la planta. Solo en la ETI ocurre una segunda explosión oxidativa disparada tras el reconocimiento de la proteína efectora por parte de la proteína R (Tsuda y Katagiri, 2010).

Ambas fases de explosión oxidativa ocurren como consecuencia de un flujo de calcio hacia el interior celular. El Ca^{2+} no solo funciona como un inductor de la explosión oxidativa, sino que también actúa como una molécula señalizadora aguas abajo de dicha explosión (Asai y Yoshioka, 2008). Se han descrito varios mecanismos que intervienen en la producción de ROS durante la interacción plata-patógeno como peroxidasas apoplásticas, amino oxidasas y, principalmente, NADPH/NADH oxidasas (Figura 8) (Torres y Dangl, 2005). Las NADPH-oxidasas están codificadas por los genes *Rboh* (*Respiratory burst oxidase homolog*) en distintas especies de plantas y tienen en el extremo amino terminal un motivo de unión a Ca^{2+}, sugiriendo que el calcio tiene un importante papel en la regulación de esta actividad (Keller *et al.*, 1998).

Además los genes *Rboh* se inducen transcripcionalmente por patógenos y elicitores fúngicos. En *Arabidopsis,* AtrbohD y AtrbohF son las NADPH-oxidasas responsables de casi toda la producción de ROS durante la PTI y ETI (Yoshioka *et al.*, 2001; Simon-Plas *et al.*, 2002; Yoshioka *et al.*, 2009; Torres, 2010).

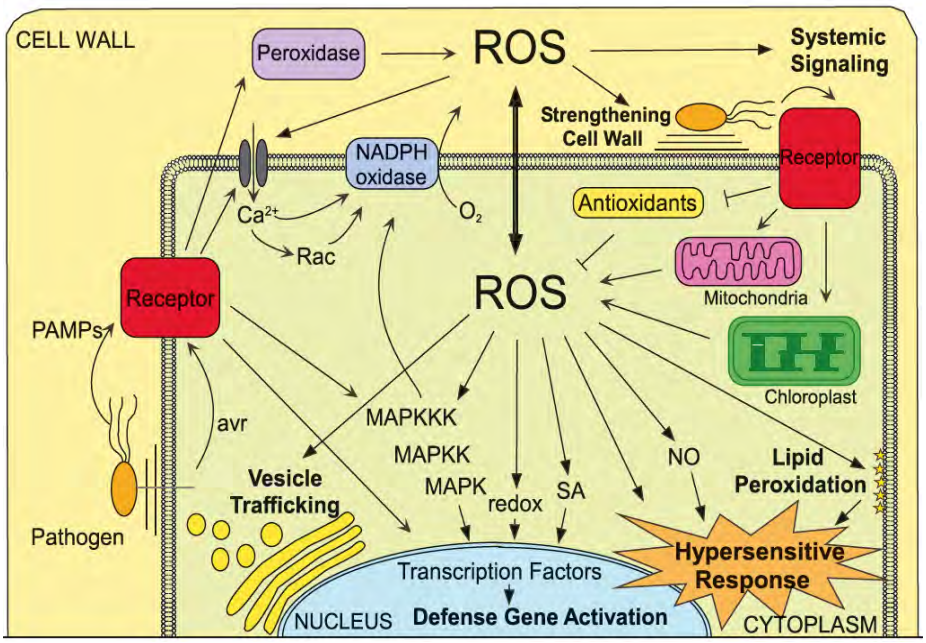

Figura 8. Producción de ROS tras el reconocimiento del patógeno y funciones asociadas a la activación de las defensas de plantas (Torres, 2010).

La segunda fase de producción de ROS promueve la respuesta hipersensible (HR) que es una forma de muerte celular programada típica de la ETI y causa en las células infectadas una muerte celular rápida que limita la expansión del patógeno biótrofo (Mur *et al.*, 2000; Delledonne *et al.*, 2001). Esta segunda fase se acompaña de una acumulación de óxido nítrico (NO) y ácido salicílico (SA) que promueven de forma coordinada la HR. Por tanto, la explosión oxidativa es necesaria pero no suficiente para disparar la HR, y se ha descrito que el NO coopera con las ROS en la activación de esta respuesta. Como se ha indicado, el NO puede reaccionar con el radical superóxido para formar peroxinitrito, que no solo interviene en la nitración de tirosinas de las proteínas para regular su actividad, sino que también es relevante para la HR y para la expresión de distintos genes de defensa (Figura 8) (Heath, 2000; Alamillo y Garcia-Olmedo, 2001; Torres *et al.*, 2006; Torres, 2010).

3.4. Función de las hormonas en la respuesta defensiva de las plantas

Las hormonas juegan un papel importante en la regulación de los procesos de desarrollo y de señalización en respuesta a un amplio rango de estreses bióticos o

abióticos. Una compleja red de señalización inducida por distintas fitohormonas regula la resistencia local y sistémica frente a la invasión por patógenos. La interacción entre las diferentes rutas de hormonas permite a la planta responder de forma apropiada a la infección en función del patógeno.

Los componentes clave en la regulación de la ruta de señalización involucrada en la respuesta de defensa a estrés biótico son el ácido salicílico (SA), el ácido jasmónico (JA) y el etileno (ET) (Durrant y Dong, 2004). Estudios recientes muestran que otras hormonas como el ácido abscísico (ABA), auxinas, giberelinas (GA), citoquininas (CK), brasinosteroides (BR) y hormonas peptídicas están también implicadas en las rutas de señalización de defensa de la planta (Bari y Jones, 2009; Pieterse *et al.*, 2009).

La acumulación de ácido salicílico y sus derivados es importante para la resistencia a los patógenos biótrofos que requieren células vivas para su reproducción. Los receptores intracelulares TIR-NBS-LRR, al reconocer las proteínas efectoras diana, estimulan la biosíntesis de SA y la señalización de forma muy efectiva a través de los reguladores EDS1 (*Enhanced Disease Susceptibility 1*) y PAD4 (*Phytoalexin Deficient 4*) que son necesarios para la acumulación de SA y controlan el alcance de la respuesta hipersensible y muerte celular en torno al foco de infección (Feys *et al.*, 2001; Wiermer *et al.*, 2005; Wirthmueller *et al.*, 2007). En el caso de los receptores CC-NBS-LRR, la señal es transmitida a través del regulador NDR1 (*Non-race-specific Disease Resistance 1*) (Aarts *et al.*, 1998). Ambas rutas convergen en PBS2 (Figura 9) (Glazebrook, 2001). El SA produce cambios en el estado redox celular que permite la activación de NPR1 (*Nonexpressor of Pathogenesis Genes 1*) mediante su disociación en monómeros por reducción de los puentes disulfuro intermoleculares. El monómero de NPR1 pasa al núcleo y modula la expresión de genes de defensa, ya que interacciona con los factores de transcripción de tipo TGA que están involucrados en la activación de los genes PR (Fan y Dong, 2002; Johnson *et al.*, 2003; Mou *et al.*, 2003; Kesarwani *et al.*, 2007). Se ha demostrado que tras la translocación de los monómeros activos de NPR1 al núcleo, se induce la expresión de los factores de transcripción WRKY que juegan un importante papel en la regulación de la respuesta de defensa dependiente de SA (Wang *et al.*, 2006). Por ejemplo, WRKY70 actúa como un regulador positivo de las defensas dependiente de SA y regula negativamente las defensas dependientes de JA (Li *et al.*, 2006).

Por otra parte, el JA y sus conjugados cooperan con el etileno para regular la resistencia a patógenos necrótrofos e insectos herbívoros. Todas las respuestas conocidas donde interviene el JA en *Arabidopsis* requieren la función de COI1 (*Coronatine Insensitive 1*), que codifica una proteína F-*box* que interviene en la proteolisis, determinando la especificidad de sustrato de la ubiquitina ligasa SCF (*Skp-Cullin-F-box*). El conjugado JA-Ile o MeJA que son las formas bioactivas del JA

percibidas por la planta, estimula la forma activa SCF^{COI1} que funciona como una ubiquitín ligasa que media la degradación de JAZ, represor de activadores de la transcripción de los genes de respuesta a JA (*PDF1.2, Thi2.1, VSP2*, etc.) (Lorenzo *et al.*, 2004; Lorenzo y Solano, 2005; Chini *et al.*, 2007; Katsir *et al.*, 2008).

El etileno es percibido por una familia de receptores de etileno asociados al retículo endoplasmático (ETRs). En ausencia de etileno, los ETRs activan al regulador negativo CTR1 que reprime al regulador positivo EIN2. En presencia de etileno, éste se une a sus receptores y CTR1 se disocia de ellos y se inactiva, de manera que se inicia la cascada regulatoria donde EIN2 favorece la acumulación del factor de transcripción EIN3. Éste induce la expresión de genes tempranos de respuesta a etileno entre los que se encuentra ERF1, que activa la expresión de los genes tardíos de respuesta al etileno como quitinasas y defensinas (Figura 9) (Berrocal-Lobo *et al.*, 2002; Kendrick y Chang, 2008; Yoo y Sheen, 2008).

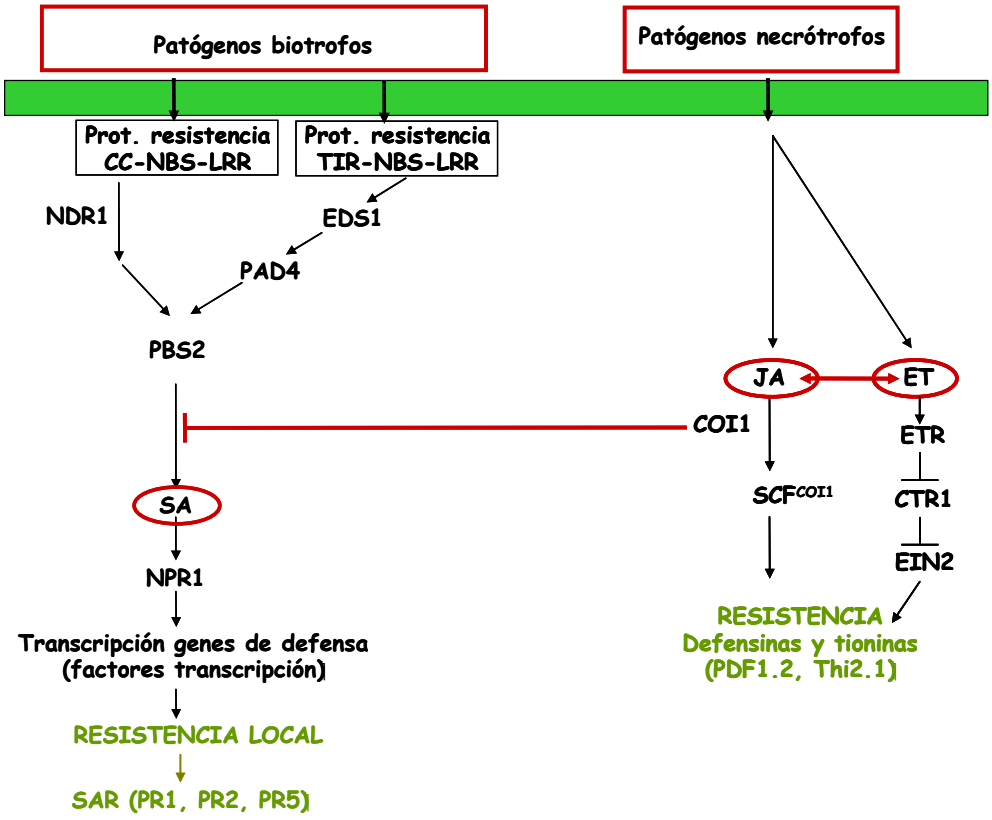

Figura 9. Esquema de las rutas de señalización mediadas por hormonas de la respuesta de defensa a estrés biótico en *Arabidopsis* (Adaptado de Dickinson, 2003).

Frente a un estímulo determinado ocurren múltiples *loops* de retroalimentación tanto positivos como negativos, que permiten ajustes rápidos a nivel celular. Las plantas modulan la abundancia relativa de SA, JA y ET y de esta forma modifican la expresión

de los genes de defensa y coordinan la interacción de la rutas de señalización que activan una respuesta de defensa efectiva frente al ataque por varios tipos de patógenos. Mientras que las respuestas a JA y ET son sinérgicas en la mayoría de los casos, entre la respuesta a SA y JA/ET se dan interacciones antagónicas, lo cual es explotado por algunos patógenos para promover la enfermedad, como en el caso de *Pseudomonas syringae* pv. *tomato* que produce la fitotoxina coronatina mimetizando a JA-Ile y de este modo suprime la respuesta de defensa dependiente de SA (Laurie-Berry *et al.*, 2006; Pieterse *et al.*, 2009).

3.5. Resistencia sistémica adquirida (SAR)

La resistencia sistémica adquirida es una forma de inducción de resistencia en tejidos sistémicos después de una infección primaria. Los patógenos avirulentos que activan la ETI y dan lugar a la respuesta hipersensible son potentes inductores de SAR (Durrant y Dong, 2004), aunque la PTI puede también dispararla (Mishina y Zeier, 2006; Tsuda *et al.*, 2008). La resistencia sistémica adquirida se ha demostrado en muchas interacciones planta-patógeno y se caracteriza por restringir el crecimiento del patógeno, suprimir el desarrollo de los síntomas de la enfermedad y proteger a la planta de la infección por otros patógenos (Hammerschmidt, 1999). Aunque la resistencia sistémica adquirida es efectiva frente a un amplio rango de patógenos como virus, bacterias, hongos y oomicetos, parece que es más efectiva frente a patógenos hemibiótrofos (Ton *et al.*, 2002). El espectro de patógenos frente al que la SAR es efectiva depende de cada especie de planta.

La resistencia sistémica adquirida se acompaña por un incremento local y sistémico de los niveles endógenos de SA. Está asociada con una activación coordinada de un conjunto específico de genes que codifican las proteínas PR, alguna de las cuales poseen actividad antimicrobiana. La aplicación exógena de SA o de sus análogos funcionales induce la SAR y activa a estos genes *PR*.

Aunque el SA es la molécula esencial para la transducción de la señal en la SAR, parece que no es el transportador sistémico de la señal. Se ha propuesto que el ácido benzoico, precursor del SA, puede ser transportado por el floema de *Nicotiana tabacum* tras la infección con el virus del mosaico del tabaco (TMV) hasta los tejidos dianas, donde posteriormente es convertido a SA por la enzima BA2H (Ácido benzoico-2-hidroxilasa) (Leon *et al.*, 1993; Shulaev *et al.*, 1995). También se ha demostrado que el metil-salicilato, sintetizado a partir del SA localmente en las hojas infectadas, es transportado a través de la planta, llegando a los tejidos diana donde se vuelve a convertir en SA (Seskar *et al.,* 1998). De hecho, en tabaco la enzima SA metil transferasa y la MeSA esterasa unida a la proteína 2 (SA-Binding proteína 2: SABP2) son esenciales para la SAR a nivel local en las hojas infectadas y en el tejido sistémico,

respectivamente (Forouhar *et al.*, 2005; Park *et al.*, 2007; Bari y Jones, 2009). Se han propuesto también otras moléculas señalizadoras como el JA, un factor derivado de glicerolípidos no definido y una proteína de transferencias de lípidos (Vlot *et al.*, 2008).

3.6. Resistencia inducida por azufre (SIR)

El azufre es uno de los macroelementos esenciales para la vida de la planta. Recientemente se ha demostrado que este macroelemento juega un papel importante en la defensa de la planta a patógenos. Este fenómeno se denomina resistencia inducida por azufre (SIR: *sulfur-induced resistance*) y también es conocido como defensa potenciada por azufre (SED: *sulfur-enhanced defense*) (Bloem *et al.*, 2007).

En plantas hay varios compuestos de defensa azufrados (SDCs: *Sulfur-containing defence compounds*) que podrían estar involucrados en la activación de SIR y tienen un fuerte impacto en la defensa potencial de la planta como glutatión, glucosinolatos, proteínas ricas en cisteína, fitoalexinas, azufre elemental y H_2S. Los SDCs sintetizados constitutivamente forman parte de los mecanismos de defensa constitutivos en la planta, mientras que los SDCs inducidos por patógenos contribuyen considerablemente a la resistencia inducida. La distribución y estructura química de los SDCs es muy variable en plantas. Aunque no se ha demostrado que los SDCs se generen frente a patógenos específicos, sí que intervienen en el curso de las interacciones específicas gen a gen entre el hospedador y el patógeno (Rausch y Wachter, 2005).

La cisteína es un componente clave, ya que actúa como indicativo de la actividad y flujo del azufre en la formación de SDCs (Hell, 1997). La biosíntesis de cisteína está muy regulada debido a su toxicidad y, de hecho, cuando se suministra a la planta un exceso de sulfato, no se incrementa la cantidad de cisteína y, en cambio sí se incrementa el contenido en glutatión (Papenbrock *et al.*, 2007). El GSH no solo protege al citosol y otros compartimentos celulares frente a las ROS o interviene en los mecanismos de destoxificación, sino que podría intervenir en la defensa frente a patógenos determinando el estado redox de NPR1, de manera que un cambio en el potencial redox del GSH después de la infección puede ser responsable de la activación de NPR1 (Mou *et al.*, 2003). Los niveles y el estado redox del GSH son importantes para determinar la capacidad de la planta de combatir a los patógenos. Durante el ataque de patógenos tanto los niveles de cisteína como de glutatión actúan como marcadores del metabolismo del azufre en la planta, siendo componentes clave para la inducción de la SIR/SED. Se ha observado que niveles elevados de Cys y GSH se correlacionan con una supresión de los síntomas de desarrollo y la carga viral en *Cucurbita pepo* (Zechmann *et al.,* 2007). Resultados similares se han obtenido en plantas de tabaco tratadas con GSH (Gullner *et al.*, 1999). Recientemente se ha demostrado la existencia

de una clara correlación entre la activación del metabolismo de la Cys y GSH y la inducción de la SIR/SED durante la interacción compatible planta-virus en *Nicotiana tabacum* (Holler *et al.*, 2010). En esta tesis hemos demostrado que la cisteína citosólica juega un papel importante en el establecimiento y señalización de la respuesta de la planta a patógenos, de manera que una regulación precisa de la homeostasis de cisteína citosólica es crítica para que se dé correctamente la respuesta de la planta a patógenos. Esta función se puede deber a la cisteína *per se* o como generador de compuestos azufrados (Alvarez *et al.*, 2011a).

Los glucosinolatos son derivados de aminoácidos y contienen al menos dos átomos de azufre por molécula. Los glucosinolatos y las tioglucosidasas operan como un componente dual en la defensa de la planta. Cuando hay daño celular los glucosinolatos citosólicos son convertidos por las tioglucosidasas vacuolares en una gran variedad de productos tóxicos volátiles como nitrilos, isotiocianatos, tiocianatos, etc. Estos productos no solo representan componentes de defensa activa frente a herbívoros sino que también suprimen el crecimiento microbiano (Lambrix *et al.*, 2001; Wittstock y Halkier, 2002; Clay *et al.*, 2009). Recientemente, se ha demostrado que los glucosinolatos presentan también actividad antifúngica (Bednarek *et al.*, 2009).

Las tioninas y defensinas son proteínas ricas en azufre que presentan estructuras complejas estabilizadas por puentes disulfuro intramoleculares (Thomma *et al.*, 2002). Algunas isoformas contribuyen a los sistemas de defensas constitutivos pero otras son fuertemente inducidas en respuesta al ataque de patógenos (Hilpert *et al.*, 2001; Nibbe *et al.*, 2002). Ensayos *in vitro* han establecido que estos péptidos son tóxicos para un amplio rango de hongos, y en algunos casos, son tóxicos para bacterias Gram positivas, levaduras, insectos o nematodos.

El ataque de patógenos y elicitores bióticos inducen la síntesis de fitoalexinas como la camalexina que es un derivado azufrado del triptófano. Las fitoalexinas son tóxicas para varias especies de hongos y bacterias (Kliebenstein, 2004).

El H_2S también interviene en la defensa de la planta, aunque su función como SDC no está clara todavía (Rausch y Wachter, 2005). La cantidad de sulfuro liberado desde el hospedador es tóxico para el patógeno dependiendo de su concentración en el sitio de ataque del patógeno y de la capacidad del patógeno para metabolizarlo. El H_2S podría actuar indirectamente induciendo la formación de ROS debido a su alto potencial oxidativo. Las ROS ejercerían un efecto tóxico directo en el crecimiento de microorganismos. Por otro lado, el azufre elemental (S^0) es el fungicida más antiguo usado por el hombre y parece que las plantas también lo usan para la defensa. Se desconoce el mecanismo de formación de S^0 en plantas. El H_2S podría ser oxidado directamente en presencia de un donador de electrones hasta S^0 o enzimas como la superóxido dismutasa podrían catalizar la oxidación del HS^- hasta S^0 (Papenbrock *et al.*, 2007). Posiblemente, el glutatión se acumula en sitios específicos y su posterior

degradación conduce a la acumulación local de S^0 (Williams y Cooper, 2004). Se ha demostrado que el S^0 se acumula en los tejidos vasculares de variedades resistentes de cacao y tomate en respuesta a la infección con *Verticillium dahliae* (Williams *et al.*, 2002).

4. AUTOFAGIA EN PLANTAS

La autofagia es un mecanismo universal presente en células eucariotas que consiste en la digestión del contenido celular para el posterior reciclaje de los nutrientes, así como en la degradación de componentes dañados o tóxicos. Es un proceso que se da de forma constitutiva, es decir, ocurre a nivel basal en células en crecimiento, permitiendo el reciclaje de proteínas y orgánulos. Las plantas inducen la autofagia bajo condiciones de estrés como limitación de nutrientes, presencia de drogas y otros tipos de estreses abióticos. Además la autofagia está involucrada en distintos fenómenos fisiológicos como el desarrollo de plantas, la respuesta inmune y la senescencia. Durante la senescencia, el reciclaje de nutrientes a través de la autofagia juega un papel importante en el mantenimiento de la viabilidad, permitiendo que los nutrientes sean utilizados eficientemente (Bassham *et al.*, 2006; Xiong *et al.*, 2007).

En plantas existen dos tipos de autofagia: microautofagia y macroautofagia. Durante la microautofagia, el material es englobado directamente por la vacuola vía invaginación del tonoplasto liberando en el lumen vacuolar una vesícula que contiene los constituyentes citoplasmáticos que son posteriormente digeridos por las hidrolasas residentes en la vacuola. La macroautofagia comienza en el citoplasma, donde los constituyentes citoplasmáticos y los orgánulos son secuestrados en una estructura de doble membrana llamada autofagosoma. La membrana externa se fusiona con el tonoplasto, liberando el cuerpo autofágico que queda rodeado por la membrana interna. Las hidrolasas vacuolares degradan el cuerpo autofágico y los productos de degradación son transportados de vuelta al citosol (Bassham *et al.*, 2006).

Durante la senescencia de células fotosintéticas de *Arabidopsis* y soja, se han observado pequeñas vacuolas asociadas a la senescencia (SAV) con una intensa actividad proteolítica, que se localizan en la periferia citoplasmática. Tienen un pH más ácido que la vacuola central y acumulan la cisteín-proteasa asociada a la senescencia SAG12, que es usada como marcador de senescencia, ya que se induce específicamente durante este proceso y es mínimamente regulada por factores ambientales (Lin y Wu, 2004). Las SAV a veces contienen agregados densos que podrían consistir en material celular parcialmente degradado y es similar al contenido de los autofagosomas tardíos. Estas estructuras son específicas de células senescentes y se utilizan como marcador de senescencia. Su origen es desconocido aunque en algunos casos se relacionan con el proceso autofágico (Otegui *et al.*, 2005). Se ha descrito recientemente en tabaco que

estas vacuolas están involucradas en la degradación de proteínas cloroplásticas durante la senescencia de la hoja (Martinez *et al.*, 2008).

4.1. Maquinaria molecular de la autofagia

Los genes *ATG* son esenciales para la formación del autofagosoma y fueron identificados por primera vez en levaduras, están muy conservados en eucariotas y han contribuido significantemente a la investigación de la autofagia en organismos superiores. En base a la homología de secuencia de las proteínas de levaduras involucradas en la autofagia se han identificado un gran número de proteínas ATG en plantas, en concreto 36 genes *ATG* en el caso de *Arabidopsis*.

Los genes esenciales para la formación del autofagosoma son *ATG1-10, 12-14, 16-18* y *31*. Sus productos pueden ser divididos en cinco grupos funcionales: el complejo quinasa ATG1, el complejo quinasa PI3, el complejo ATG9 y dos sistemas parecidos a los de ubiqutinación (*ubiquitination-like*).

La autofagia tiene lugar a través de cinco fases distintas: iniciación, nucleación, expansión y finalización de vesículas, fusión del autofagosoma/vacuola y degradación del contenido (Figura 12) (Gozuacik y Kimchi, 2004; Thompson y Vierstra, 2005; Mitou *et al.*, 2009; Yoshimoto *et al.*, 2010).

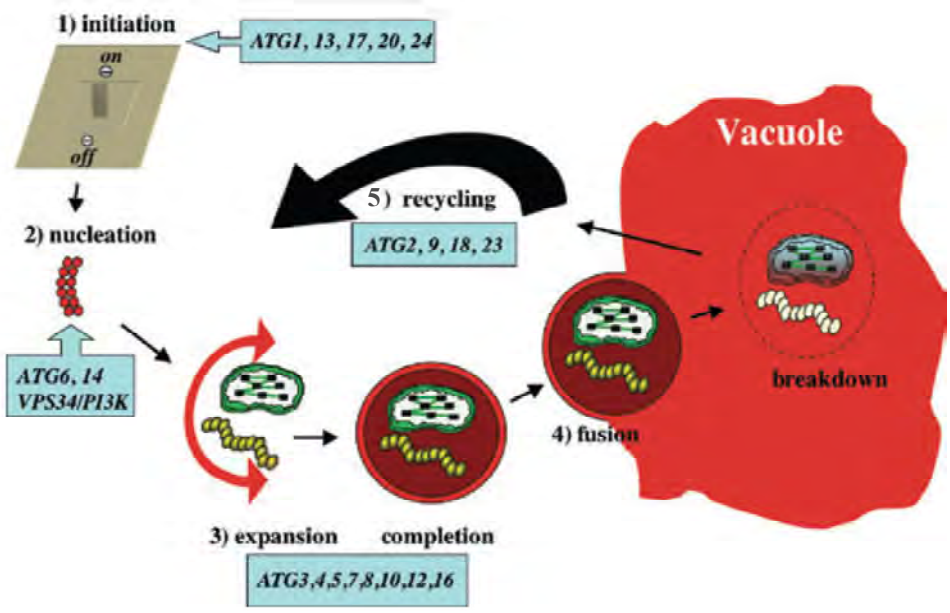

Figura 12. Fases de la autofagia en plantas. Se encuentran representados los genes *ATG* requeridos para cada paso (Seay *et al.*, 2006).

Los grupos funcionales y la maquinaria básica de la autofagia existente en levaduras se pueden extrapolar a plantas para cada una de las fases de la autofagia que se detallan a continuación:

1. Iniciación: La ruta de señalización de TOR y su efecto en autofagia se conserva en plantas (Diaz-Troya *et al.*, 2008; Liu y Bassham, 2010). En este paso interviene la proteína TOR (*target of rapamycin*) que es una serina/treonina quinasa regulada en respuesta a variaciones en aminoácidos, ATP y factores de crecimiento. La represión de la actividad TOR se correlaciona con la estimulación de la autofagia (Noda y Ohsumi, 1998). En levaduras, la inactivación de TOR induce la autofagia mediante al menos dos mecanismos. El primero involucra la activación de los factores de transcripción GCN4 y GLN3 conduciendo a la inducción transcripcional de algunos genes *ATG* (Natarajan *et al.*, 2001). En el segundo mecanismo se produce la modificación dependiente de TOR del complejo de autofagia ATG1/ATG13. En condiciones normales, TOR induce la hiperfosforilación de ATG13 inhibiendo su asociación con ATG1. La inactivación de TOR conduce a una rápida desfosforilación de ATG13 y un incremento en su afinidad por la proteína ATG1. La asociación ATG1-ATG13 induce la autofosforilación y activación de ATG1, promoviendo la autofagia (Kamada *et al.*, 2000; Codogno, 2004).

2. Nucleación: la nucleación de los autofagosomas es iniciada por un complejo proteico que incluye VPS34 (PI3K, fosfatidilinositol 3-quinasa) y ATG6/VPS30. ATG6 interviene en la regulación de la actividad de VPS34. La actividad PI3K de VPS34 conduce a la acumulación de fosfatidilinositol 3-fosfato (PI3P). El PI3P producido sirve como punto de partida para la formación del autofagosoma donde intervienen ATG18 y ATG2 (Petiot *et al.*, 2000; Kihara *et al.*, 2001; Xie y Klionsky, 2007).

3. Expansión y finalización de las vesículas: los dos sistemas de ubiquitinación mencionados anteriormente juegan un papel importante en la biogénesis del autofagosoma. En la primera reacción, ATG12 se conjuga con ATG5 de forma covalente. La reacción de conjugación comienza con la activación de ATG12 por la proteína ATG7 que interviene como una enzima E1 de activación de la ubiquitina. Posteriormente, ATG12 es transferida a ATG10 que actúa como una enzima E2 que conjuga ubiquitina (Shintani *et al.*, 1999; Tanida *et al.*, 1999). Finalmente, ATG12 es covalentemente conjugada a ATG5, dicha conjugación permite la formación y estabilización del complejo formado por ATG12, ATG5 y ATG16 (Figura 13b) (Mizushima *et al.*, 1999). Este complejo proteico es necesario para la segunda reacción de *ubiquitination-like* y permite la elongación de la membrana del autofagosoma. Este complejo se localiza en la membrana externa del autofagosoma en formación y se disocia tan pronto como la vesícula se completa, de manera que tiene un papel regulador más que estructural (Mizushima *et al.*, 2001). En la segunda reacción de *ubiquitination-like* interviene la proteína ATG8 que es procesada a nivel del extremo carboxilo terminal por la cisteín-proteasa ATG4 y deja accesible el residuo de glicina para la conjugación lipídica de ATG8. ATG7 activa a ATG8 y la transfiere a ATG3 que actúa como una proteína E2 específica en la conjugación de ATG8 con el lípido

fosfatidiletanolamina (PE) (Figura 13a). Esta conjugación lipídica es esencial para la formación del autofagosoma, de forma que ATG8 queda anclado covalentemente en la membrana del autofagosoma y es la única proteína autofágica que no es excluida del auofagosoma una vez completado (Figura 13c). Por tanto, ATG8 es un marcador ideal de autofagia. En plantas existen varias isoformas de ATG8, y concretamente en *A.thaliana* hay 9 diferentes. No está claro por qué existen distintos parálogos, puede que se induzcan por diferentes estímulos o en diferentes tejidos y estadíos o que exista una redundancia funcional (Doelling *et al.*, 2002; Ketelaar *et al.*, 2004; Fujioka *et al.*, 2008).

4. Fusión del autofagosoma/vacuola: el autofagosoma se fusiona con la vacuola liberando al interior de la vacuola el cuerpo autofágico. Se conocen algunos factores específicos implicados en este paso. En la organización del sitio de fusión están involucrados un complejo VPS, proteínas RAB GTPasas y las proteínas SNAREs que forman un complejo que sirve de puente entre los dos orgánulos (Darsow *et al.*, 1997; Ungermann y Langosch, 2005).

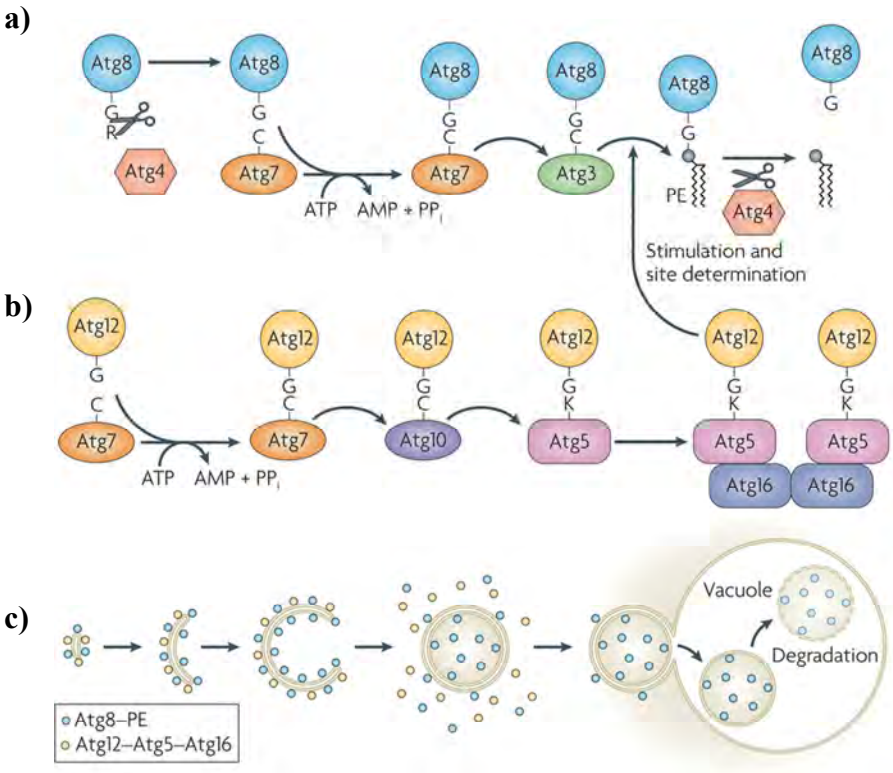

Figura 13. Sistemas de conjugación *ubiquitination-like*. a), Sistema de conjugación de la proteína ATG8. **b)**, Sistema de conjugación de la proteína ATG12. **c)**, Localización de los dos sistemas de conjugación *ubiquitination-like* durante la elongación de la membrana del autofagosoma y fusión con la vacuola.

5. Degradación del contenido y reciclaje: en el lumen de la vacuola, lipasas como ATG15 en levaduras degradan las membranas autofágicas y posteriormente el contenido es catabolizado por enzimas líticas. Los productos de degradación que se obtienen pasan al citosol para ser reutilizados (Kim *et al.*, 2007). ATG9 es una proteína integral de membrana requerida para la distribución y reclutamiento de la membrana en la formación del autofagosoma. En levaduras y *Arabidopsis*, ATG9 interacciona con ATG2 y ATG18 para la correcta localización de ambas proteínas (Bassham, 2007).

4.2. Funciones fisiológicas de la autofagia en plantas

El papel de la autofagia en el desarrollo de la planta fue estudiado usando distintos mutantes en los genes *ATG (atg*). En condiciones ricas en nutrientes, las plantas defectuosas en la autofagia presentan un desarrollo embrionario, germinación, desarrollo de los cotiledones, elongación de la raíz, floración y generación de semillas normales. En cambio, cuando estas plantas crecen en condiciones de déficit de nutrientes (nitrógeno o carbono), se observa una aceleración en la floración, un incremento de la clorosis, una disminución en la producción de semillas, y una inhibición de la elongación de la raíz, entre otros efectos. Cuando se induce la senescencia artificialmente con oscuridad, los niveles de ARNm de marcadores de senescencia como SEN1 o SAG12 incrementan más rápidamente en estos mutantes *atg* que en plantas silvestres, de modo que se observa una aceleración de la senescencia. Todo ello sugiere que la autofagia juega un importante papel en el mantenimiento de una eficiente movilización de los nutrientes en condiciones limitantes de nutrientes o durante la senescencia (Doelling *et al.*, 2002; Hanaoka *et al.*, 2002; Phillips *et al.*, 2008).

Estudios recientes han revelado que la autofagia puede estar involucrada en la degradación de los cloroplastos, que son parcialmente movilizados a la vacuola mediante autofagia a través de cuerpos esféricos llamados RCBs (*Rubisco-containing bodies*) (Ishida *et al.*, 2008).

La autofagia también interviene durante el estrés oxidativo. Plantas transgénicas silenciadas para *ATG18a* (RNAi-*AtATG18a)* son más sensibles al estrés oxidativo y acumulan más proteínas oxidadas en comparación con plantas silvestres. Por tanto, la autofagia juega un papel importante en el transporte a la vacuola y la degradación de componentes celulares dañados u oxidados producidos durante el estrés oxidativo (Xiong *et al.*, 2005; Xiong *et al.*, 2007).

El modo en que la autofagia contribuye a la respuesta inmune es muy diverso. Distintos estudios demuestran que durante la respuesta hipersensible frente a patógenos, la autofagia interviene regulando negativamente la muerte celular programada, limitando así la HR en el sitio de infección del patógeno. En plantas de tabaco donde se

había silenciado el gen *ATG6,* la muerte celular causada por el Virus del Mosaico del Tabaco (TMV) se propagaba desde el sitio de infección a otras partes de la hoja no infectadas, e incluso a hojas superiores, aunque el virus por sí mismo no se movía del sitio de infección. Estos resultados muestran que ATG6 o procesos regulados por ATG6 restringen la muerte celular durante la HR (Liu *et al.*, 2005). Se ha observado un fenotipo similar en plantas de *Arabidopsis* infectadas con *Pseudomonas syringae pv. tomato avrRpm1* donde se había silenciado también el gen *ATG6.* Todo ello sugiere que la autofagia interviene reteniendo la muerte celular, posiblemente a través de la degradación de señales celulares que promueven la muerte celular (Lenz *et al.*, 2011). Los mutantes *atg7* y *atg9* de *Arabidopsis* presentan una PCD más reducida que las plantas silvestres durante la respuesta hipersensible inducida por *Pseudomonas syringae pv. tomato* que expresa el gen de avirulencia *avrRps4*. Esto sugiere, por el contrario, que la autofagia funciona potenciando la muerte celular durante la HR. Todos estos resultados indican que el papel de la autofagia en la ejecución de la muerte celular en plantas durante la ETI es dependiente del tipo de receptor involucrado en el reconocimiento del efector (Hofius *et al.*, 2009). La autofagia también interviene regulando negativamente la muerte celular durante las infecciones que dan lugar a formación de lesiones o un daño considerable en el hospedador. En mutantes *atg* infectados con patógenos virulentos biótrofos como *Hyaloperonospora arabidopsidis,* que no produce daños celulares, no se observaron diferencias con respecto a las plantas silvestres. En cambio, cuando la infección se realizaba con el hongo necrótrofo *Alternaria brassicicola,* la necrosis se extendió considerablemente desde el sitio de infección en los mutantes (Lenz *et al.*, 2011).

Como se ha descrito anteriormente el SA es un elemento clave en la respuesta de la planta al estrés y al ataque por patógenos. Además se ha descrito que el SA controla la expresión de distintos genes durante el desarrollo de la senescencia. La concentración de SA endógeno es cuatro veces superior en hojas senescentes de *Arabidopsis* que en hojas no senescenctes, e induce la expresión de varios genes asociados a la senescencia (*SAGs*) como *PR1*, *quitinasas*, *SAG12*, etc. Estos SAGs requieren la presencia de SA para alcanzar la máxima expresión (Morris *et al.*, 2000). En condiciones ricas en nutrientes, varios mutantes *atg* exhiben un fenotipo de senescencia temprana debido a una aceleración de la muerte celular programada. El fenotipo de senescencia temprana, así como la expansión de la HR en estos mutantes, es suprimido en mutantes defectuosos en la biosíntesis de SA o en la señalización por SA. Estos resultados indican que la excesiva señalización por SA es la causa principal del fenotipo de muerte celular temprana. Se ha propuesto que la autofagia es inducida por el SA vía NPR1 y a su vez que la autofagia regula negativamente la señalización por SA limitando la senescencia y la muerte celular inducida por patógenos (Figura 14) (Yoshimoto *et al.*, 2009; Yoshimoto *et al.*, 2010; Lenz *et al.*, 2011).

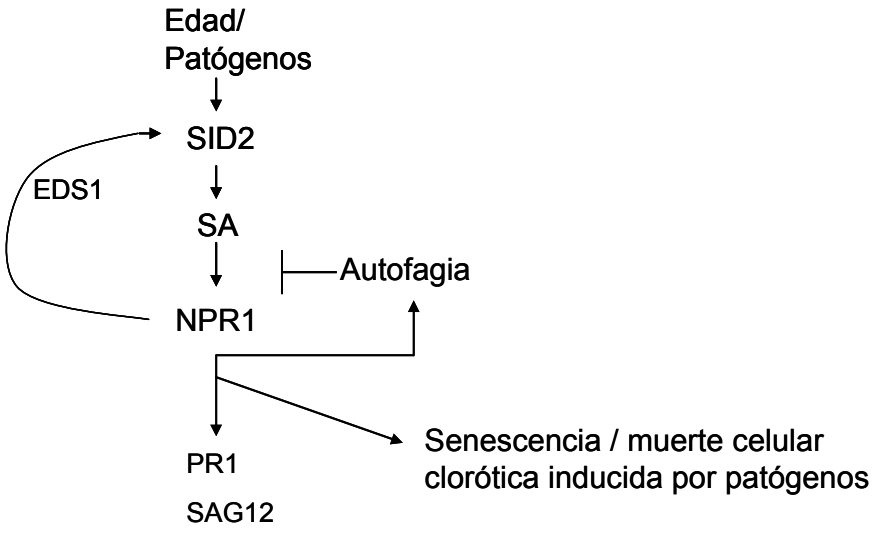

Figura 14. Modelo hipotético del papel de la autofagia durante senescencia y la muerte celular inducida por el ataque de patógenos (Yoshimoto *et al*., 2009).

Objetivos

El principal objetivo de nuestro grupo es clarificar la dinámica de la ruta de biosíntesis de cisteína en cada compartimento subcelular y la función fisiológica de la cisteína en la respuesta de la planta a señales intracelulares y medioambientales. El hecho de que la biosíntesis de cisteína sea llevada a cabo por una compleja variedad de isoformas que están presentes en el citosol, los cloroplastos y las mitocondrias, resultando en diferentes conjuntos o "pools" subcelulares de cisteína, sugiere que estas isoformas específicas pueden tener funciones desconocidas particulares en el metabolismo y señalización de la célula. En base a todo ello se establecieron los siguientes objetivos:

1. Caracterización de una isoforma minoritaria con homología a las OASTLs, localizada en el citosol de *A.thaliana*, denominada CS-LIKE, y análisis detallado de los mutantes insercionales deficientes en esta proteína.

2. Determinación de la implicación de la cisteína citosólica en las respuestas de la planta a la infección por patógenos, mediante el análisis de los mutantes deficientes en la OASTL mayoritaria OAS-A1 y en la proteína DES1.

3. Caracterización de la modificación post-traduccional de la proteína OAS-A1 mediante nitración.

Capítulo I

Una nueva enzima con actividad L-Cys desulfhidrasa regula la homeostasis de cisteína en *A.thaliana*

An *O*-Acetylserine(thiol)lyase Homolog with L-Cysteine Desulfhydrase Activity Regulates Cysteine Homeostasis in Arabidopsis[1][C][W]

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Cysteine (Cys) occupies a central position in plant metabolism due to its biochemical functions. Arabidopsis (*Arabidopsis thaliana*) cells contain different *O*-acetylserine(thiol)lyase (OASTL) enzymes that catalyze the biosynthesis of Cys. Because they are localized in the cytosol, plastids, and mitochondria, this results in multiple subcellular Cys pools. Much progress has been made on the most abundant OASTL enzymes; however, information on the less abundant OASTL-like proteins has been scarce. To unequivocally establish the enzymatic reaction catalyzed by the minor cytosolic OASTL isoform CS-LIKE (for Cys synthase-like; At5g28030), we expressed this enzyme in bacteria and characterized the purified recombinant protein. Our results demonstrate that CS-LIKE catalyzes the desulfuration of L-Cys to sulfide plus ammonia and pyruvate. Thus, CS-LIKE is a novel L-Cys desulfhydrase (EC 4.4.1.1), and we propose to designate it DES1. The impact and functionality of DES1 in Cys metabolism was revealed by the phenotype of the T-DNA insertion mutants *des1-1* and *des1-2*. Mutation of the *DES1* gene leads to premature leaf senescence, as demonstrated by the increased expression of senescence-associated genes and transcription factors. Also, the absence of DES1 significantly reduces the total Cys desulfuration activity in leaves, and there is a concomitant increase in the total Cys content. As a consequence, the expression levels of sulfur-responsive genes are deregulated, and the mutant plants show enhanced antioxidant defenses and tolerance to conditions that promote oxidative stress. Our results suggest that DES1 from Arabidopsis is an L-Cys desulfhydrase involved in maintaining Cys homeostasis, mainly at late developmental stages or under environmental perturbations.

Sulfur is a macronutrient that is essential for plant growth and development. The most abundant form of sulfur in nature and the source of sulfur for plants is sulfate; this form is reduced and assimilated into Cys. In addition to its role as an amino acid in proteins, Cys functions as a precursor for a huge number of essential biomolecules, such as vitamins and cofactors (Droux, 2004; Wirtz and Droux, 2005), antioxidants like glutathione, which is regarded as the major determinant of cellular redox homeostasis (Meyer and Hell, 2005; Mullineaux and Rausch, 2005), and many defense compounds (Rausch and Wachter, 2005). All of these biomolecules contain sulfur moieties that act as functional groups and are derived from Cys. The biosynthesis of Cys is accomplished through two sequential reactions catalyzed by the enzymes Ser acetyltransferase (SAT; EC 2.3.1.30), which synthesizes the intermediary product *O*-acetylserine (OAS), and *O*-acetylserine(thiol)lyase (OASTL; EC 2.5.1.47), which combines sulfide with OAS to produce Cys. Together, these two enzymes form the heterooligomeric Cys synthase complex, which was first described in bacteria and has since been extensively studied in plants (Droux et al., 1998; Wirtz and Hell, 2006). Plant cells contain different SAT and OASTL enzymes localized in the cytosol, plastids, and mitochondria, resulting in a complex variety of isoforms and different subcellular Cys pools. Arabidopsis (*Arabidopsis thaliana*) is the best investigated plant system; its genome encodes five different *SAT* (Howarth et al., 2003) and eight *OASTL* (Wirtz et al., 2004) genes.

The OASTL enzymes belong to the superfamily of β-substituting Ala synthases (Hatzfeld et al., 2000; Supplemental Table S1). The proteins encoded by *OAS-A1* (At4g14880), *OAS-B* (At2g43750), and *OAS-C* (At3g59760) are considered authentic OASTLs that are located in the cytosol, plastids, and mitochondria of Arabidopsis cells, respectively (Wirtz et al., 2004). A property that defines an authentic OASTL is the ability to interact with SAT, and this can be demonstrated using different approaches (Bogdanova and Hell,

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[C] Some figures in this article are displayed in color online but in black and white in the print edition.

[W] The online version of this article contains Web-only data.

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1997; Droux et al., 1998; Wirtz et al., 2001; Bonner et al., 2005; Heeg et al., 2008). The OASTL family also includes another isoform located in the mitochondria encoded by *ATCYS-C1* (At3g61440), which actually functions as a β-cyanoalanine synthase (CAS; EC 4.4.1.9; Hatzfeld et al., 2000; Yamaguchi et al., 2000). According to the Genevestigator microarray database (www.genevestigator.com) and numerous studies from a large number of laboratories, the three OASTL isoforms OAS-A1, OAS-B, and OAS-C and the CAS isoform ATCYSC1 are the most highly expressed in Arabidopsis cells. The remaining OASTL-like proteins located in the cytosol (encoded by *ATCYS-D1* [At3g04940], *ATCYS-D2* [At5g28020], and *CS-LIKE* [At5g28030]) and in the plastid (encoded by *CS26* [At3g03630]) are expressed at much lower levels and have not been characterized enzymatically. Their functions in plant sulfur metabolism are currently unknown. In particular, the CS-LIKE protein was identified by sequence homology upon the completion of the sequencing of the Arabidopsis genome. Because of its cytosolic localization, it is thought to have an auxiliary function with respect to the major cytosolic isoform OAS-A1.

While Cys and glutathione are usually considered to be components of cellular antioxidant systems, emerging evidence points to the existence of reactive sulfur species. Thiols are easily oxidized to species with sulfur in higher oxidation states. These species subsequently inhibit enzymes; therefore, they can be considered to be a new class of oxidative stress agents (Giles and Jacob, 2002). Furthermore, thiol groups can generate reactive oxygen species (ROS) through the Fenton reaction catalyzed by iron. Evidence has shown that free Cys, but not glutathione, exacerbates the prooxidant properties of ferrous iron (Vanlerberghe et al., 2002; Jacob et al., 2003; Park and Imlay, 2003). Therefore, under nonstress conditions, Cys levels should remain low; these levels have been calculated to be at least 10 times lower than those of glutathione, which ranges in the millimolar order (Meyer et al., 2001; Krueger et al., 2009).

Several candidate Cys-degrading enzymes have been reported to exist in different plant species (Papenbrock et al., 2007). One class of these enzymes is the L-Cys desulfhydrases (DESs; EC 4.4.1.1), which catalyze the formation of sulfide, ammonia, and pyruvate in a stoichiometric ratio of 1:1:1 and require pyridoxal phosphate (PLP) as a cofactor. Such activity has been found in plants; however, the corresponding genes and pure proteins have remained unisolated (Schmidt, 2005). In addition to DESs, D-Cys desulfhydrases (EC 4.4.1.15) that specifically use D-Cys as their substrate and are completely distinct from their L-counterparts have been shown to exist in several plant species (Rennenberg et al., 1987). In Arabidopsis, two genes have been identified, At1g48420 and At3g26115, that encode proteins possessing this activity (Papenbrock et al., 2007). Another Cys desulfuration reaction catalyzed by the L-Cys desulfurases (EC 2.8.1.7) occurs in iron-sulfur cluster biosynthesis and involves the formation of L-Ala and elemental sulfur or H_2S from Cys, also with PLP as a cofactor. These proteins are now known as NifS-like proteins (Schmidt, 2005), and they likely provide sulfur for the formation of biotin and Fe-S cluster assembly for mitochondrial and cytosolic proteins and for thiamine synthesis in the chloroplast (Van Hoewyk et al., 2008).

In this work, our aim was to characterize in detail the protein named CS-LIKE. We expressed this protein in bacteria and found that CS-LIKE is a DES enzyme (EC 4.4.1.1). We confirmed this assertion by characterizing T-DNA-tagged mutants, which were found to have increased levels of Cys and glutathione that allow the mutants to tolerate oxidative stress conditions.

RESULTS

Expression and Functional Characterization of the CS-LIKE Recombinant Protein

With the aim of identifying the enzymatic reaction that is catalyzed by the minor cytosolic OASTL isoform in Arabidopsis, CS-LIKE, we expressed the corresponding full-length cDNA (*At5g28030*) in *Escherichia coli* as a 6× His N-terminally tagged recombinant protein. The recombinant protein was purified to apparent homogeneity by affinity chromatography using nickel-nitrilotriacetic acid agarose (Ni-NTA) resin under nondenaturing conditions to preserve the enzymatic activity. We selected the elution fractions containing the unique protein band of estimated size as visualized by SDS-PAGE (Supplemental Fig. S1) and discarded the elution fractions containing any other protein bands to be sure that the enzymatic reaction we were measuring was due to the recombinant CS-LIKE. We were able to recover 0.16 mg of purified protein per 100 mL of *E. coli* culture with a yield of 55% (Table I).

Different enzymatic assays were carried out with the bacterial crude extracts and purified recombinant protein. While we were unable to detect β-cyanoalanine synthase activity, we measured both OASTL and DES activities, both in bacterial extracts and with the purified recombinant CS-LIKE. After the purification process, the specific DES activity of the protein increased 4.5-fold and the specific OASTL activity diminished 7.3-fold (Table I). These results indicate that CS-LIKE protein predominantly catalyzes the degradation of Cys rather than its synthesis and that the OASTL reaction is a side reaction of the DES reaction. We also measured the D-Cys desulfhydrase activity of the recombinant protein and determined a specific activity of 4.7 units mg^{-1}, which is about 10-fold lower than the measured DES activity.

The DES activity was quantified as the release of sulfide from L-Cys as described previously (Riemenschneider et al., 2005a); however, it has been

Table I. *Purification of CS-LIKE expressed in E. coli*

The gene encoding CS-LIKE was cloned in the pDEST17 vector using the Gateway technology. The protein was expressed in *E. coli* and purified using the Ni-NTA purification system as described in "Materials and Methods." DES and OASTL activities were measured as described.

Purification Step	Protein	Specific Activity		Total Activity		Purification Factor		Yield	
		DES	OASTL	DES	OASTL	DES	OASTL	DES	OASTL
	mg	*units mg*$^{-1}$		*units*				*%*	
Crude extract	1.29	10.0	8.8	13.0	11.3	–	–	–	–
Ni-NTA chromatography	0.16	44.5	1.2	7.1	0.2	4.5	0.1	54.6	1.7

reported that the degradation of L-Cys can release, besides sulfide, L-Ala or pyruvate plus ammonium (Rennenberg et al., 1987; Zheng et al., 1993). In characterizing the products of the enzymatic reaction catalyzed by CS-LIKE, we failed to detect L-Ala by HPLC, but we easily detected pyruvate and ammonium. The formation of pyruvate and ammonium in the reaction mixture containing L-Cys and recombinant CS-LIKE protein was detected as consumption of NADH by reactions catalyzed by lactate dehydrogenase and Glu dehydrogenase, respectively, as described in "Materials and Methods." In 1 mL of reaction mixture containing 8 μg of the recombinant protein in the presence of a saturating concentration of Cys, we detected the formation of 96.44 nmol of ammonium and 104.17 nmol of pyruvate. Furthermore, in the same reaction mixture, we colorimetrically quantified the release of 105.18 nmol of sulfide. Thus, we conclude that CS-LIKE catalyzes the desulfuration of L-Cys to equimolar amounts of pyruvate plus NH_3 and SH_2 and that CS-LIKE is a DES.

The conclusion that CS-LIKE is a DES is supported by the alignment of CS-LIKE protein with the major OASTL isoforms (Supplemental Fig. S2). Residues 74 to 78 of OAS-A1, with the sequence TSGNT, form a loop that is involved in sulfur incorporation into Cys (Bonner et al., 2005). This loop is highly conserved across the major OASTL isoforms, but in CS-LIKE, Gly replaces Ser-75. Furthermore, CS-LIKE has nonconservative amino acid changes in the β8A-β9A loop, which is highly conserved in true OASTL enzymes and has been shown to be important for the interaction with SAT (Bonner et al., 2005). In contrast, all of the residues that are responsible for PLP anchoring are conserved in CS-LIKE, suggesting that the desulfuration of L-Cys is dependent on PLP. We have observed that 100 μM aminooxyacetate, a known inhibitor of PLP-dependent enzymes, causes an 80% reduction in DES enzyme activity of recombinant CS-LIKE. This result demonstrates that PLP is a required cofactor for this activity, as has been reported previously for other plant systems (Rennenberg et al., 1987).

We characterized the DES and OASTL reactions catalyzed by purified recombinant CS-LIKE by performing substrate saturation experiments (Supplemental Fig. S3). The K_m value for L-Cys in the DES reaction is 13-fold lower than that for OAS in the OASTL reaction (Table II), indicating a much higher affinity of CS-LIKE for L-Cys as a substrate. Although V_{max} is higher for the Cys synthesis reaction, the catalytic efficiencies (k_{cat}/K_m) of CS-LIKE for both substrates, L-Cys and OAS, are in the same order of magnitude. These results further reinforce the idea that CS-LIKE protein functions as a DES with a side OASTL reaction.

Based on the experimental data described above, we propose to rename Arabidopsis CS-LIKE protein DES1, and we will use this nomenclature for the rest of this article.

Identification and Characterization of Arabidopsis *des1* Mutants

With the aim of determining the function of the DES1 protein in Arabidopsis, T-DNA insertion mutants from the existing collections were screened. The SALK_103855 and RATM13-2715-1_G mutants, designated *des1-1* and *des1-2*, were selected for further

Table II. *Catalytic properties of the CS-LIKE recombinant protein for the DES and OASTL enzymatic reactions*

The substrate saturation experiments were performed with CS-LIKE recombinant protein and Cys as a substrate for the DES reaction; OAS and H_2S were used as cosubstrates for the OASTL reaction. The kinetic constants were calculated from the Hanes plots of substrate concentration versus substrate concentration/initial velocity.

DES Reaction			OASTL Reaction			
K_{mcys}	V_{max}	k_{cat}/K_{mcys}	K_{mOAS}	K_{mH2S}	V_{max}	k_{cat}/K_{mOAS}
mM	*μmol min^{-1} mg^{-1}*	*mM^{-1}min^{-1}*	*mM*	*mM*	*μmol min^{-1} mg^{-1}*	*mM^{-1}min^{-1}*
0.4 ± 0.1	0.04 ± 0.003	3.5	5.2 ± 0.6	0.25 ± 0.01	1.8 ± 0.04	5.9

analysis (Supplemental Fig. S4). Reverse transcription (RT)-PCR analysis was conducted on the homozygous mutant plants using specific primers, and no *DES1* transcript was detected. This result suggests that both *des1-1* and *des1-2* contain knockout mutations. A Southern-blot analysis was also performed to determine the number of T-DNA insertions. A single unique T-DNA insertion site was confirmed to exist in the *des1-1* genome with the appearance of a single hybridization band (Supplemental Fig. S4).

We screened the *des1-1* and *des1-2* mutant plants for potential growth defects and found no significant phenotypic differences from wild-type plants under normal growth conditions, either in soil or on solid Murashige and Skoog (MS) medium in petri dishes. It was apparent, however, that these mutants flowered before wild-type plants. It is well established that late-flowering plants form more leaves (Koornneef et al., 1991). Therefore, we determined the number of rosette leaves of the mutant and wild-type plants at flowering time after being grown under long-day conditions. The mutants produced 14.0 ± 1.5 rosette leaves, while the wild-type plants produced 17.5 ± 1.8 rosette leaves, under our experimental conditions. This result suggests that the *des1* mutants have an early-flowering phenotype. Based on this phenotype, we propose that the mutation in the *DES1* gene leads to premature leaf senescence. Many aspects of the regulatory network controlling leaf senescence in Arabidopsis have been identified, including the increased expression of senescence-associated genes and transcription factors (Lim et al., 2007). To confirm that *des1* mutants initiate leaf senescence earlier, we performed real-time quantitative (q) RT-PCR for the senescence-associated genes *SEN1* and *SAG21* and for *NAP*, which is a member of the NAC transcription factor gene family. We observed an induction of the expression levels of these senescence gene markers in leaves of *des1-1* and *des1-2* plants when compared with their respective wild-type plants at the same developmental stage (Fig. 1), thus confirming the onset of leaf senescence in these mutant plants.

Biochemical characterization of both *des1-1* and *des1-2* plants revealed that the total DES activity was reduced by 20% to 25% in both mutants relative to their respective wild types (Table III). Interestingly, the reduction in total DES activity was the same regardless of the growth stage of the plant, as much in mature plants as in seedlings. This result further confirms that the DES1 protein is a DES enzyme. This finding also suggests that cytosolic DES1 contributes considerably to the total L-Cys desulfuration of the plant. Accordingly, the total amounts of Cys in the *des1-1* and *des1-2* mutants were about 20% and 25% higher, respectively, than those found in their respective wild-type ecotypes, both in mature plants and seedlings. In addition, the glutathione content was also significantly higher (Table III).

We also performed qRT-PCR on the OASTL gene family in the *des1-1* mutant and wild-type plants. We

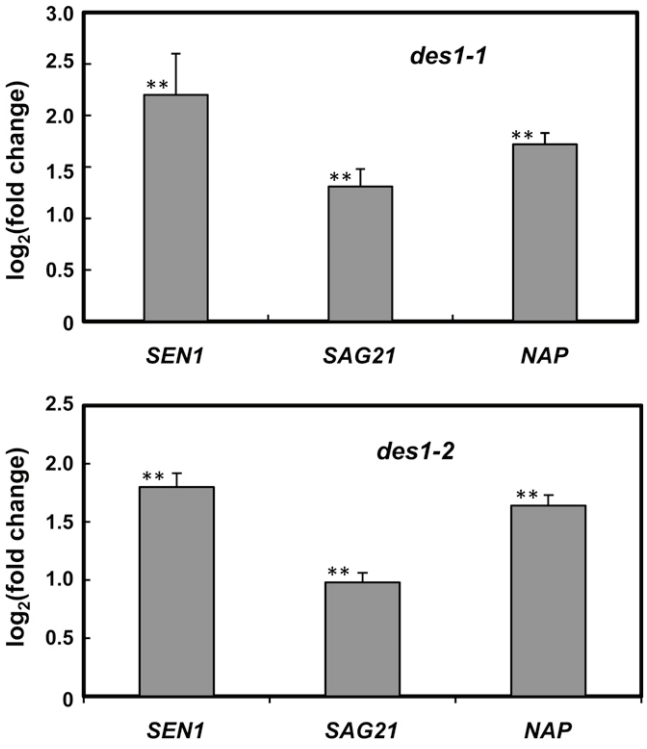


Figure 1. Relative expression levels of senescence-associated genes in *des1* mutant plants. Real-time RT-PCR analysis of the expression of *SEN1* (*At4g35770*), *SAG21* (*At4g02380*), and *NAP* (*At1g69490*) genes was performed in leaves from Col-0 and No-0 wild-type and *des1-1* and *des1-2* mutant plants grown for 5 weeks under control conditions. The transcript levels were normalized to the internal control, the constitutive *UBQ10* gene. Data shown are means + SD of three independent analyses made from RNA obtained from different plants grown in different pots at the same time, and they represent the transcript level of each gene in the mutant plants relative to the transcript level in the corresponding wild-type ecotype. ANOVA was performed using the software OriginPro 7.5. ** $P < 0.05$.

observed opposing regulatory patterns in the transcript levels of the different gene members: a slight repression of the most abundant *OAS-A1* and *OAS-B* genes and a significant induction of the three β-cyanoalanine synthase-like-encoding genes (Fig. 2). The induction of *CYS-C1*, *CYS-D1*, and *CYS-D2* could be an indication that these enzymes possess similar functions to DES1. To check this hypothesis, we measured the total DES activity in their respective previously described deficient mutants (Watanabe et al., 2008a). None of the mutants showed a reduction in DES activity relative to wild-type plants, as was shown for the two *des1* mutants.

Many studies on the regulation of the sulfate assimilatory pathway have shown that there is negative regulation by reduced sulfur-containing compounds and positive regulation by OAS (Hawkesford, 2000; Hawkesford and De Kok, 2006; Kopriva, 2006). Some components of the pathway have been shown to be specifically sensitive to this regulation at the transcript level (Hirai et al., 2003; Nikiforova et al., 2003). Thus, a

Table III. *Enzyme activity and thiol content*

DES enzyme activity and total levels of Cys and glutathione were measured in the leaves of Col-0 and No-0 wild-type and *des1* mutant plants grown for 5 weeks in soil under control conditions and in whole 2-week-old seedlings grown on MS solid medium. Values are means ± SD of four independent experiments made from different plants and seedlings grown in different pots and plates, respectively. ANOVA was performed using the software OriginPro 7.5. ** $P < 0.05$

Plant Line	DES Activity	Total Cys	Total Glutathione
	nmol min^{-1} mg^{-1}	*nmol g^{-1} fresh wt*	
Leaves from 5-week-old plants grown in soil			
Col-0	12.4 ± 0.8	29.8 ± 2.3	302.8 ± 21.8
des1-1	10.1 ± 0.7**	36.1 ± 4.6**	345.2 ± 12.3**
No-0	15.5 ± 0.9	29.0 ± 3.4	322.2 ± 11.3
des1-2	13.1 ± 0.8**	36.2 ± 7.3**	390.0 ± 16.2**
Whole 2-week-old seedlings grown on MS medium			
Col-0	6.9 ± 0.05	47.7 ± 4.1	730.7 ± 51.2
des1-1	5.9 ± 0.1**	58.0 ± 3.8**	867.2 ± 10.2**
No-0	7.15 ± 0.28	50.3 ± 2.4	606.6 ± 14.6
des1-2	6.2 ± 0.4**	62.9 ± 2.8**	693.3 ± 13.7**

lower intracellular content of sulfate, Cys, and glutathione is concomitant with increasing transporter activity predominantly at the level of the mRNA (Buchner et al., 2004). To demonstrate that the 20% to 25% increase in the Cys level found in the *des1* mutants is significant, and therefore to demonstrate the importance of DES1 in plant metabolism, we studied the regulation of sulfate transporter genes. Both ecotype Columbia (Col-0) wild-type and *des1-1* plants were grown simultaneously in soil irrigated with sufficient-sulfate solution (described as control conditions) and low-sulfate solution. Then, we determined the levels of different sulfate transporter genes by qRT-PCR in leaves of both genotypes grown in the two different sulfate conditions. The expression of all of the analyzed transporters responded very differently to sulfur nutrition in *des1-1* mutant plants than in wild-type plants (Fig. 3). In Col-0 plants, a significant increase in expression of the tested sulfate transporters was observed in low-sulfate compared with control conditions, as reported previously (Takahashi et al., 2000; Yoshimoto et al., 2002; Buchner et al., 2004; Kataoka et al., 2004). However, in *des1-1* plants under sulfur limitation, the *SULTR2.1* and *SULTR3.1* transporters were down-regulated, *SULTR2.2* expression was unaffected, and *SULTR1.2* was induced, but to a significantly lower extent than in the wild type.

5′-Adenylylsulfate (APS) reductase is also susceptible to negative control by thiols and positive regulation by sulfur limitation at the mRNA level (Vauclare et al., 2002). Accordingly, we extended the qRT-PCR analysis in Col-0 wild-type and *des1-1* mutant plants

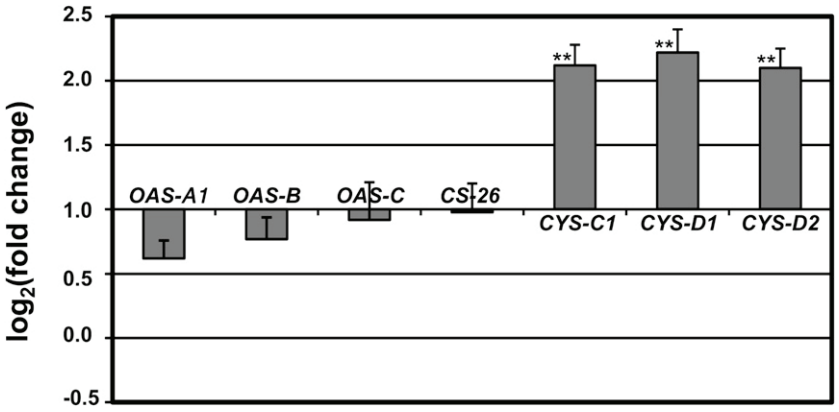


Figure 2. Relative expression levels of the OASTL gene family in the *des1-1* mutant plants. Real-time RT-PCR analysis of the expression of the *OAS-A1* (*At4g14880*), *OAS-B* (*At2g43750*), *OAS-C* (*At3g59760*), *CS-26* (*At3g03630*), *CYS-C1* (*At3g61440*), *CYS-D1* (*At3g04940*), and *CYS-D2* (*At5g28020*) genes was performed in leaves from the wild-type Col-0 and *des1-1* mutant plants grown for 5 weeks under control conditions. The transcript levels were normalized to the constitutive *UBQ10* gene. Data shown are means + SD of three independent analyses made from RNA obtained from different plants grown in different pots at the same time, and they represent the transcript level of each gene in the mutant plants relative to the transcript level in the wild-type plants. ANOVA was performed using the software OriginPro 7.5. ** $P < 0.05$.

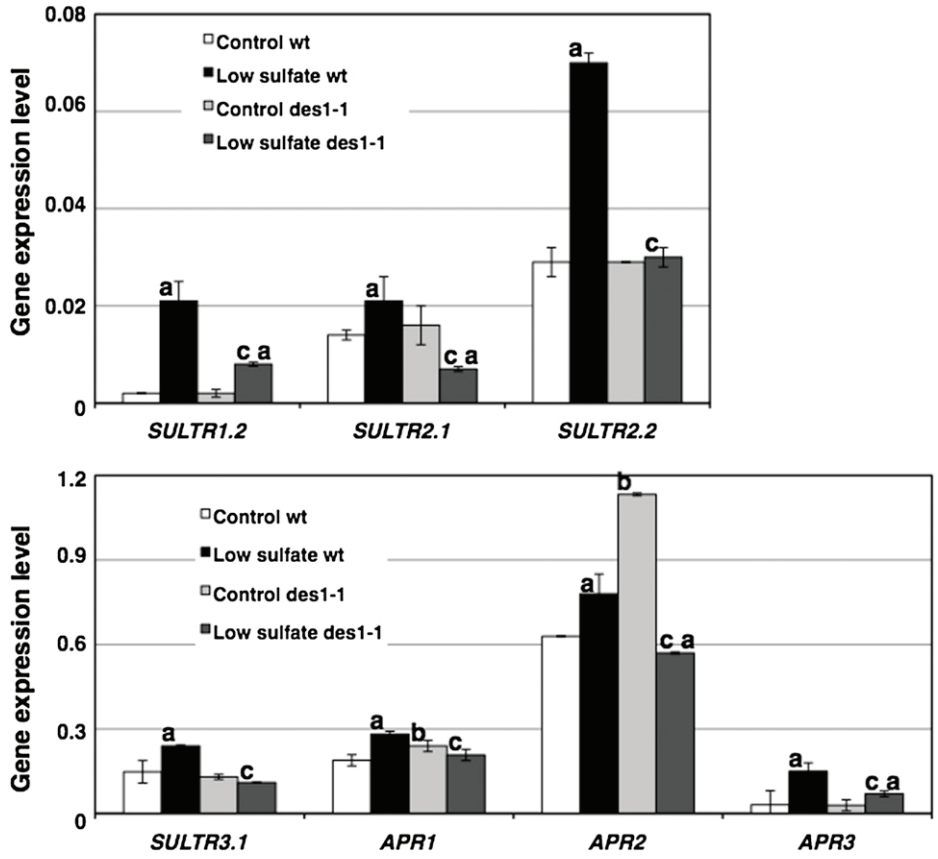


Figure 3. Sulfate regulation of the expression levels of sulfur-responsive genes in the Col-0 wild-type (wt) and *des1-1* mutant plants. Real-time RT-PCR analysis of expression of the *SULTR1.2* (*At1g78000*), *SULTR2.1* (*At5g10180*), *SULTR2.2* (*At1g77990*), *SULTR3.1* (*At3g51895*), *APR1* (*At4g04610*), *APR2* (*At1g62180*), and *APR3* (*At4g21990*) genes was performed in leaves from the Col-0 wild-type and *des1-1* mutant plants grown in soil and irrigated with Hoagland medium (control conditions) or deionized water (low-sulfate conditions). The transcript levels were normalized using the constitutive *UBQ10* gene as an internal control. Data shown are means ± SD of three independent analyses made from RNA obtained from different plants grown in different pots at the same time and are divided into two different graphs due to the different scales. ANOVA was performed using the software OriginPro 7.5. Significant differences between each plant line grown under low-sulfate and control conditions are indicated by the letter a ($P < 0.05$). Significant differences between *des1-1* and wild-type plants grown under control conditions are indicated by the letter b ($P < 0.05$). Significant differences between *des1-1* and wild-type plants grown under low-sulfate conditions are indicated by the letter c ($P < 0.05$).

grown in the two different sulfate concentrations to the three members of the APS reductase gene family from Arabidopsis (Fig. 3). Under conditions of sufficient sulfate, *APR1* and *APR2* expression levels were increased in the *des1-1* mutant with respect to the wild type. Moreover, we observed opposite responses in the wild type and the mutant in the regulation of the expression levels of *APR1* and *APR2*. Both transcripts were up-regulated in the wild type and down-regulated in the *des1-1* mutant under low-sulfate conditions. *APR3* gene expression was induced in both genotypes, but considerably less so in the mutant.

Tolerance of *des1* Mutants to Abiotic Stress

We showed previously that depleting the cytosolic levels of Cys alters the antioxidative capacity of the cytosol in Arabidopsis; as a consequence, there is an increased sensitivity to cadmium (Cd; Lopez-Martin et al., 2008a). We next wanted to study how an increase in the levels of cytosolic Cys, as in the *des1* mutants, would affect Cd sensitivity. We performed a Cd tolerance test comparing the *des1-1* and *des1-2* mutant plants with their respective wild-type ecotypes. Seeds were germinated on solid MS medium containing two concentrations of $CdCl_2$ and allowed to grow for 14 d. On 175 μM Cd, a high proportion of the Col-0 and ecotype Nossen (No-0) wild-type seeds failed to germinate; those that germinated developed chlorotic leaves. In contrast, seeds of both mutants were able to germinate on this Cd concentration and produced green leaves. At 250 μM, the highest tested $CdCl_2$ concentration, the difference between the wild-type ecotypes and their respective mutants was much more remarkable (Fig. 4A). We quantified the tolerance as the ratio between the total number of live seedlings

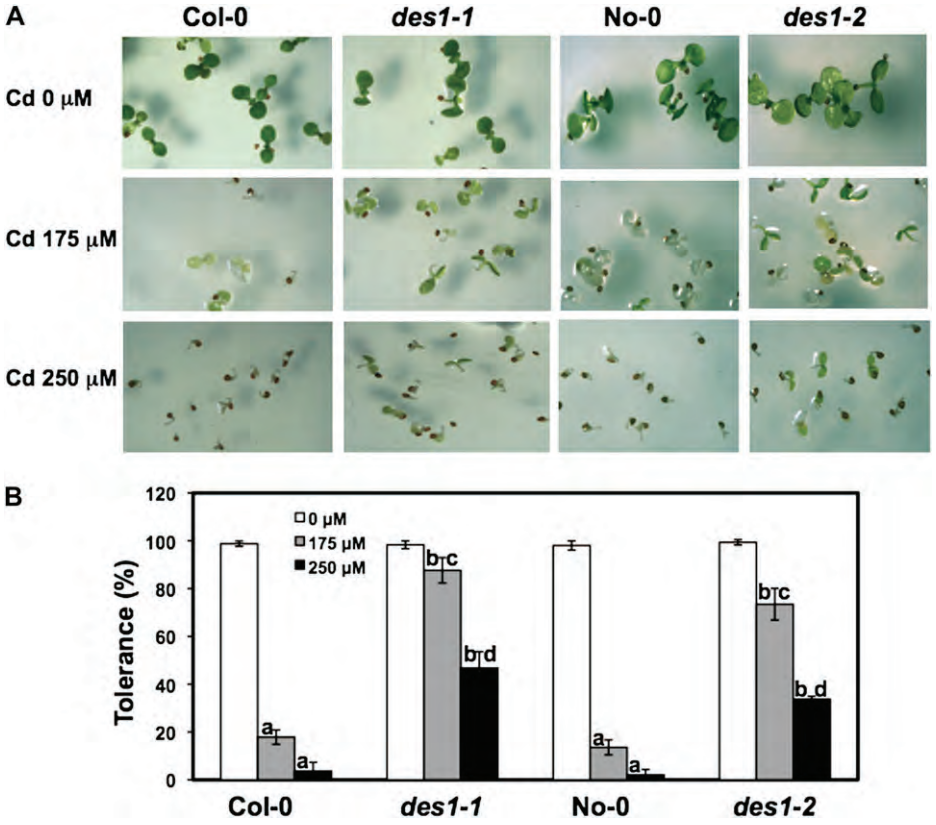


Figure 4. Sensitivity of the *des1-1* and *des1-2* mutant plants to Cd stress. A, Col-0 and No-0 wild-type and mutant seeds were germinated on solid MS medium containing varying concentrations of $CdCl_2$ as stated, and photographs were taken after 14 d of growth. B, The tolerance to Cd was calculated as the ratio of the total number of live seedlings to the total number of sown seeds after 14 d of growth in the presence of different concentrations of Cd. Data shown are means ± SD of three independent analyses made in separate batches at different times. ANOVA was performed using the software OriginPro 7.5. Significant differences between the wild-type plants grown in the presence and absence of Cd are indicated by the letter a ($P < 0.05$). Significant differences between the mutant plants grown in the presence and absence of Cd are indicated by the letter b ($P < 0.05$). Significant differences between the mutant plants and their respective wild-type plants grown in 175 μM Cd are indicated by the letter c ($P < 0.05$). Significant differences between the mutant plants and their respective wild-type plants grown in 250 μM Cd are indicated by the letter d ($P < 0.05$). [See online article for color version of this figure.]

and the total number of sown seeds after 14 d of growth. With this metric, the Col-0 and No-0 wild types showed 18% and 14% tolerance, respectively, to 175 μM Cd and the *des1-1* and *des1-2* mutants showed 88% and 75% tolerance, respectively. At 250 μM Cd, we observed only about 4% and 2% tolerance in the wild-type ecotypes; in contrast, the tolerance of both mutant strains was between 47% and 34% (Fig. 4B). These data show that the *des1* mutants have an increased tolerance to Cd.

A large number of reports have shown that Cd treatment generates oxidative stress in different plant systems (Sandalio et al., 2001; Schutzendubel et al., 2001; Romero-Puertas et al., 2007). We wanted to know if the enhanced Cd tolerance in the *des1* mutants would correlate with enhanced antioxidative defenses. Imaging of ROS in vivo in plant tissues by confocal laser microscopy is a very useful technique (Sandalio et al., 2008); we specifically stained for hydrogen peroxide (H_2O_2) in roots of *des1-1* mutant seedlings after a short treatment with 250 μM $CdCl_2$ and compared the staining with Col-0 wild-type roots (Fig. 5A). In wild-type roots, we observed an increase in fluorescence emission resulting from the oxidation of the nonfluorescent 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) to the highly fluorescent product; this signal reflects significant production of H_2O_2. In contrast, roots of the *des1-1* mutant had a much lower fluorescence emission than wild-type roots. In green tissues, chlorophyll autofluorescence interfered with the H_2O_2 detection. The significant decrease in H_2O_2 production observed in the *des1-1* mutant roots compared with the wild type upon Cd treatment was further confirmed in whole seedlings by spectrofluorimetric quantification (Fig. 5B). The concentrations of H_2O_2 in Col-0 and *des1-1* seedlings were identical in the absence of Cd. In contrast, ROS increased 64% more in the Col-0 seedlings after Cd treatment but remained almost unchanged (only 7% more) in the *des1-1* seedlings.

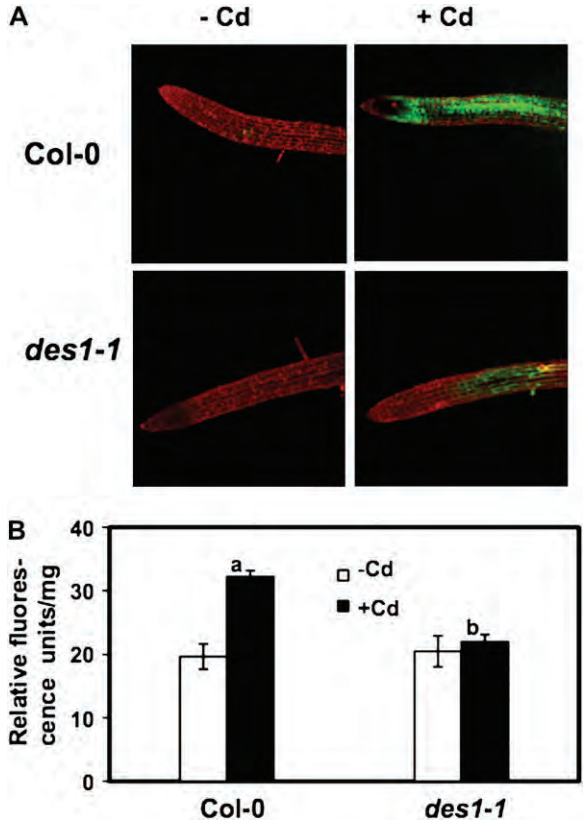


Figure 5. Fluorescence microscopic detection of H_2O_2 in root tissues and quantification in whole seedlings after Cd treatment. A, Roots from 5-d-old wild-type Col-0 and *des1-1* mutant plants grown on solid MS medium were soaked for 10 min in the absence or presence of 250 μM $CdCl_2$, rinsed with water, and then loaded with H_2DCFDA for 5 min in the presence of propidium iodide to visualize cell walls (pseudocolored in red) by confocal microscopy. H_2O_2 is pseudocolored in green. The experiments were repeated using roots from plants grown on different plates at least five times with similar results. B, Whole 2-week-old wild-type Col-0 and *des1-1* seedlings grown on solid MS medium were subjected to the same Cd treatment, then harvested and ground, and H_2O_2 was quantified by H_2DCFDA fluorescence as described in "Materials and Methods." Data shown are means ± SD of three independent analyses made in seedlings grown on different plates. ANOVA was performed using the software OriginPro 7.5. Significant differences between Cd-treated and untreated wild-type plants are indicated by the letter a ($P < 0.05$). Significant differences between Cd-treated *des1-1* mutant and Cd-treated wild-type plants are indicated by the letter b ($P < 0.05$).

We further tested the tolerance of *des1-1* and *des1-2* mutant plants by exogenously applying H_2O_2 to the growth medium. At concentrations of 4 mM and lower, no phenotypic differences could be observed between the wild-type ecotypes and their respective mutant seeds after 14 d of growth. In the presence of 6 mM H_2O_2, however, seeds of both mutants germinated and produced green leaves better than the Col-0 and No-0 wild types; the seedlings remained alive but with a significantly reduced size compared with seedlings grown in the absence of H_2O_2 (Fig. 6A). By quantifying the tolerance to H_2O_2, we found that in medium containing 6 mM H_2O_2 the *des1-1* and *des1-2* seedlings had 71% and 84% tolerance, respectively, whereas the Col-0 and No-0 wild types had only 13% and 39% tolerance, respectively (Fig. 6B).

We also examined the activities of antioxidant enzymes in the leaves. We found that these enzymes were significantly induced in both *des1-1* and *des1-2* mutant plants as compared with wild-type plants; this induction is likely responsible for the antioxidant protection against the abiotic stresses and the significant decrease in H_2O_2 production described above. We visualized one isoform of ascorbate peroxidase (APX) activity by nondenaturing PAGE. The APX activity was shown to qualitatively increase in gel band intensity; we calculated a roughly 2-fold increase in APX activity over wild-type levels in both *des1* mutants. In general, different peroxidases are known to use different substrates to metabolize H_2O_2. When guaiacol was used as a substrate, peroxidase activities were also found to be 2-fold enhanced in the both *des1* mutant plants (Fig. 7).

DISCUSSION

Cys occupies a central position in plant primary and secondary metabolism due to its biochemical functions. Cys residues are essential for protein structure and many protein functions, including catalytic activities, binding capacities, and redox modulations. In addition to its role in proteins, Cys is the precursor molecule of many essential biomolecules and defense molecules that are involved in plant protection (Rausch and Wachter, 2005; Wirtz and Droux, 2005). These diverse functions rely on the very high reactivity of the thiol moiety, but because of this reactivity, Cys can be a very toxic molecule when present above a certain concentration threshold (Park and Imlay, 2003). Thus, it is essential to maintain Cys homeostasis.

In recent years, the enzymes involved in Cys biosynthesis have been heavily studied in plants. Very recently, much progress has been made in understanding the OASTL enzymes from Arabidopsis, with the focus being mainly on the functional characterization of the most abundant enzymes (Heeg et al., 2008; Lopez-Martin et al., 2008a; Watanabe et al., 2008a). Less is known about the low abundance OASTL-like proteins; these proteins have been suggested to play an auxiliary role in Cys biosynthesis under particular conditions. Our data have demonstrated, however, that the minor cytosolic OASTL protein CS-LIKE catalyzes the enzymatic desulfuration of L-Cys to sulfide, ammonia, and pyruvate. This conclusion is mainly inferred from the affinity of CS-LIKE for L-Cys as a substrate, which was found to be more than 10 times higher than that for OAS. The three major OASTLs from Arabidopsis have K_m values for OAS that are at least five times lower than what we report for CS-LIKE (Jost et al., 2000). Also, their K_m values for sulfide are 2 orders of magnitude lower than that determined for

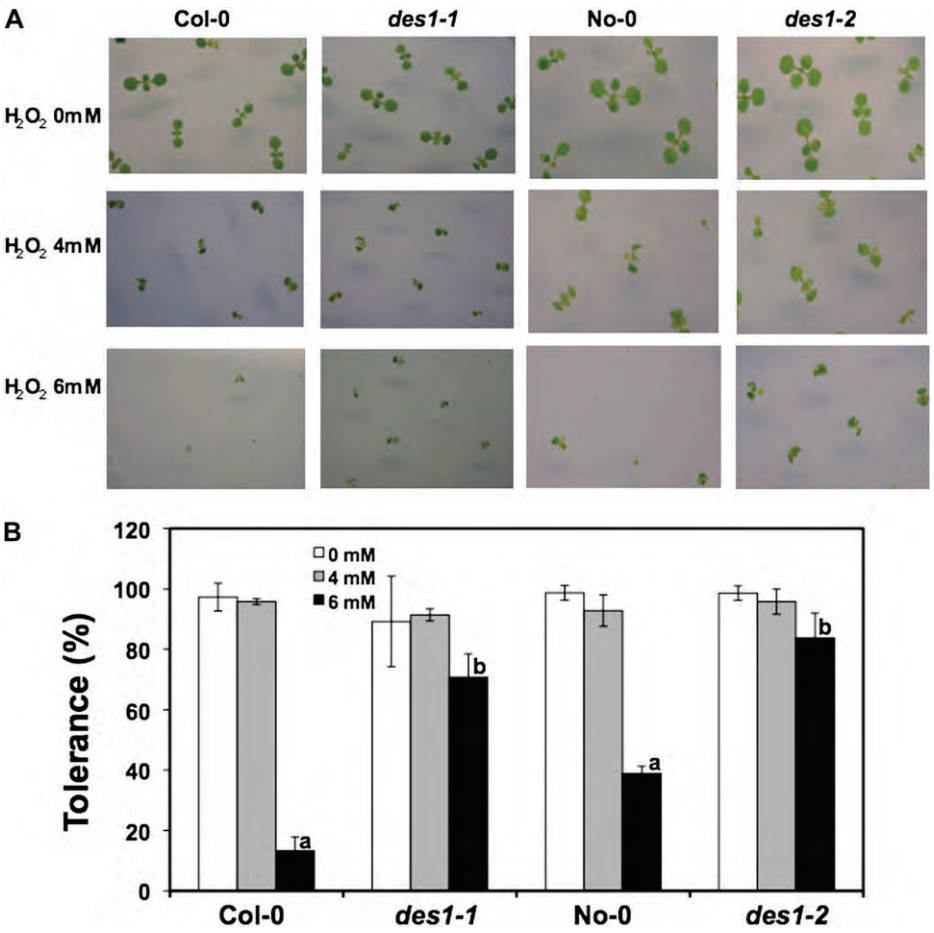
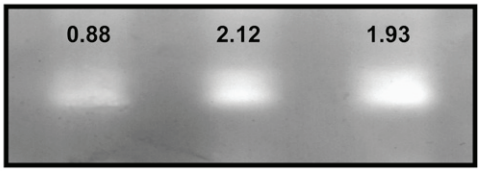


Figure 6. Sensitivity of the *des1-1* and *des1-2* mutant plants to oxidative stress. A, Col-0 and No-0 wild-type and mutant seeds were germinated on solid MS medium containing varying concentrations of H_2O_2 as stated, and photographs were taken after 10 d of growth. B, The tolerance to H_2O_2 was calculated as the ratio of the total number of live seedlings to the total number of sown seeds after 10 d of growth in the presence of different concentrations of H_2O_2. Data shown are means ± SD of three independent analyses made in separate batches at different times. ANOVA was performed using the software OriginPro 7.5. Significant differences between wild-type plants grown in 6 mM H_2O_2 and in the absence of H_2O_2 are indicated with the letter a ($P < 0.05$). Significant differences between the mutant plants and their respective wild-type plants grown in 6 mM H_2O_2 are indicated with the letter b ($P < 0.05$). [See online article for color version of this figure.]

recombinant CS-LIKE (Wirtz et al., 2004). It seems apparent, therefore, from our studies on the recombinant protein that CS-LIKE is not a true OASTL isoform, as has been suggested previously. One main characteristic of the OASTL proteins is the ability to interact with SAT, as has been described in different plant species (Droux et al., 1992, 1998; Ruffet et al., 1994; Bogdanova and Hell, 1997; Wirtz et al., 2001; Berkowitz et al., 2002; Wirtz and Hell, 2006, 2007). The highly conserved β8A-β9A surface loop has been proposed to be the site of interaction with SAT (Bonner et al., 2005). The sequence alignment of CS-LIKE with the cytosolic OASTL shows several amino acid differences in this loop, reinforcing our conclusion that CS-LIKE is not a true OASTL enzyme. Its inability to interact with SAT, apparently due to its structural features, was experimentally demonstrated when Arabidopsis OASTL proteins were purified on SAT immobilized resin and then separated by two-dimensional PAGE and identified by matrix-assisted laser-desorption ionization time of flight mass spectrometry (Heeg et al., 2008). Therefore, our enzymatic characterization of recombinant CS-LIKE protein, together with an analysis of its structural features and experimental data from other investigators, suggest that CS-LIKE is a novel DES located in the cytoplasm. We propose to designate it as DES1. Interestingly, the existence of enzymes with H_2S-releasing activity that are localized in the cytosol and distinct from OASTL proteins was suggested previously during the characterization of RNA interference-transgenic potato (*Solanum tuberosum*) plants with reduced OASTL (Riemenschneider et al., 2005b).

The function of the DES1 enzyme and its impact on plant metabolism are further revealed by the phenotype of the null mutants. In both mutant alleles, the absence of DES1 significantly reduces the total Cys desulfhydrase activity both in the leaves of mature plants and in seedlings, and this results in a concomitant increase in the total Cys content. As a consequence, the expression levels of genes in the sulfate assimilation pathway, which are susceptible to sulfur availability, are deregulated. We clearly observed differential responses of the different sulfate transporter and APS reductase genes to sulfur limitation conditions in the *des1-1* mutant compared with wild-type plants. These results demonstrated that mutation of DES1 has a significant effect on the two main regulatory points of the sulfate assimilatory pathway (Hawkesford, 2000; Hawkesford and De Kok, 2006; Kopriva, 2006), highlighting the importance of this enzyme.

A Relative quantification of APX activity in gel



B

Plant line	GPX activity (U/mg)
Col-0	0.098 ± 0.021
des1-1	0.192 ± 0.028 (196%)**
No-0	0.121 ± 0.009
des1-2	0.209 ± 0.021 (173%)**

Figure 7. APX and guaiacol peroxidase (GPX) activity levels in the *des1-1* and *des1-2* mutant plants. A, In-gel activity of APX in leaf extracts. Leaf protein extracts (30 μg) were loaded in parallel onto two nondenaturing polyacrylamide gels; after electrophoresis, one gel was stained to develop the APX activity and the other was stained with Coomassie Brilliant Blue as described in "Materials and Methods." The Coomassie Brilliant Blue-stained gel is shown in Supplemental Figure S5. The quantification of each APX activity band relative to the Coomassie Brilliant Blue-stained band was calculated with Quantity One software. The experiment was repeated at least three times with similar results. B, Guaiacol peroxidase activity determined in leaf extracts from Col-0 and No-0 wild-type and mutant plants as described in "Materials and Methods." Values are means ± SD from three independent experiments made using leaf extracts obtained from different plants grown in different pots. Percentages with regard to the respective wild type are given in parentheses. ANOVA was performed using the software OriginPro 7.5. ** $P < 0.05$.

In addition, the *des1-1* and *des1-2* mutant plants show enhanced antioxidant defenses, reflected in a significant decrease in ROS production and an ability to tolerate conditions of oxidative stress. This apparent contradiction, an increase in the concentration of a reducing agent triggering antioxidant defenses, can be explained by considering that intracellular Cys reduces ferric iron with exceptional speed and promotes oxidative damage through the Fenton reaction (Park and Imlay, 2003). Thus, although it is normally viewed as a reducing agent, Cys can induce the expression of antioxidant enzymes; for instance, cytosolic copper/zinc superoxide dismutase is induced by glutathione, Cys, and dithiothreitol (Herouart et al., 1993). Furthermore, transgenic tobacco (*Nicotiana tabacum*) plants that express the wheat (*Triticum aestivum*) OASTL gene *cys1* have higher intracellular levels of Cys and glutathione and also display enhanced ROS-scavenging systems, such as elevated copper/zinc superoxide dismutase transcript levels and superoxide dismutase activities, and a greater tolerance to methyl viologen-induced photooxidative damage (Youssefian et al., 2001). Overexpression of the *E. coli* SAT gene *cysE* in tobacco also increases the levels of Cys and concomitantly increases the resistance to oxidative stress generated by exogenous H_2O_2. Plants with the bacterial SAT targeted to the cytosol are more resistant than plants with the protein targeted to the chloroplasts (Blaszczyk et al., 1999). Also, we have demonstrated that knockout *oas-a1* mutants that are deficient in the most abundant form of cytosolic OASTL show phenotypic characteristics that are apparently opposite those of the *des1* mutants (Lopez-Martin et al., 2008a). These data from the *oas-a1* mutants, together with our data here on the *des1* mutants, support our claim that Cys in the cytosol is an important determinant of antioxidative capacity in Arabidopsis (Lopez-Martin et al., 2008b).

We propose that DES1 contributes to the maintenance of Cys homeostasis under certain conditions or growth stages. The Genevestigator data show that the highest expression of *DES1* is observed in the senescent growth stages, suggesting a requirement for DES1 at a late developmental stage, when it probably plays a role in the mobilization of sulfur. Accordingly, it has been determined previously in Arabidopsis plants that the OASTL activity decreases with increasing age, whereas the DES activity increases in older plants (Burandt et al., 2001). Leaf senescence is associated with an intense proteolytic activity that produces the release of Cys, and Cys desulfuration must be an essential way to avoid the accumulation of toxic levels of Cys in the cell. This is in agreement with the observed phenotype of premature leaf senescence in the *des1* mutants as a consequence of their inability to desulfurate Cys, and it is confirmed by our studies of the expression of senescence-associated genes. Also, our analysis of the expression levels of the different members of the OASTL gene family showed a significant induction of *CYS-C1*, *CYS-D1*, and *CYS-D2* expression, which could be reasonable for CYS-C1 because this β-cyanoalanine synthase is involved in the detoxification of cyanide and cyanide is coproduced during ethylene synthesis in senescent plants (Lim et al., 2007).

Therefore, Cys homeostasis should be precisely maintained in the cytosol, which is the compartment where Cys biosynthesis mainly occurs. This conclusion is confirmed by the repression in the *des1* mutants of the major OASTL enzymes that catalyze the synthesis of Cys, which can be explained as a strategy to avoid the accumulation of Cys. We have previously demonstrated that the cytosolic OASTL isoform OAS-A1 is the major contributor to Cys biosynthesis (Lopez-Martin et al., 2008a). Independent investigations on the Arabidopsis OASTL and SAT gene families have also demonstrated that the cytosol is the major site of Cys synthesis (Haas et al., 2008; Heeg et al., 2008; Watanabe et al., 2008a, 2008b). Cys concentrations in the cytosol are estimated to be over 300 μM, whereas the other cell compartments contain below 10 μM Cys (Krueger et al., 2009). Thus, Cys synthesis and degradation in the cytosol should be coordinated through OAS-A1 and DES1 activities throughout the life cycle of the plant.

In conclusion, although we cannot rule out a role in a secondary metabolite biosynthetic pathway, we suggest that DES1 (previously known as CS-LIKE) of Arabidopsis is a DES involved in precisely maintaining Cys homeostasis in the cytosol at some specific developmental stage or under environmental perturbations.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Stress Treatments

Arabidopsis (*Arabidopsis thaliana*) wild-type ecotypes Col-0 and No-0 and the SALK_03855 and RIKEN RATM13-27151_G mutants were used in this work (Alonso et al., 2003; Ito et al., 2005). The plants were grown either on solid MS medium in petri dishes or in soil. Plants grown in soil were irrigated with Hoagland II medium (Jones, 1982) containing 0.75 mM sulfate, and these conditions were referred to as control conditions; alternatively, they were irrigated with deionized water, referred to as low-sulfate conditions. Plants were grown under a photoperiod of 16 h of white light (120 μE m^{-2} s^{-1}) at 20°C and 8 h of dark at 18°C.

Cd or H_2O_2 stress was imposed on plants growing on solid medium with the addition of $CdCl_2$ or H_2O_2 to the medium at the indicated concentrations. The tolerance to the chemical was calculated as the ratio between the total number of live seedlings and the total number of sown seeds after growth in the presence of different concentrations of the chemical.

Expression of CS-LIKE in *Escherichia coli* Using Gateway Technology

To construct the expression clone, initially the *CS-LIKE* (*At5g28030*) cDNA was directionally cloned into the pENTR/D-TOPO vector for entry into the Gateway System (Invitrogen). Total RNA was extracted from Arabidopsis wild-type leaves using the RNeasy Plant Mini Kit (Qiagen) and reverse transcribed using an oligo(dT) primer and the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Subsequently, a 972-bp sequence encoding the full-length CS-LIKE protein was amplified by PCR using the primers GW-CSL-F and GW-CSL-R (Supplemental Table S2) and the proofreading Platinum Pfx DNA polymerase (Invitrogen). PCR conditions were as follows: a denaturation cycle of 2 min at 94°C, followed by 30 amplification cycles of 15 s at 94°C, 30 s at 60°C, and 1 min at 68°C. The amplified cDNA was then ligated into the pENTR/D-TOPO vector using the pENTR Directional TOPO Cloning Kit (Invitrogen) following the manufacturer's instructions. Positive clones were identified by PCR and chosen for plasmid DNA isolation. The *CS-LIKE* cDNA was then cloned into the expression vector pDEST17 using the *E. coli* Expression Systems with Gateway Technology (Invitrogen), which allowed us to generate a fusion protein with an N-terminal 6× His tag confirmed by sequencing, and its expression was inducible by L-Ara in BL21-AI *E. coli* cells.

Purification of Recombinant CS-LIKE Protein

The 6× His-tagged recombinant protein was isolated from 100 mL of BL21-AI *E. coli* cells cultured at 28°C to an optical density at 600 nm (OD_{600}) of 0.4 and then induced with 0.1% L-Ara overnight at 22°C. The purification was performed under nondenaturing conditions by affinity to nickel resin using the Ni-NTA Purification System (Invitrogen) according to the manufacturer's instructions. The purification process was visualized by SDS-PAGE using 12% (w/v) polyacrylamide gels and Coomassie Brilliant Blue staining.

Determination of Enzyme Activities

Plant leaf material was ground in 50 mM phosphate buffer (pH 7.5), 1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride using a mortar and pestle with liquid nitrogen. After centrifugation at 15,000*g* for 15 min at 4°C, the resulting supernatant was used as plant soluble extract for OASTL activity measurements. Plant leaf material ground in 20 mM Tris-HCl (pH 8) as described above was used for plant soluble extract for DES activity measurements. The total amount of protein in the extracts was determined by the Bradford (1976) method.

OASTL activity was measured using the method described previously (Barroso et al., 1995) in soluble plant, soluble bacterial, or purified protein extracts.

DES activity was measured by the release of sulfide from L-Cys as described previously (Riemenschneider et al., 2005a). The assay contained in a total volume of 1 mL: 1 mM dithiothreitol, 1 mM L-Cys, 100 mM Tris-HCl, pH 8.0, and enzyme extract. The reaction was initiated by the addition of L-Cys. After incubation for 15 min at 37°C, the reaction was terminated with the addition of 100 μL of 30 mM $FeCl_3$ dissolved in 1.2 N HCl and 100 μL of 20 mM *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride dissolved in 7.2 N HCl. The formation of methylene blue was determined at 670 nm, and the enzyme activity was calculated using the extinction coefficient of 15×10^6 cm^2 mol^{-1} (Papenbrock and Schmidt, 2000). D-Cys desulfhydrase activity was determined in the same way, but D-Cys was used instead of L-Cys (Riemenschneider et al., 2005c).

The substrate saturation experiments were performed with CS-LIKE recombinant protein and Cys as the substrate for the DES reaction by varying the Cys concentration from 0.03 to 2 mM. OAS and H_2S were cosubstrates for the OASTL reaction; the OAS concentration was varied from 0.05 to 12 mM, and the H_2S concentration was varied from 10 to 1,000 μM. Data from at least four replicate experiments were pooled and analyzed. The kinetic parameters for the DES and OASTL reactions were determined from a Hanes plot of s/vi against s, where s is the substrate concentration and vi is the initial velocity.

CAS activity was measured by the release of sulfide from L-Cys in the presence of cyanide following the method and extinction coefficient described earlier (Meyer et al., 2003).

Glu dehydrogenase activity was assayed by following the oxidation of NADH at 340 nm as described previously (Glevarec et al., 2004). The assay contained in a total volume of 850 μL: 120 mM Tris-HCl, pH 9, 12 mM 2-oxoglutarate, 0.25 mM NADH, and 500 μL of the DES reaction. The reaction was initiated by the addition of 6 units of bovine liver Glu dehydrogenase (Sigma), and the OD_{340} (extinction coefficient of 6.2 mM^{-1} cm^{-1}) was recorded for a period of 15 min.

Lactate dehydrogenase activity was assayed using the spectrophotometric method of following the oxidation of NADH at 340 nm (Riemenschneider et al., 2005c). The reaction was performed in 1 mL containing 50 mM potassium phosphate, pH 8.0, 0.23 mM NADH, and 500 μL of the DES reaction. The reaction was initiated by the addition of 6 units of rabbit muscle lactate dehydrogenase (Sigma), and the OD_{340} was recorded for a period of 15 min.

APX activity was detected on native PAGE gels according to the method described earlier (Mittler and Zilinskas, 1993). Equal amounts of soluble protein (30 μg) were separated on a discontinuous polyacrylamide gel under nondenaturing conditions at 4°C as described earlier (Laemmli, 1970); specific activity staining followed. The stacking and separating gels contained 4% and 10% polyacrylamide, respectively. The carrier buffer contained 2 mM ascorbate, and a prerun of 30 min was performed to let ascorbate enter the gel before the samples were loaded. After protein separation, the gel was equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 30 min, followed by 20 min of incubation in 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM ascorbate and 2 mM H_2O_2. The gel was then washed with the buffer for 1 min and visualized by immersion in 50 mM sodium phosphate buffer (pH 7.8) containing 28 mM tetramethyl ethylene diamine and 2.5 mM nitroblue tetrazolium. The APX activity appeared as an achromatic band on a purple-blue background. An identical gel was run in parallel and stained with Coomassie Brilliant Blue to detect all protein bands. The resulting two gels were scanned, and the APX activity band was quantified relative to the Coomassie Brilliant Blue-stained band using the Quantity One software (Bio-Rad).

Guaiacol peroxidase activity was assayed colorimetrically with guaiacol as a substrate. The reaction mixture contained 50 mM phosphate buffer (pH 6.1), 0.25 mM H_2O_2, 6.25 mM guaiacol, and enzyme extract. The linear increase in absorption at 470 nm, due to the formation of tetraguaiacol, was followed for 2 min (extinction coefficient of 26.6 mM^{-1} cm^{-1}).

Mutant Isolation by PCR Screening

To identify individuals that were homozygous for the insertion, genomic DNA was extracted from either 30 mg mL^{-1} kanamycin-resistant (SALK mutant) or 25 mg mL^{-1} hygromycin-resistant (RIKEN mutant) seedlings and

subjected to PCR genotyping using the following primer pairs (Supplemental Table S2): LBb1/GW-CSL-R, GW-CSL-F/GW-CSL-R, and GW-CSL-F/LBb1 for the SALK mutant and DS3-4/GW-CSL-R and GW-CSL-F/GW-CSL-R for the RIKEN mutant. PCR conditions were as described previously (Lopez-Martin et al., 2008a).

RNA Isolation and RT-PCR Analysis

Total RNA was extracted from Arabidopsis leaves using the RNeasy Plant Mini Kit (Qiagen) and reverse transcribed using an oligo(dT) primer and the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). An aliquot of the cDNA was amplified in subsequent PCRs using the following primers: GW-CSL-F and GW-CSL-R for the *DES1* (*At5g28030*) gene and UBQ10F and UBQ10R for the constitutive *UBQ10* gene, which was used as a control (Supplemental Table S2). PCR conditions were as described above.

DNA Isolation and Southern-Blot Analysis

Total DNA was isolated from Arabidopsis leaves and digested with *Xba*I, a restriction enzyme that does not cut inside the *DES1* gene, following a previously described method (Lopez-Martin et al., 2008a).

Real-Time RT-PCR

qRT-PCR was used to analyze the expression of senescence-associated genes, the OASTL gene family, and sulfur-responsive genes in the *des1-1* mutant plants. First-strand cDNA was synthesized as described above. Gene-specific primers for each gene were designed using the Vector NTI Advance 10 software (Invitrogen; Supplemental Table S2). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad); the signals were detected on an iCYCLER (Bio-Rad) according to the manufacturer's instructions. The cycling profile consisted of 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. A melt curve from 60°C to 90°C was run following the PCR cycling. The expression levels of the genes of interest were normalized to that of the constitutive *UBQ10* gene by subtracting the cycle threshold (CT) value of *UBQ10* from the CT value of the gene (ΔCT). The fold change was calculated as $2^{-(\Delta CT\ mutant\ -\ \Delta CT\ wild\ type)}$. The results shown are means ± SD of at least three independent RNA samples.

Quantification of Thiol Compounds

To quantify the total Cys and glutathione contents, thiols were extracted, reduced with $NaBH_4$, and quantified by reverse-phase HPLC after derivatization with monobromobimane (Molecular Probes) as described previously (Dominguez-Solis et al., 2001).

Detection and Quantification of H_2O_2

For the fluorimetric detection of H_2O_2, roots from 1-week old seedlings grown on vertical MS plates were treated with 250 μM $CdCl_2$ for 5 min, rinsed with water, and then incubated for 5 min with 10 mM H_2DCFDA (Molecular Probes) in the presence of 10 mM propidium iodide to visualize the cell walls. The samples were observed using a TCS SP2 spectral confocal microscope (Leica Microsystems) with the following settings: excitation, 488 nm; emission, 500 to 550 nm for fluorescein detection and 600 to 650 nm for propidium detection.

The quantification of H_2O_2 was performed following the protocol described previously (Joo et al., 2005). Basically, frozen plant tissue was hand ground in liquid nitrogen, and the powder was weighed and immediately taken up in 10 mM Tris-HCl buffer, pH 7.5. The extract was centrifuged twice at 15,000*g* for 10 min. We performed each measurement on two equal aliquots, to one of which we added 100 mM ascorbate, and they were allowed to react for 15 min. Then ROS levels were assayed by adding H_2DCFDA in dimethyl sulfoxide to both aliquots to a final concentration of 25 μM and incubating at 30°C for 30 min. Fluorescence was measured using a Cary Eclipse fluorescence spectrophotometer (Varian) with excitation/emission wavelengths set to 485 and 525 nm, respectively. We then subtracted the ascorbate-insensitive background from each experimental value. Total protein was quantified using the method of Bradford (1976). The average fluorescence value obtained from three successive measurements was divided by the protein content and expressed as relative fluorescence units per milligram of protein.

Supplemental Data

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

Supplemental Figure S1. Purification of the CS-LIKE recombinant protein as shown by SDS-PAGE.

Supplemental Figure S2. Amino acid alignment of CS-LIKE (CSL) and the major OASTL isoforms in Arabidopsis: cytosolic OAS-A1, plastid OAS-B, and mitochondrial OAS-C.

Supplemental Figure S3. Michaelis-Menten plots for DES and OASTL reactions with the substrates L-Cys and OAS, respectively.

Supplemental Figure S4. Intron-exon organization of the *DES1* gene (*At5g28030*) in the *des1* mutants, and RT-PCR and Southern-blot analyses.

Supplemental Figure S5. Nondenaturing polyacrylamide gel stained with Coomassie Brilliant Blue in parallel with the gel shown in Figure 7A as a control for protein loading.

Supplemental Table S1. Arabidopsis OASTL gene family.

Supplemental Table S2. Sequences of oligonucleotides used in this work.

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LITERATURE CITED

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, et al (2003) Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science **301:** 653–657

Barroso C, Vega JM, Gotor C (1995) A new member of the cytosolic O-acetylserine(thiol)lyase gene family in Arabidopsis thaliana. FEBS Lett **363:** 1–5

Berkowitz O, Wirtz M, Wolf A, Kuhlmann J, Hell R (2002) Use of biomolecular interaction analysis to elucidate the regulatory mechanism of the cysteine synthase complex from Arabidopsis thaliana. J Biol Chem **277:** 30629–30634

Blaszczyk A, Brodzik R, Sirko A (1999) Increased resistance to oxidative stress in transgenic tobacco plants overexpressing bacterial serine acetyltransferase. Plant J **20:** 237–243

Bogdanova N, Hell R (1997) Cysteine synthesis in plants: protein-protein interactions of serine acetyltransferase from Arabidopsis thaliana. Plant J **11:** 251–262

Bonner ER, Cahoon RE, Knapke SM, Jez JM (2005) Molecular basis of cysteine biosynthesis in plants: structural and functional analysis of O-acetylserine sulfhydrylase from Arabidopsis thaliana. J Biol Chem **280:** 38803–38813

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Buchner P, Takahashi H, Hawkesford MJ (2004) Plant sulphate transporters: co-ordination of uptake, intracellular and long-distance transport. J Exp Bot **55:** 1765–1773

Burandt P, Schmidt A, Papenbrock J (2001) Cysteine synthesis and cysteine desulfuration in Arabidopsis plants at different developmental stages and light conditions. Plant Physiol Biochem **39:** 861–870

Dominguez-Solis JR, Gutierrez-Alcala G, Vega JM, Romero LC, Gotor C (2001) The cytosolic O-acetylserine(thiol)lyase gene is regulated by heavy metals and can function in cadmium tolerance. J Biol Chem **276:** 9297–9302

Droux M (2004) Sulfur assimilation and the role of sulfur in plant metabolism: a survey. Photosynth Res **79:** 331–348

Droux M, Martin J, Sajus P, Douce R (1992) Purification and characteri-

zation of O-acetylserine (thiol) lyase from spinach chloroplasts. Arch Biochem Biophys **295:** 379–390

Droux M, Ruffet ML, Douce R, Job D (1998) Interactions between serine acetyltransferase and O-acetylserine (thiol) lyase in higher plants: structural and kinetic properties of the free and bound enzymes. Eur J Biochem **255:** 235–245

Giles GI, Jacob C (2002) Reactive sulfur species: an emerging concept in oxidative stress. Biol Chem **383:** 375–388

Glevarec G, Bouton S, Jaspard E, Riou MT, Cliquet JB, Suzuki A, Limami AM (2004) Respective roles of the glutamine synthetase/glutamate synthase cycle and glutamate dehydrogenase in ammonium and amino acid metabolism during germination and post-germinative growth in the model legume Medicago truncatula. Planta **219:** 286–297

Haas FH, Heeg C, Queiroz R, Bauer A, Wirtz M, Hell R (2008) Mitochondrial serine acetyltransferase functions as a pacemaker of cysteine synthesis in plant cells. Plant Physiol **148:** 1055–1067

Hatzfeld Y, Maruyama A, Schmidt A, Noji M, Ishizawa K, Saito K (2000) β-Cyanoalanine synthase is a mitochondrial cysteine synthase-like protein in spinach and Arabidopsis. Plant Physiol **123:** 1163–1171

Hawkesford MJ (2000) Plant responses to sulphur deficiency and the genetic manipulation of sulphate transporters to improve S-utilization efficiency. J Exp Bot **51:** 131–138

Hawkesford MJ, De Kok LJ (2006) Managing sulphur metabolism in plants. Plant Cell Environ **29:** 382–395

Heeg C, Kruse C, Jost R, Gutensohn M, Ruppert T, Wirtz M, Hell R (2008) Analysis of the *Arabidopsis O*-acetylserine(thiol)lyase gene family demonstrates compartment-specific differences in the regulation of cysteine synthesis. Plant Cell **20:** 168–185

Herouart D, Van Montagu M, Inze D (1993) Redox-activated expression of the cytosolic copper/zinc superoxide dismutase gene in Nicotiana. Proc Natl Acad Sci USA **90:** 3108–3112

Hirai MY, Fujiwara T, Awazuhara M, Kimura T, Noji M, Saito K (2003) Global expression profiling of sulfur-starved Arabidopsis by DNA macroarray reveals the role of O-acetyl-l-serine as a general regulator of gene expression in response to sulfur nutrition. Plant J **33:** 651–663

Howarth JR, Dominguez-Solis JR, Gutierrez-Alcala G, Wray JL, Romero LC, Gotor C (2003) The serine acetyltransferase gene family in Arabidopsis thaliana and the regulation of its expression by cadmium. Plant Mol Biol **51:** 589–598

Ito T, Motohashi R, Kuromori T, Noutoshi Y, Seki M, Kamiya A, Mizukado S, Sakurai T, Shinozaki K (2005) A resource of 5,814 dissociation transposon-tagged and sequence-indexed lines of Arabidopsis transposed from start loci on chromosome 5. Plant Cell Physiol **46:** 1149–1153

Jacob C, Giles GI, Giles NM, Sies H (2003) Sulfur and selenium: the role of oxidation state in protein structure and function. Angew Chem Int Ed Engl **42:** 4742–4758

Jones JB (1982) Hydroponics: its history and use in plant nutrition studies. J Plant Nutr **5:** 1003–1030

Joo JH, Wang S, Chen JG, Jones AM, Fedoroff NV (2005) Different signaling and cell death roles of heterotrimeric G protein alpha and beta subunits in the *Arabidopsis* oxidative stress response to ozone. Plant Cell **17:** 957–970

Jost R, Berkowitz O, Wirtz M, Hopkins L, Hawkesford MJ, Hell R (2000) Genomic and functional characterization of the oas gene family encoding O-acetylserine (thiol) lyases, enzymes catalyzing the final step in cysteine biosynthesis in Arabidopsis thaliana. Gene **253:** 237–247

Kataoka T, Hayashi N, Yamaya T, Takahashi H (2004) Root-to-shoot transport of sulfate in Arabidopsis: evidence for the role of SULTR3;5 as a component of low-affinity sulfate transport system in the root vasculature. Plant Physiol **136:** 4198–4204

Koornneef M, Hanhart CJ, van der Veen JH (1991) A genetic and physiological analysis of late flowering mutants in Arabidopsis thaliana. Mol Gen Genet **229:** 57–66

Kopriva S (2006) Regulation of sulfate assimilation in Arabidopsis and beyond. Ann Bot (Lond) **97:** 479–495

Krueger S, Niehl A, Lopez-Martin MC, Steinhauser D, Donath A, Hildebrandt T, Romero LC, Hoefgen R, Gotor C, Hesse H (2009) Analysis of cytosolic and plastidic serine acetyltransferase mutants and subcellular metabolite distributions suggests interplay of the cellular compartments for cysteine biosynthesis in Arabidopsis. Plant Cell Environ **32:** 349–367

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Lim PO, Kim HJ, Nam HG (2007) Leaf senescence. Annu Rev Plant Biol **58:** 115–136

Lopez-Martin MC, Becana M, Romero LC, Gotor C (2008a) Knocking out cytosolic cysteine synthesis compromises the antioxidant capacity of the cytosol to maintain discrete concentrations of hydrogen peroxide in Arabidopsis. Plant Physiol **147:** 562–572

Lopez-Martin MC, Romero LC, Gotor C (2008b) Cytosolic cysteine in redox signaling. Plant Signal Behav **3:** 880–881

Meyer AJ, Hell R (2005) Glutathione homeostasis and redox-regulation by sulfhydryl groups. Photosynth Res **86:** 435–457

Meyer AJ, May MJ, Fricker M (2001) Quantitative in vivo measurement of glutathione in Arabidopsis cells. Plant J **27:** 67–78

Meyer T, Burow M, Bauer M, Papenbrock J (2003) Arabidopsis sulfurtransferases: investigation of their function during senescence and in cyanide detoxification. Planta **217:** 1–10

Mittler R, Zilinskas BA (1993) Detection of ascorbate peroxidase activity in native gels by inhibition of the ascorbate-dependent reduction of nitroblue tetrazolium. Anal Biochem **212:** 540–546

Mullineaux PM, Rausch T (2005) Glutathione, photosynthesis and the redox regulation of stress-responsive gene expression. Photosynth Res **86:** 459–474

Nikiforova V, Freitag J, Kempa S, Adamik M, Hesse H, Hoefgen R (2003) Transcriptome analysis of sulfur depletion in Arabidopsis thaliana: interlacing of biosynthetic pathways provides response specificity. Plant J **33:** 633–650

Papenbrock J, Riemenschneider A, Kamp A, Schulz-Vogt HN, Schmidt A (2007) Characterization of cysteine-degrading and H2S-releasing enzymes of higher plants: from the field to the test tube and back. Plant Biol **9:** 582–588

Papenbrock J, Schmidt A (2000) Characterization of a sulfurtransferase from Arabidopsis thaliana. Eur J Biochem **267:** 145–154

Park S, Imlay JA (2003) High levels of intracellular cysteine promote oxidative DNA damage by driving the Fenton reaction. J Bacteriol **185:** 1942–1950

Rausch T, Wachter A (2005) Sulfur metabolism: a versatile platform for launching defence operations. Trends Plant Sci **10:** 503–509

Rennenberg H, Arabatzis N, Grundel I (1987) Cysteine desulphydrase activity in higher plants: evidence for the action of L- and D-cysteine specific enzymes. Phytochemistry **26:** 1583–1589

Riemenschneider A, Nikiforova V, Hoefgen R, De Kok LJ, Papenbrock J (2005a) Impact of elevated H(2)S on metabolite levels, activity of enzymes and expression of genes involved in cysteine metabolism. Plant Physiol Biochem **43:** 473–483

Riemenschneider A, Riedel K, Hoefgen R, Papenbrock J, Hesse H (2005b) Impact of reduced *O*-acetylserine(thiol)lyase isoform contents on potato plant metabolism. Plant Physiol **137:** 892–900

Riemenschneider A, Wegele R, Schmidt A, Papenbrock J (2005c) Isolation and characterization of a D-cysteine desulfhydrase protein from Arabidopsis thaliana. FEBS J **272:** 1291–1304

Romero-Puertas MC, Corpas FJ, Rodriguez-Serrano M, Gomez M, Del Rio LA, Sandalio LM (2007) Differential expression and regulation of antioxidative enzymes by cadmium in pea plants. J Plant Physiol **164:** 1346–1357

Ruffet ML, Droux M, Douce R (1994) Purification and kinetic properties of serine acetyltransferase free of *O*-acetylserine(thiol)lyase from spinach chloroplasts. Plant Physiol **104:** 597–604

Sandalio LM, Dalurzo HC, Gomez M, Romero-Puertas MC, del Rio LA (2001) Cadmium-induced changes in the growth and oxidative metabolism of pea plants. J Exp Bot **52:** 2115–2126

Sandalio LM, Rodriguez-Serrano M, Romero-Puertas MC, Del Rio LA (2008) Imaging of reactive oxygen species and nitric oxide in vivo in plant tissues. Methods Enzymol **440:** 397–409

Schmidt A (2005) Metabolic background of H_2S release from plants. *In* LJ De Kok, E Schnug, eds, Proceedings of the 1st Sino-German Workshop on Aspects of Sulfur Nutrition of Plants. Landbauforschung Völkenrode, Braunschweig, Germany, pp 121–129

Schutzendubel A, Schwanz P, Teichmann T, Gross K, Langenfeld-Heyser R, Godbold DL, Polle A (2001) Cadmium-induced changes in antioxidative systems, hydrogen peroxide content, and differentiation in Scots pine roots. Plant Physiol **127:** 887–898

Takahashi H, Watanabe-Takahashi A, Smith FW, Blake-Kalff M,

Hawkesford MJ, Saito K (2000) The roles of three functional sulphate transporters involved in uptake and translocation of sulphate in Arabidopsis thaliana. Plant J **23:** 171–182

Van Hoewyk D, Pilon M, Pilon-Smits EAH (2008) The functions of NifS-like proteins in plant sulfur and selenium metabolism. Plant Sci **174:** 117–123

Vanlerberghe GC, Robson CA, Yip JY (2002) Induction of mitochondrial alternative oxidase in response to a cell signal pathway down-regulating the cytochrome pathway prevents programmed cell death. Plant Physiol **129:** 1829–1842

Vauclare P, Kopriva S, Fell D, Suter M, Sticher L, von Ballmoos P, Krahenbuhl U, den Camp RO, Brunold C (2002) Flux control of sulphate assimilation in Arabidopsis thaliana: adenosine 5′-phosphosulphate reductase is more susceptible than ATP sulphurylase to negative control by thiols. Plant J **31:** 729–740

Watanabe M, Kusano M, Oikawa A, Fukushima A, Noji M, Saito K (2008a) Physiological roles of the β-substituted alanine synthase gene family in Arabidopsis. Plant Physiol **146:** 310–320

Watanabe M, Mochida K, Kato T, Tabata S, Yoshimoto N, Noji M, Saito K (2008b) Comparative genomics and reverse genetics analysis reveal indispensable functions of the serine acetyltransferase gene family in *Arabidopsis*. Plant Cell **20:** 2484–2496

Wirtz M, Berkowitz O, Droux M, Hell R (2001) The cysteine synthase complex from plants: mitochondrial serine acetyltransferase from Arabidopsis thaliana carries a bifunctional domain for catalysis and protein-protein interaction. Eur J Biochem **268:** 686–693

Wirtz M, Droux M (2005) Synthesis of the sulfur amino acids: cysteine and methionine. Photosynth Res **86:** 345–362

Wirtz M, Droux M, Hell R (2004) O-Acetylserine (thiol) lyase: an enigmatic enzyme of plant cysteine biosynthesis revisited in Arabidopsis thaliana. J Exp Bot **55:** 1785–1798

Wirtz M, Hell R (2006) Functional analysis of the cysteine synthase protein complex from plants: structural, biochemical and regulatory properties. J Plant Physiol **163:** 273–286

Wirtz M, Hell R (2007) Dominant-negative modification reveals the regulatory function of the multimeric cysteine synthase protein complex in transgenic tobacco. Plant Cell **19:** 625–639

Yamaguchi Y, Nakamura T, Kusano T, Sano H (2000) Three Arabidopsis genes encoding proteins with differential activities for cysteine synthase and beta-cyanoalanine synthase. Plant Cell Physiol **41:** 465–476

Yoshimoto N, Takahashi H, Smith FW, Yamaya T, Saito K (2002) Two distinct high-affinity sulfate transporters with different inducibilities mediate uptake of sulfate in Arabidopsis roots. Plant J **29:** 465–473

Youssefian S, Nakamura M, Orudgev E, Kondo N (2001) Increased cysteine biosynthesis capacity of transgenic tobacco overexpressing an *O*-acetylserine(thiol)lyase modifies plant responses to oxidative stress. Plant Physiol **126:** 1001–1011

Zheng L, White RH, Cash VL, Jack RF, Dean DR (1993) Cysteine desulfurase activity indicates a role for NIFS in metallocluster biosynthesis. Proc Natl Acad Sci USA **90:** 2754–2758

Supplemental Data

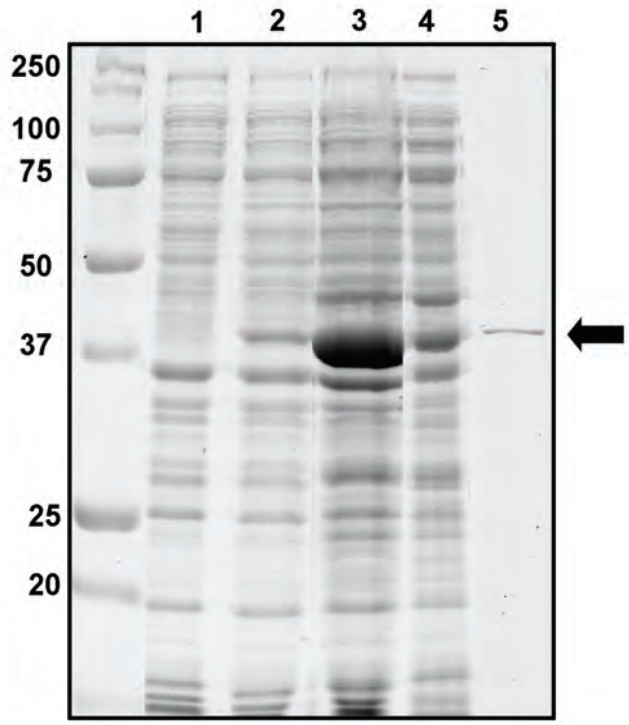


Figure S1. Purification of the CS-LIKE recombinant protein as shown by SDS-PAGE. Proteins were separated on a 12% polyacrylamide gel and stained with Coomassie Blue. Lanes 1, 2 and 3, fractions of un-induced cell culture, 1 h induction and overnight induction with L-arabinose, respectively. Lane 4, crude extract fraction of the overnight induced culture. Lane 5, fraction eluted from the Ni-NTA agarose column. The molecular markers are also shown.

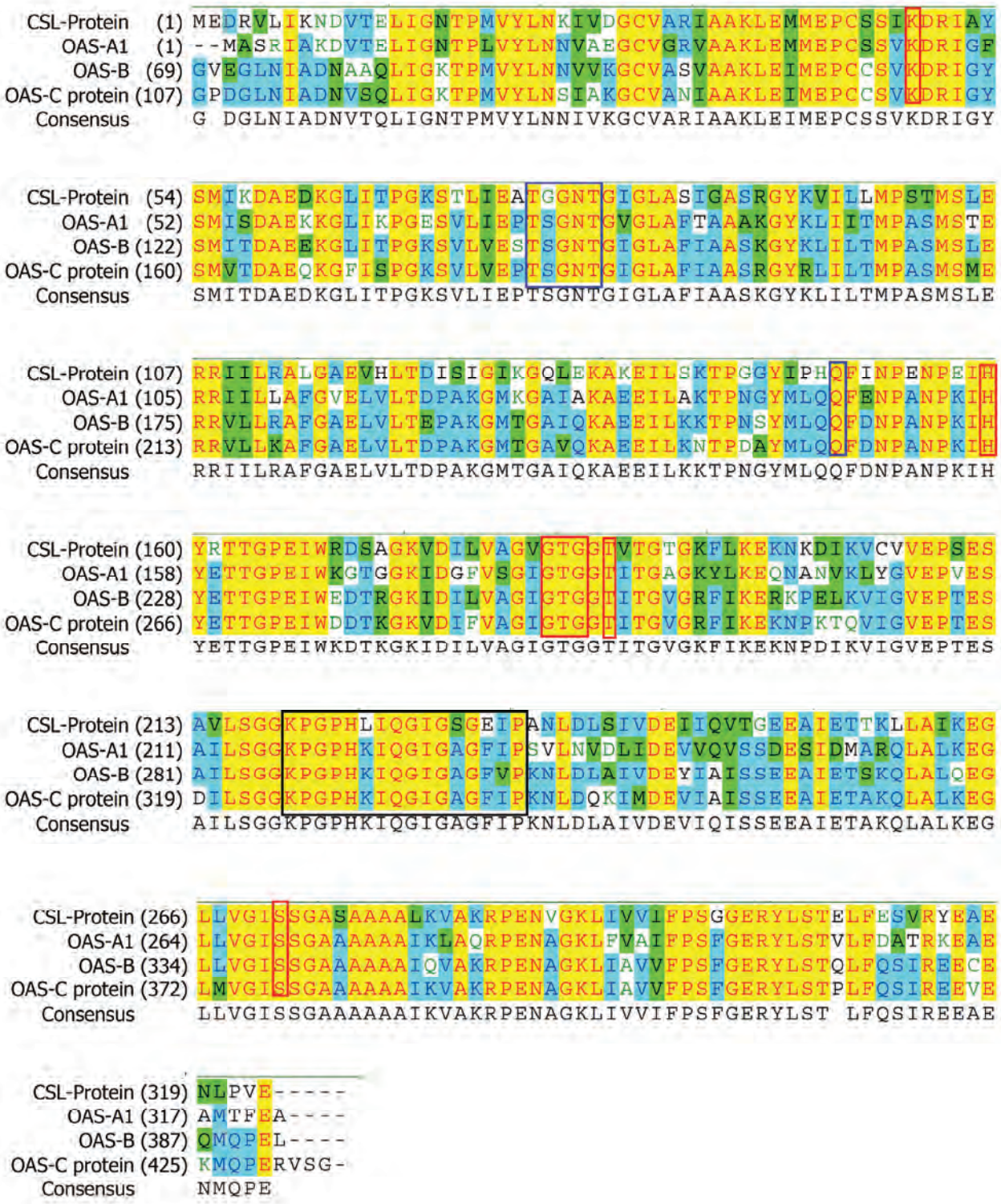
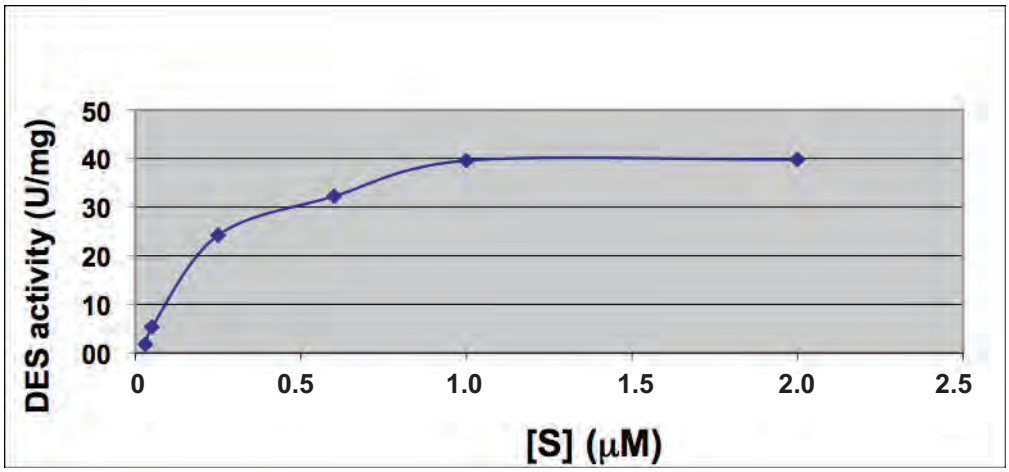


Figure S2. Amino acid alignment of CS-LIKE (CSL) and the major OASTL isoforms in *A. thaliana*: cytosolic OAS-A1, plastid OAS-B and mitochondrial OAS-C. The alignment was created with Vector NTI software. The PLP-binding sites are shown in red, the substrate binding site is in blue and the SAT protein-interaction site is in black.



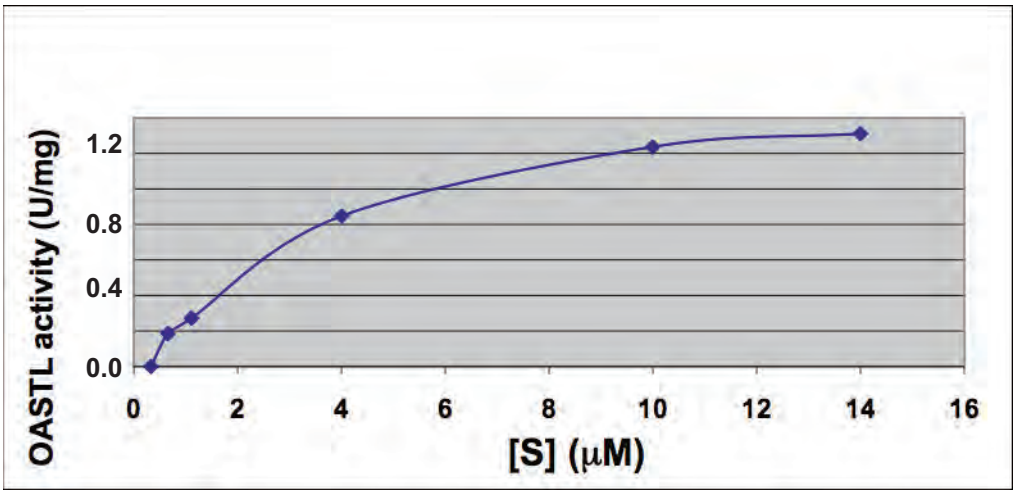


Figure S3. Michaelis-Menten plots for DES and OASTL reactions with the substrates L-cysteine and OAS, respectively. A representative replicate is shown.

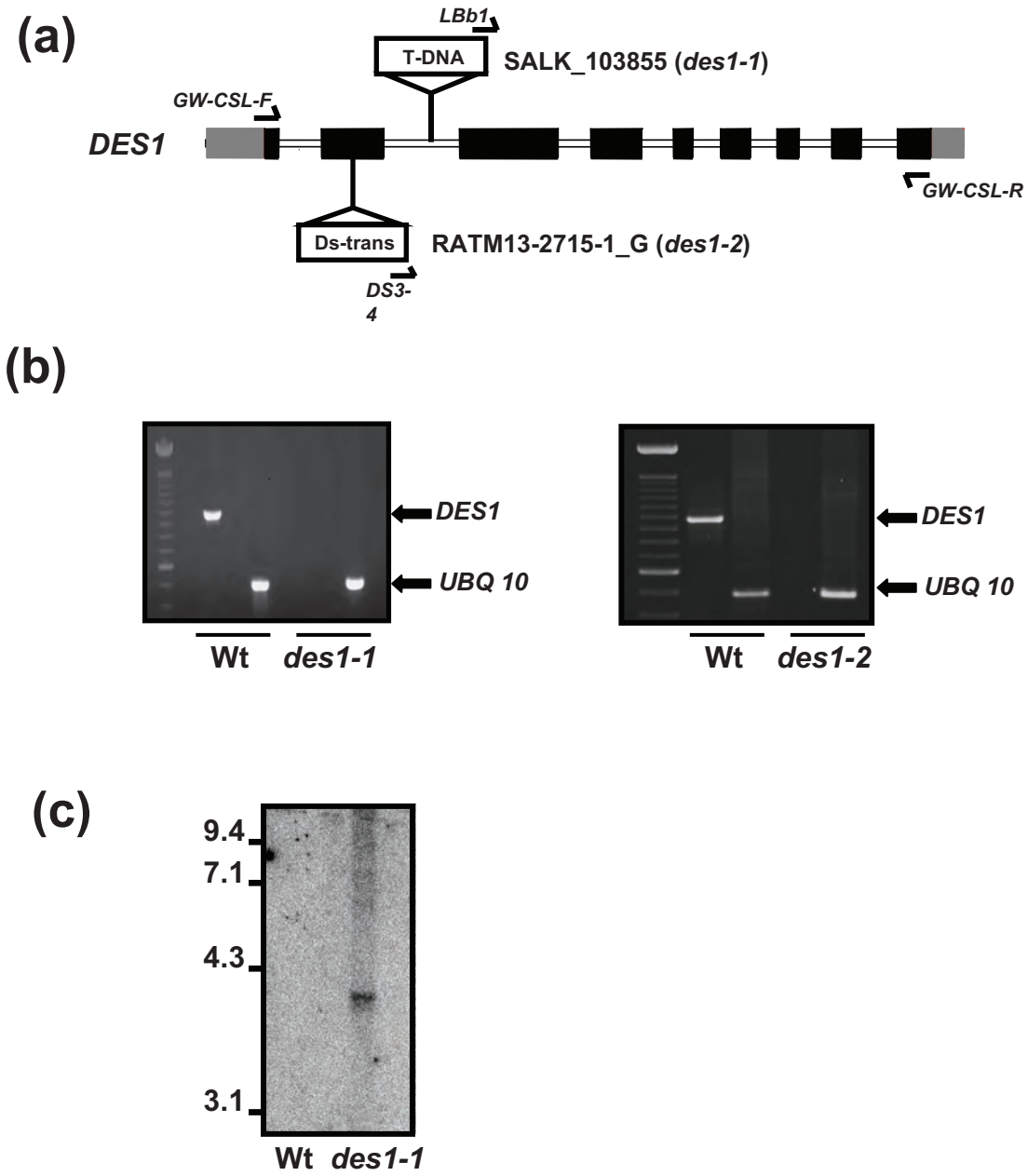


Figure S4. A, Intron-exon organization of the *DES1* gene *(At5g28030)* in the SALK__103855 and RATM13-2715-1_G insertion mutants, named *des1-1* and *des1-2*, respectively. *LBb1*, *DS3-4, F* and *R* show the locations and directions of primers used in the screening for mutant plants. The gene structure and T-DNA region are not drawn to scale. B, RT-PCR analysis of the mutant plants. RNA samples were prepared from the leaves of two-week-old plants, and primers specific for *DES1* and constitutive *UBQ10* transcripts were used. The *des1-1* and *des1-2* mutants are null mutants. C, Southern blot analysis of the *des1-1* mutant plant. Genomic DNA prepared from the leaves was digested with *XbaI* and hybridized with a T-DNA probe. The *des1-1* mutant plants contain a unique T-DNA insertion.

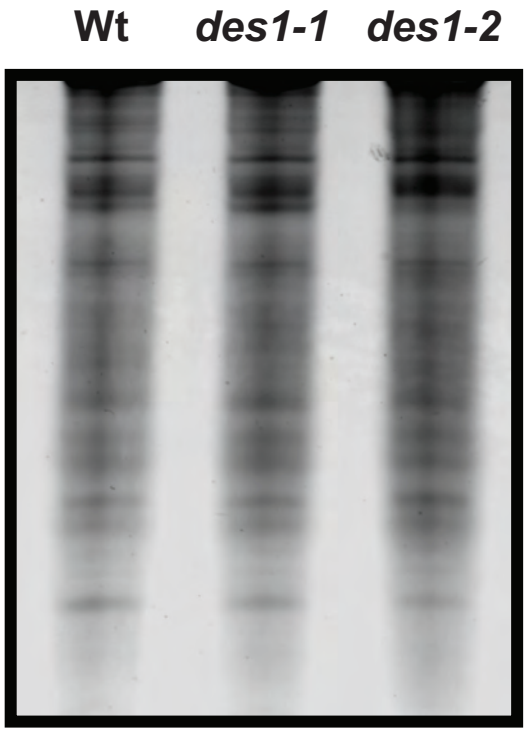


Figure S5. Non-denaturing polyacrylamide gel stained with Coomassie blue performed in parallel with the gel shown in Figure 7A as a control for protein loading.

Table S1. Arabidopsis O-acetylserine(thiol)lyase gene family

Isoform	AGI Code	Subcellular Localization	In Vitro Activity	References
OAS-A1	At4g14880	Cytosol	OASTL	(1-4)
OAS-A2	At3g22460	Not determined	Not determined	(2)
OAS-B	At2g43750	Plastid	OASTL	(2-4)
OAS-C	At3g59760	Mitochondria	OASTL	(2-4)
CYS-C1	At3g61440	Mitochondria	CAS	(3, 5, 6)
CYS-D1	At3g04940	Cytosol	OASTL	(3, 5, 6)
CYS-D2	At5g28020	Cytosol	OASTL	(3, 5, 6)
CS-26	At3g03630	Plastid	Not determined	(3)
CS-Like	At5g28030	Cytosol	Not determined	(3)

1. C. Barroso, J. M. Vega, C. Gotor, *FEBS letters* **363**, 1 (1995); 2. R. Jost *et al.*, *Gene* **253**, 237 (2000); 3. M. Watanabe *et al.*, *Plant Physiology* **146**, 310 (2008); 4. C. Heeg *et al.*, *Plant Cell* **20**, 168 (2008); 5. Y. Hatzfeld *et al.*, *Plant Physiology* **123**, 1163 (2000); 6. Y. Yamaguchi, T. Nakamura, T. Kusano, H. Sano, *Plant & Cell Physiology* **41**, 465 (2000).

Table S2. Sequences of oligonucleotides used in this work

Gen	Oligo name	Sequence (5´-3´)
Left border T-DNA (SALK)	LBb1	GCGTGGACCGCTTGCTGCAACT
Ds-transposon (RIKEN)	DS3-4	CCGTCCCGCAAGTTAAATATG
UBQ10 (At4g05320)	UBQ10F	GATCTTTGCCGGAAAACAATTGGAGGATGGT
	UBQ10R	CGACTTGTCATTAGAAAGAAAGAGATAACAGG
DES1 (At5g28030)	F	CACCATGGAAGACCGCGTCTTGAT
	R	TCATTCAACTGGCAAATTCTCAGCTT

For Real-time RT-PCR analysis

UBQ10 (At4g05320)	qUBQ10F	GGCCTTGTATAATCCCTGATGAATAAG
	qUBQ10R	AAAGAGATAACAGGAACGGAAACATAGT
SEN1 (At4g35770)	qSEN1F	AACATGTGGATCTTTCAAGTGCC
	qSEN1R	GTCGTTGCTTTCCTCCATCG
SAG21 (At4g02380)	qSAG21F	ATTACAGACCCGAAACCG
	qSAG21R	GCACCAGATCATTACCCA
NAP (At1g69490)	qNAPF	CGTCGATGAAACTTCCAAGG
	qNAPR	CCGAACCAACTAGACTCCGAAT
OAS-A1 (At4g14880)	qOAS-A1F	ACTTTTATTCCAGTACAACCGGATT
	qOAS-A1R	TGACTTTTATTGGCACACAAGAGAA
OAS-B (At2g43750)	qOAS-BF	AAACAGCGACGTCGTTTTGCAGCTACAGTG
	qOAS-BR	CTCTTCTCGAATCGACTGGAAAAGCTGGGT
OAS-C (At3g59760)	qOAS-CF	AGTAGGATCTGGTGGTGGCGAATGAATGGCTTC
	qOAS-CR	TGTTCATACCTCAGGCTGATCATTTTCTCC
DES1 (At5g28030)	qDES1F	TCGAGTCAAGTCAGATATGAAGCT
	qDES1R	TGTAACCTTGGTACCAACATCTCT
CS26 (At3g03630)	qCS26F	CGTCTCCTTCGCTCCGTCTTCTTCCTCAGT
	qCS26R	GGTTGGGCTCGAGACATGTTTATCTCTCAG
CYS-C1 (At3g61440)	qC1F	GATGCAATGTATTTGTCTCTTTTTCTCATC
	qC1R	TTTCTTTTTATAATCTTTTGCTCCCGAGAT

CYS-D1 *(At3g04940)*	qD1 F	AGAACAGTTCTCTCTTTTTGTCGACAGTCT
	qD1 R	GGACAGTCCACCTTAGAGGCTAGAGATTAG
CYS-D2 *(At5g28020)*	qD2 F	GAAACAATATATAACCAAAAAATTGCCAAGAT
	qD2 R	GTCCAACATTCATTCAATAGGCAAATTCTC
SULTR1.2 *(At1g78000)*	SULTR1.2F	GGATCCAGAGATGGCTACATGA
	SULTR1.2R	TCGATGTCCGTAACAGGTGAC
SULTR2.1 *(At5g10180)*	SULTR2.1F	ATTGTTGCTCTAACCGAGGCGATT
	SULTR2.1R	TGTACCCTTTTATTCCGGCGAACG
SULTR2.2 *(At1G77990)*	SULTR2.2F	CGACATGTCTTGCGTGATGGGCG
	SULTR2.2R	GCTCGCTTCAATTTGTGAAGTACCCT
SULTR3.1 *(At3g51895)*	SULTR3.1F	CCAGATCCAAATTCATCGGTGA
	SULTR3.1R	CGGTTCGGTTTTAAACGTGTG
APR1 *(At4g04610)*	APR1F	CATTGGAGCCAAAAGTTTCGC
	APR1R	TCCTCAATCTCAACCACATCAAC
APR2 *(At1g62180)*	APR2F	GAGCTCCACGGGCTATTAAGTA
	APR2R	TTCTCTTTCATTCTTCATCACCC
APR3 *(At4g21990)*	APR3F	GGAAGAGATCCTCCGTGAAAGC
	APR3R	CTGTAACCTCAGAAGCAACAATGGA

Capítulo II

El sulfuro generado en el citosol regula negativamente la autofagia y modula el perfil transcripcional de *A. thaliana*

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Cysteine-Generated Sulfide in the cytosol negatively regulates autophagy and modulates the transcriptional profile in *Arabidopsis*

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Abstract

In *Arabidopsis thaliana*, DES1 is the only identified L-cysteine desulfhydrase located in the cytosol, and it is involved in the degradation of cysteine and the concomitant production of H_2S in this cell compartment. Detailed characterization of the T-DNA insertion mutants *des1-1* and *des1-2* has provided insight into the role of sulfide metabolically generated in the cytosol as a signaling molecule. Mutations of the *DES1* gene impede H_2S generation in the *Arabidopsis* cytosol and strongly affect plant metabolism. Senescence-associated vacuoles are detected in mesophyll protoplasts of *des1* mutants. Additionally, DES1 deficiency promotes the accumulation and lipidation of the ATG8 protein, which is associated with the process of autophagy. The transcriptional profile of the *des1-1* mutant corresponds to its premature senescence and autophagy-induction phenotypes, and restoring H_2S generation has been shown to eliminate the phenotypic defects of *des1* mutants. However, sulfide is able to reverse ATG8 protein accumulation, even in wild-type plants when autophagy is induced by carbon starvation, suggesting a general effect of sulfide on autophagy that is unrelated to nutrient limitation stress. Our results suggest that cysteine-generated sulfide in the cytosol negatively regulates autophagy and modulates the transcriptional profile of *Arabidopsis*. The possible mechanisms of H_2S action and its molecular targets are discussed.

INTRODUCTION

Cysteine (Cys) occupies a central position in plant primary and secondary metabolism due to its biochemical functions. Beyond its importance as an amino acid, Cys is a precursor for a large number of essential bio-molecules, including the proteinogenic amino acid methionine; vitamins and co-factors (Droux, 2004; Wirtz and Droux, 2005); and antioxidants such as glutathione, which has been regarded as the major determinant of cellular redox homeostasis (Meyer and Hell, 2005; Mullineaux and Rausch, 2005). Recently, however, it has been suggested that Cys itself is an important determinant of the antioxidative capacity of the cytosol in *Arabidopsis thaliana* (Lopez-Martin et al., 2008a; Lopez-Martin et al., 2008b). Many plant defense compounds produced under a variety of adverse environmental conditions, including biotic and abiotic stress, derive from Cys (Rausch and Wachter, 2005) and contain sulfur moieties as functional groups. The biosynthesis of Cys involves the sequential reaction of two enzymes, serine acetyltransferase (SAT, EC 2.3.1.30), which synthesizes the intermediary product O-acetylserine (OAS) from acetyl-CoA and serine, and O-acetylserine(thiol)lyase (OASTL, EC 2.5.1.47), which incorporates the sulfide derived from the assimilatory reduction of sulfate with OAS to produce Cys. Together, the SAT and OASTL enzymes form the hetero-oligomeric Cys synthase complex, which was first described in bacteria and has been studied extensively in plants (Droux et al., 1998; Wirtz and Hell, 2006). Plant cells contain different SAT and OASTL enzymes in the cytosol, plastids and mitochondria, resulting in a complex variety of isoforms and different subcellular Cys pools. In the model plant *A. thaliana*, five *SAT* (Howarth et al., 2003) and nine *OASTL* genes (Wirtz et al., 2004) have been identified.

The OASTL enzymes belong to the super-family of β-substituting alanine synthases. In *Arabidopsis*, the most abundantly transcribed *OASTL* genes encode the cytosolic OAS-A1 (At4g14880), plastidial OAS-B (At2g43750) and mitochondrial OAS-C (At3g59760) isoforms. These proteins are recognized as authentic OASTLs due to their ability to interact with SAT, which has been demonstrated using

several different approaches (Bogdanova and Hell, 1997; Droux et al., 1998; Wirtz et al., 2001; Bonner et al., 2005; Heeg et al., 2008). The OASTL family also includes a mitochondrial isoform encoded by *CYS-C1* (At3g61440) that functions as a β-cyanoalanine synthase (CAS, EC 4.4.1.9) (Hatzfeld et al., 2000; Yamaguchi et al., 2000; Garcia et al., 2010) and is highly expressed in *Arabidopsis* cells. Another gene, *OAS-A2*, does not produce a functional protein due to the presence of an in-frame stop codon and an unspliced intron. The remaining OASTL-like proteins located in the cytosol, encoded by *CYS-D1* (At3g04940), *CYS-D2* (At5g28020) (Yamaguchi et al., 2000) and *DES1* (At5g28030) (Alvarez et al., 2010), along with the protein product of *CS26* (At3g03630) in the plastid (Bermudez et al., 2010) were identified by sequence homology and are expressed at much lower levels.

In recent years, much progress has been made in understanding the OASTL enzymes in *Arabidopsis*, with a primary focus on the most abundant enzymes as a result of their involvement in the primary sulfate assimilation pathway. We recently investigated DES1, a minor OASTL-like protein located in the cytosol and presented findings pertaining to its metabolic function. We showed that DES1 catalyzes the desulfuration of L-cysteine to sulfide plus ammonia and pyruvate, rather than promoting Cys biosynthesis; thus, DES1 is a novel L-cysteine desulfhydrase (EC 4.4.1.1) (Alvarez et al., 2010). A role of DES1 in plant metabolism was evidenced by the phenotypes of the T-DNA insertion mutants *des1-1* and *des1-2*, which exhibit premature leaf senescence along with increased expression of senescence-associated genes and transcription factors. Disrupted DES1 function also significantly reduces the total Cys desulfuration activity in leaves, with a concomitant increase in the total cysteine content (Alvarez et al., 2010). With respect to the response of these knockout mutants to plant pathogens, *des1* mutants were found to behave as constitutive SAR mutants, exhibiting high resistance to biotrophic and necrotrophic pathogens, SA accumulation and *WRKY54* and *PR1* induction (Alvarez et al., 2011). Taken together, these data highlight the importance of DES1 in the signaling and regulation of plant responses to various processes.

Hydrogen sulfide has increasingly been recognized as an important signaling molecule, functioning as a physiologic gasotransmitter of comparable importance to NO and CO in mammalian systems. The list of biological roles of H_2S in various systems of the human body has rapidly expanded and is the subject of several recent reviews (Lowicka and Beltowski, 2007; Szabo, 2007; Li and Moore, 2008; Gadalla and Snyder, 2010; Kabil and Banerjee, 2010). The majority of this research has emphasized the physiological effects of this gas in the cardiovascular and nervous systems. Hydrogen sulfide is weakly acidic and dissociates in aqueous solution. Under physiological pH conditions (pH 7.4), one-third of the H_2S present is undissociated, and the remaining two-thirds dissociate into H^+ and HS^-. HS^- may subsequently dissociate into H^+ and S^{2-}, but this dissociation requires high pH conditions. Similar to NO and CO, H_2S is lipophilic and permeates plasma membranes freely, although the ionized form HS^- cannot permeate the membranes (Lowicka and Beltowski, 2007; Kabil and Banerjee, 2010). H_2S is endogenously produced in mammalian tissues by enzymatic reactions of L-cysteine, primarily via two cytoplasmic enzymes, cystathionine-γ-lyase (CSE, EC 4.4.1.1) and cystathionine-β-synthetase (CBS, EC 4.2.1.22), which both use pyridoxal-5'-phosphate as a cofactor, with ammonia and pyruvate as by-products (Gadalla and Snyder, 2010). To our knowledge, DES1 is the only L-cysteine desulfhydrase present in the *Arabidopsis* cytosol that catalyzes the desulfuration of L-cysteine to sulfide plus ammonia and pyruvate and requires pyridoxal-5'-phosphate as a cofactor (Alvarez et al., 2010). DES1 may therefore be responsible for the release of metabolically regulated sulfide in this cellular compartment. The L-cysteine desulfurases (EC 2.8.1.7) also catalyze Cys desulfuration required for iron-sulfur cluster, thiamine, biotin and molybdenum cofactor synthesis, but they instead generate L-Ala and elemental sulfur (Van Hoewyk et al., 2008).

Emerging data over the recent years suggest that H_2S may be a new signaling molecule equally important to plants as NO and H_2O_2. H_2S has been found to mediate increases in tolerance and protection against certain plant stresses. For example, sulfide alleviates the inhibitory effects of copper and aluminum stress on wheat germination and is associated with antioxidative defense (Zhang et al., 2008; Zhang et al., 2010). Similarly, the inhibitory effect of boron on cucumber root elongation is substantially reduced by H_2S treatment, which up-regulates the cell wall-associated proteins and expansins (Wang et al., 2010). It has also been reported that sulfur fertilization increases plant resistance against fungal pathogens, and it has been proposed that H_2S is involved in the mechanisms of the Sulfur-Induced Resistance (SIR), or Sulfur-Enhanced Defense (SED), phenomenon (Rausch and Wachter, 2005). A field experiment demonstrated that *Brassica napus* is able to react to fungal infection and releases H_2S as a result of increased L-cysteine desulfhydrase activity (Bloem et al., 2004). We recently demonstrated that cytosolic cysteine plays a role in the establishment and signaling of the plant response to pathogens. This function could be due to cysteine itself or to its function as generator of a

particular sulfur compound (Alvarez et al., 2011). Hydrogen sulfide has been recently discovered as a component of the ABA signaling network in guard cells (Garcia-Mata and Lamattina, 2010; Lisjak et al., 2010). It has also been shown to modulate photosynthesis in *Spinacia oleracea* seedlings by regulating the expression of genes involved in photosynthesis and thiol redox modification (Chen et al., 2011).

In this work, we observed senescence-associated vacuoles and an induced autophagy phenotype in *des1* mutants. Additionally, we found that these phenotypes could be rescued by the exogenous application of H_2S. An in-depth study of the effects of exogenous H_2S revealed a specific role of sulfide as a general repressor of autophagy and a transcriptional regulator in *Arabidopsis*.

RESULTS

Senescence-Associated Vacuoles and Induced Autophagy in *des1* Mutants

We previously demonstrated that a mutation in the *DES1* gene causes premature leaf senescence, as evidenced by increased transcript levels of senescence-associated genes (Alvarez et al., 2010). To further dissect this particular phenotype, we aimed to determine how senescence was induced at the cellular level in this mutant. Plants contain different types of vacuoles, and the senescence-associated vacuoles (SAVs) are formed *de novo* during leaf senescence. SAVs are small in size and exhibit a lower pH than the central vacuole (Zouhar and Rojo, 2009). *des1-1* mutant plants and their respective wild-type Col-0, were grown for four weeks side by side under long-day photoperiod and nutrient-sufficient conditions. The identification of vacuolar compartments associated with leaf senescence was performed in mesophyll protoplasts using Lysotracker Red, a fluorescent marker for acidic organelles that has been used to identify SAVs developing in the periphery of *Arabidopsis* mesophyll cells (Otegui et al., 2005). After incubating the protoplasts prepared from *des1-1* and wild-type leaves with Lysotracker Red, small fluorescent structures were clearly visible in the cytoplasmic periphery around the plastids in *des1-1* protoplasts but not in wild-type protoplasts (Fig. 1A). Further evidence indicating that this fluorescence signal was due to the mutation in *DES1* was the presence of these structures, albeit at lower abundance, in a second mutant allele, *des1-2*, and their absence in No-0 plants, the respective wild-type ecotype (Fig. 1A).

Because cellular components are degraded and the released nutrients are mobilized for reuse during leaf senescence (Lim et al., 2007), we evaluated whether the SAVs observed in the *des1* mutants were related to an autophagic mechanism. Autophagy is a ubiquitous eukaryotic process by which the cytoplasm and organelles can be internalized into vacuoles for degradation. Associations between carbon and nitrogen availabilities, leaf senescence and autophagy have been established (Hanaoka et al., 2002). Proteins involved in the autophagy process (ATG proteins) have been used to monitor autophagic activity in plants, such as ATG8 accumulation and lipidation. The ATG8 protein is covalently conjugated to a lipid molecule, and its lipidation is required for the formation of the double membrane-bound structures called autophagosomes, which are the most remarkable feature of autophagy (Nakatogawa et al., 2009). We have used polyclonal antibodies raised against the recombinant ATG8 protein from *Chlamydomonas reinhardtii*, which has been shown to be functionally conserved and therefore useful as a molecular autophagy marker in *Arabidopsis* (Perez-Perez et al., 2010). Four-week-old *des1* mutant plants and their respective wild-type ecotypes were grown as described above, and leaf tissues from these plants were homogenized. Total protein samples were generated by low-speed centrifugation at 500 g, and the resulting supernatants were subjected to immunoblot analysis (Fig. 1B). The antibodies detected two groups of ATG8 proteins, with a protein-banding pattern similar to those previously observed in *Arabidopsis* (Yoshimoto et al., 2004; Chung et al., 2010). The slower-mobility group corresponds to unmodified protein forms, while the group with faster mobility includes ATG8 proteins conjugated with PE. The SDS-PAGE profiles of the ATG8 proteins revealed increased accumulation in both *des1* mutant alleles relative to the wild-type plants, predominantly for the modified ATG8 forms (Fig. 1B). Collectively, these results suggest that deficient DES1 protein function promotes early senescence that can be visualized at the cellular level and autophagy activation.

Sulfide Rescues the Senescence-Associated Vacuole and Autophagy Phenotypes of the *des1* Mutants

We recently demonstrated that DES1 catalyzes the enzymatic desulfuration of L-Cys to sulfide plus ammonia and pyruvate in the cytosol of *Arabidopsis* and proposed that DES1 is involved in maintaining Cys homeostasis primarily at late developmental stages (Alvarez et al., 2010). Accordingly, disruption of the *DES1* gene provokes a 20-25% increase in the amount of Cys (Alvarez et al., 2010), but it may also reduce the capacity of the cytosol to release H_2S. To assess the latter, we used an H_2S-selective electrode to measure endogenous H_2S concentrations in leaf

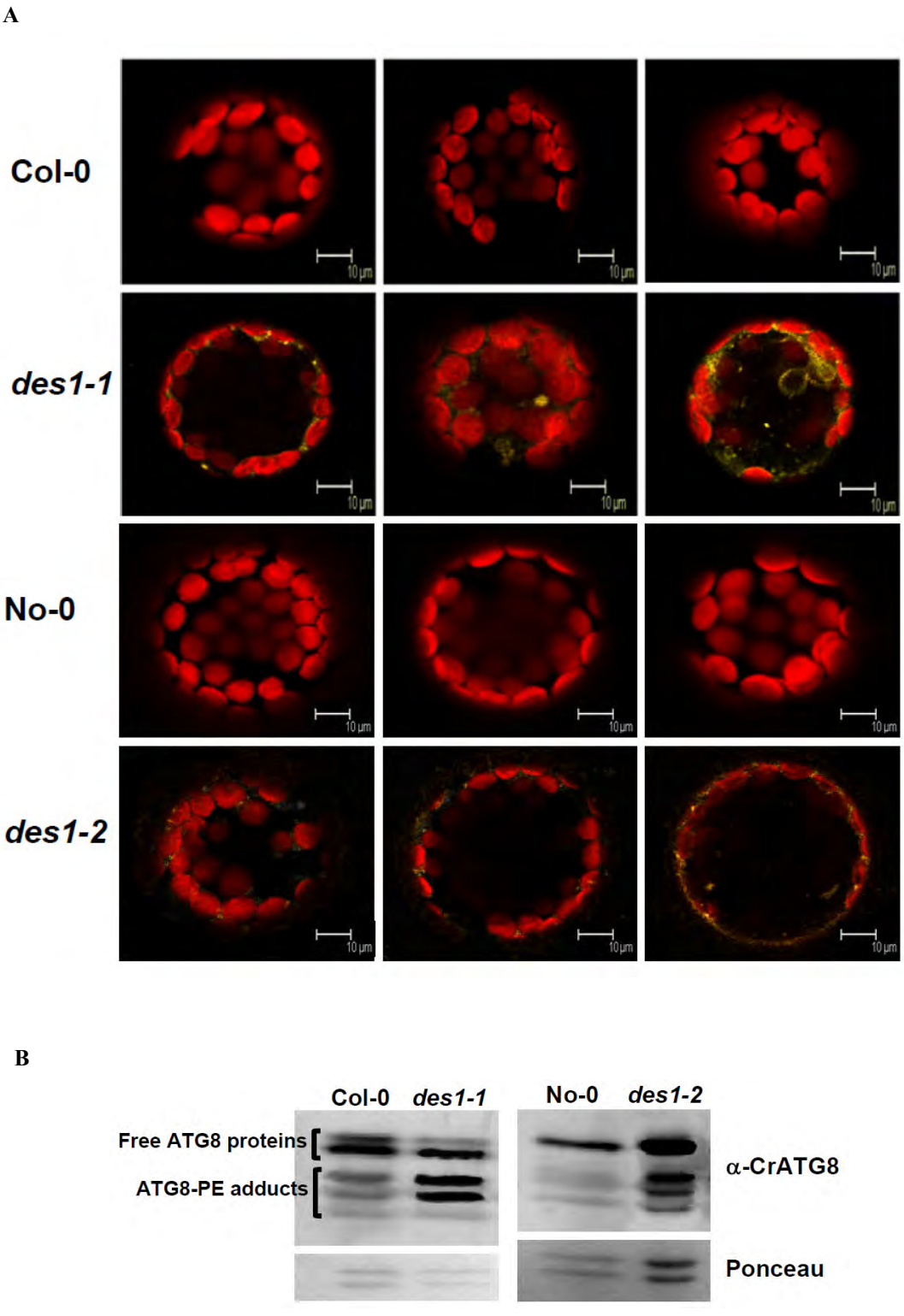

Figure 1. Early senescence and induced autophagy phenotypes of *des1* mutant plants. **(A)** Wild-type Col-0 and No-0 ecotypes and *des1* mutant plants were grown in soil for four weeks, and senescence-associated vacuoles were visualized by staining of protoplasts isolated from leaves with Lysotracker Red. The yellow fluorescence due to specific staining is superimposed with the chlorophyll-specific red fluorescence. The experiment was repeated at least four times with different batches of plants, and the results were consistent across all replicates. **(B)** Immunoblot analysis of ATG8 accumulation in leaves of Col-0 and No-0 wild-type and *des1* mutant plants. The total lysates prepared from leaves were centrifuged at low speed, and the supernatants were resolved by 15% SDS-PAGE and subjected to immunoblot analysis with anti-CrATG8 antibodies. The Ponceau staining is shown as the protein loading control. The experiment was repeated at least four times with different batches of plants, and a representative image is shown.

extracts from *des1* and wild-type plants grown in soil for four weeks under a long-day photoperiod (Fig. 2).

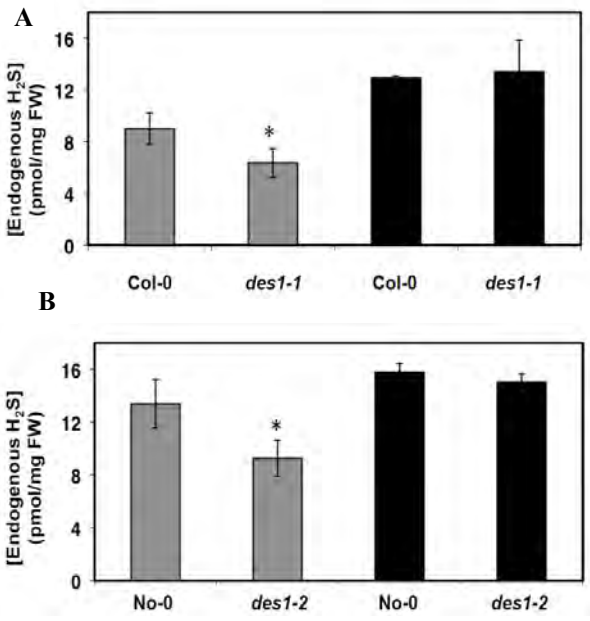

Figure 2. Endogenous H_2S content in leaf extracts. Twenty-day-old Col-0 and *des1-1* **(A)** and No-0 and *des1-2* **(B)** plants grown in soil under physiological conditions were divided into two batches. One batch was maintained at the same conditions (grey bars), and the other was irrigated with 200 μM Na_2S for 10 additional days (black bars). After this period, leaf extracts were prepared, and electrochemical detection of H_2S was performed using an H_2S-selective electrode as described in the materials and methods. Data are the mean ± SD for three independent experiments. *, $P< 0.05$.

Both *des1* alleles showed a 30% reduction in endogenous sulfide compared to their respective wild-type plants, highlighting the essential role of DES1 in regulating endogenous sulfide levels in leaves. We also observed that the endogenous H_2S concentration in leaves is dependent on ecotype in *Arabidopsis*, with a 45-49% higher sulfide content in No-0 than Col-0. When plants grown under physiological conditions were treated exogenously with 200 μM Na_2S for 10 days, we observed an increase in the endogenous H_2S content that was limited to approximately 13 pmol/mgFW in Col-0 and *des1-1* plants and approximately 15 pmol/mgFW in No-0 and *des1-2* plants (Fig. 2). No phenotypic damage was observed during the period of the treatment; therefore, these values do not exceed the sulfide toxicity thresholds that the plant can tolerate without compromising viability.

To determine if exogenously applied sulfide could completely or partially rescue the phenotypic characteristics observed in the *des1* mutants, 20-day-old plants grown with sufficient sulfur nutrition were treated for 10 days with 200 μM Na_2S and compared with untreated plants grown adjacent to them. The sulfide treatment clearly eliminated the phenotypic differences of the *des1-1* mutant. After mesophyll protoplasts were prepared from the leaves of sulfide-treated plants and analyzed for the presence of SAVs, yellow fluorescent structures corresponding to SAVs stained with Lysotracker Red were not detectable in the *des1-1* protoplasts, which were visualized in high number (Fig. 3A). We also observed a reduced accumulation of ATG8, primarily the lipidated ATG8 form, in the Na_2S-treated *des1-1* plants, suggesting that exogenous sulfide addition can inhibit the autophagy process (Fig. 3B).

Studies assessing the role of hydrogen sulfide in mammalian systems typically involve the administration of exogenous H_2S generated *in vitro* from both Na_2S and NaHS at micromolar concentrations (Szabo, 2007). Because the NaHS compound is more widely used as the hydrogen sulfide donor, we performed experiments similar to those described above on 20-day-old plants treated with different concentrations of NaHS. We observed that exogenous addition of NaHS rescued the autophagy activation phenotype of the *des1-1* mutant. Immunoblot analysis of ATG8 showed that even with a low NaHS concentration of 50 μM, the significant accumulation of modified ATG8 was lost; the protein banding patterns were similar at all NaHS concentrations (Fig. 4).

To confirm that the observed phenotype of the *des1-1* mutant plants was indeed due to the disruption of the *DES1* gene and a reduction of cytosolic endogenous sulfide, complementation analysis was performed using the full-length *DES1* cDNA fragment. We first measured and compared the endogenous H_2S concentrations in leaf extracts of the complemented *des1-1*:P35S-DES1 line, the wild-type and the *des1-1* mutant. The wild-type and complemented line contained similar levels of endogenous sulfide, 10.5 and 10.1 pmol/mgFW, respectively, while the *des1-1* mutant displayed a reduced level of 6.8 pmol/mgFW. Additionally, the *des1-1*:P35S-DES1 line showed the same pattern of ATG8 accumulation observed in the wild-type. The ATG8-PE adducts observed in the *des1-1* mutant were virtually undetectable, especially considering the higher protein loading in the lanes corresponding to the wild-type and complemented line (Fig. 5). Therefore, complementation of *des1-1* with the P35S-DES1 construct resulted in wild-type levels of endogenous sulfide and also reversed the autophagy-induced phenotype.

In plants, autophagy is recognized as a critical factor in nutrient remobilization when the environmental nutrient supply is limited. Several studies have reported autophagy induction by carbon and nitrogen starvation, which is observed in many

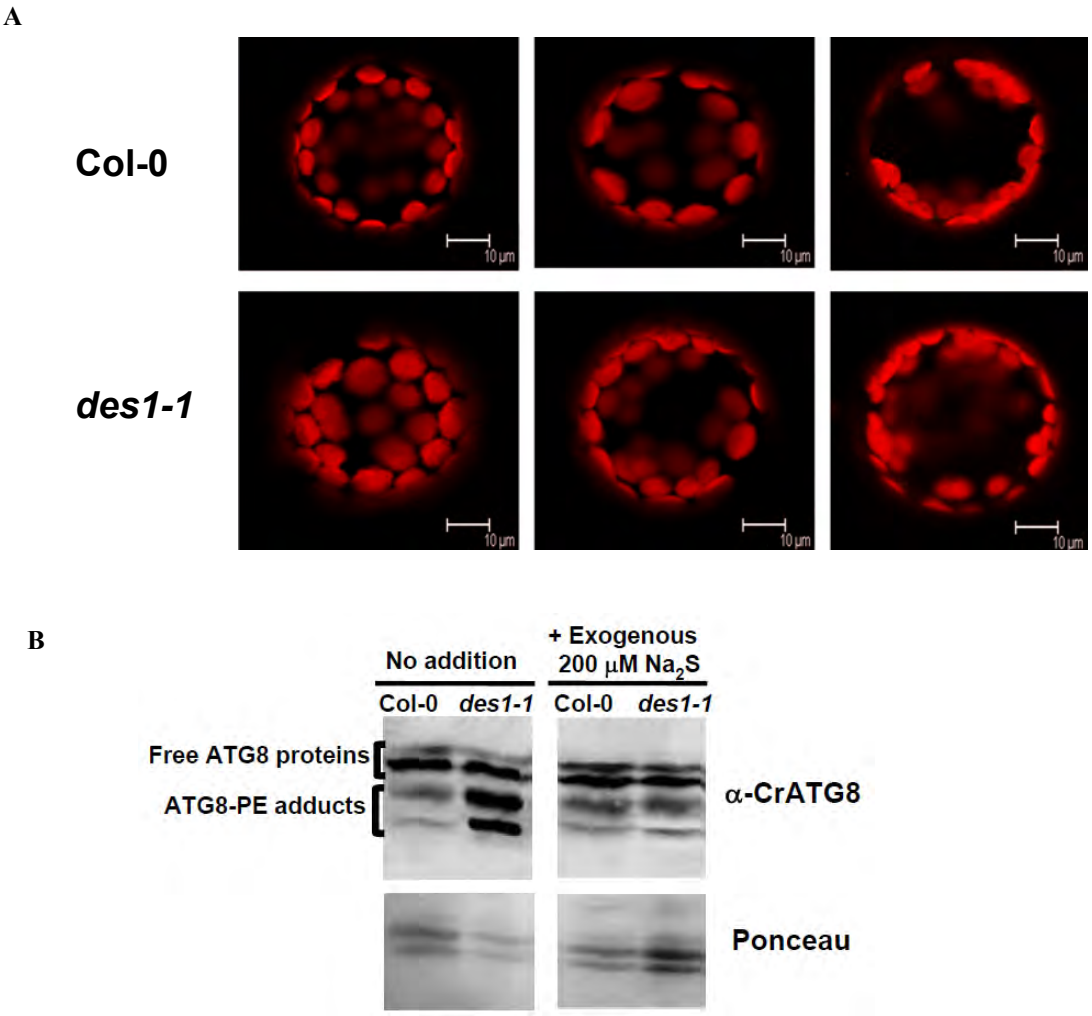

Figure 3. Effect of exogenous sulfide on the SAVs and induced autophagy phenotype of *des1-1* mutant plants. **(A)** Twenty-day-old Col-0 and *des1-1* plants were grown in soil and irrigated with 200 µM Na_2S in water for 10 days, and senescence-associated vacuoles were visualized by Lysotracker Red staining of protoplasts isolated from leaves. The experiment was repeated at least four times with different batches of plants, and the results were consistent across replicates. A representative image is shown. **(B)** Immunoblot analysis of ATG8 accumulation in leaves of Col-0 and *des1-1* plants treated with 200 µM Na_2S for 10 days and untreated plants (no Na_2S addition) grown side by side. The total lysates prepared from leaves were centrifuged at low speed, and the supernatants were resolved by 15% SDS-PAGE and subjected to immunoblot analysis with anti-CrATG8 antibodies. The Ponceau staining is shown as the protein loading control. The experiment was repeated at least four times with different batches of plants, and a representative image is shown.

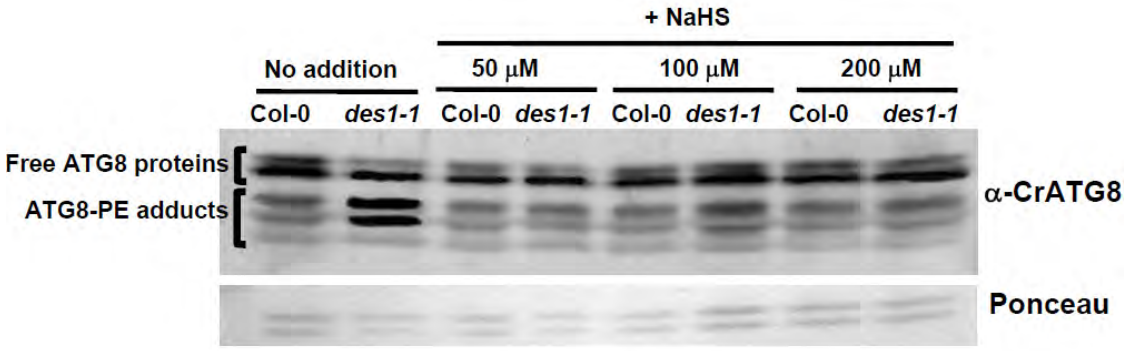

Figure 4. Effect of exogenous NaHS on the induced autophagy phenotype of the *des1-1* mutant. Twenty-day-old Col-0 and *des1-1* plants grown in soil were irrigated with water (no addition) or with different concentrations of NaHS in water for 10 days, and immunoblot analysis of ATG8 accumulation in leaves was performed. The total lysates were centrifuged at low speed, and the supernatants were resolved by 15% SDS-PAGE and subjected to immunoblot analysis with anti-CrATG8 antibodies. The Ponceau staining is shown as the protein loading control. The experiment was repeated at least four times with different batches of plants, and a representative image is shown.

cases as a substantial increase in ATG8 protein levels (Thompson et al., 2005; Chung et al., 2009).

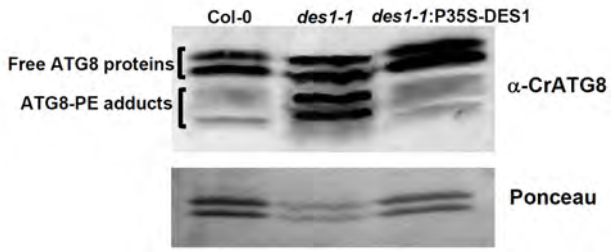

Figure 5. Genetic complementation of the induced autophagy phenotype of the *des1-1* mutant. Immunoblot analysis of ATG8 accumulation in the leaves of four-day-old Col-0, *des1-1* and the complemented *des1-1*:P35S-DES1 plants. The total lysates were centrifuged at low speed, and the supernatants were resolved by 15% SDS-PAGE and subjected to immunoblot analysis with anti-CrATG8 antibodies. The Ponceau staining is shown as the protein loading control. The experiment was repeated at least two times with different batches of plants, and a representative image is shown.

As indicated above, increased ATG8 protein levels were observed in *des1* mutants when the plants were grown with a sufficient C, N and S nutrient supply; thus, the effect of sulfide treatment does not appear to depend on the nutrient supply. To ensure that sulphur depletion was not a contributing factor in our plant system, we analyzed the transcript levels of genes in the sulfate-assimilation pathway that are regulated by sulfur availability. Col-0 wild-type plants were grown for 20 days under the same conditions used for the previous experiments and irrigated with water or 200 μM Na_2S for the next 10 days. The expression of sulfate transporter genes and APS reductases was analyzed. The transcript levels of the four sulfate transporter genes and the three APS reductases analyzed by qRT-PCR were similar for both sulfur nutrition conditions (see Supplemental Fig. 1A online). These results suggest that sulfide inhibition of the autophagy process (as measured by ATG8 protein accumulation) is unrelated to nutrient mobilization.

Sulfide Rescues the Autophagy Activation Resulting from Dark-Induced Carbon Starvation in *Arabidopsis* Plants

Previous studies have shown that ATG8 mRNA preferentially accumulates when detached leaves or whole plants are placed in extended darkness to deplete the available sugars (Doelling et al., 2002; Slavikova et al., 2005; Thompson et al., 2005; Phillips et al., 2008) and during sucrose starvation of cultured cells (Contento et al., 2004; Rose et al., 2006). Similarly, large increases in ATG8 protein levels under dark-induced carbon starvation have also been observed in *Arabidopsis* wild-type plants and in the *atg5* and *atg7* mutants, in which the two conjugation systems involved in autophagosome formation are compromised (Thompson et al., 2005). To test if the effects of sulfide as an autophagy inhibitor were observable under conditions not affecting sulfur nutrition, wild-type and *des1* mutant plants grown in soil were placed in darkness to induce carbon starvation and then allowed to recover (Thompson et al., 2005). In the absence of sulfide, enhanced chlorosis of the cotyledons and leaves was evident after the dark treatment in both the wild-type and *des1* mutant plants. In contrast, sulfide addition during the dark treatment and the recovery period apparently reduced the extent of chlorosis in all plant lines, resulting in a healthier phenotype (see Supplemental Fig. 2A online). To examine the effect of exogenous sulfide at the molecular level, the leaves were collected and homogenized, and total protein samples were obtained by low-speed centrifugation and subjected to immunoblot analysis. Consistent with the data previously described, ATG8 protein levels, primarily the conjugated ATG8 form, increased in Col-0 wild-type plants under dark-induced carbon starvation compared to plants grown under normal physiological conditions; in the *des1-1* background, a further increase in ATG8 protein accumulation was not observed (Fig. 6A). Remarkably, the addition of sulfide not only reduced the levels of ATG8 proteins in the *des1-1* plants grown under physiological conditions to the same levels observed in wild-type plants but also reduced ATG8 protein accumulation in both Col-0 and *des1-1* plants under carbon starvation (Fig. 6A). Nearly identical results were obtained in wild-type No-0 and *des1-2* mutant plants. No-0 plants under dark-induced carbon starvation had higher levels of the different ATG8 forms compared to the same plants under normal physiological growth conditions; the ATG8 protein levels were further increased in the *des1-2* mutant (Fig. 6B). Sulfide also reduced the accumulation of ATG8 proteins in carbon-starved No-0 and *des1-2* plants and physiologically grown *des1-2* plants to the levels observed in wild-type No-0 grown under physiological conditions (Fig. 6B). Further analysis of the transcript levels of four sulfate transporter genes and three APS reductase genes in Col-0 wild-type plants subjected to carbon-starvation in the absence and presence of sulfide revealed no significant differences for the two sulfur conditions (see Supplemental Fig. 1B online). This finding further supported that the effect of sulfide was not related to sulfur limitation.

DES1 catalyzes the desulfuration of L-Cys to sulfide plus ammonia and pyruvate (Alvarez et al., 2010), and several reports have shown that different

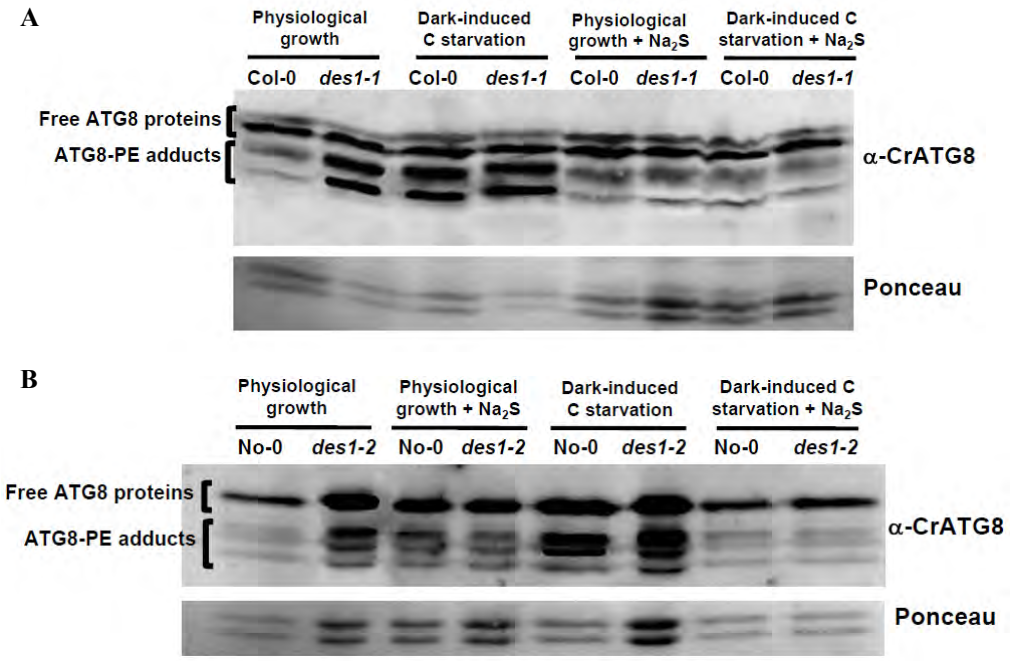

Figure 6. Effect of exogenous sulfide on the induced autophagy phenotype of *Arabidopsis* plants subjected to dark-induced carbon starvation. Twenty-day-old Col-0 and *des1-1* **(A)** and No-0 and *des1-2* **(B)** plants were grown in soil under physiological conditions and irrigated with water or 200 μM Na_2S for ten days before leaves were collected. Another batch of the same plant lines were subjected to carbon starvation in the absence or presence of 200 μM Na_2S by placing them in darkness for 3 days and allowing them to recover for 5 days before the leaves were collected. Total lysates prepared from leaves were centrifuged at low speed, and the supernatants were resolved by 15% SDS-PAGE and subjected to immunoblot analysis with anti-CrATG8 antibodies. The Ponceau staining is shown as the protein loading control. The experiment was repeated at least three times with different batches of plants, and a representative image is shown.

ATG proteins, including ATG8, are induced during nitrogen starvation in *Arabidopsis* (Thompson et al., 2005; Xiong et al., 2005). We therefore performed the dark-induced carbon starvation experiments in the presence of ammonium to confirm the specific role of sulfide as a repressor of autophagy. Unlike sulfide, ammonium was unable to reduce chlorosis in any plant lines (see Supplemental Fig. 2B online) and was unable to reduce the enhanced accumulation of ATG8 proteins in both Col-0 and *des1-1* mutant

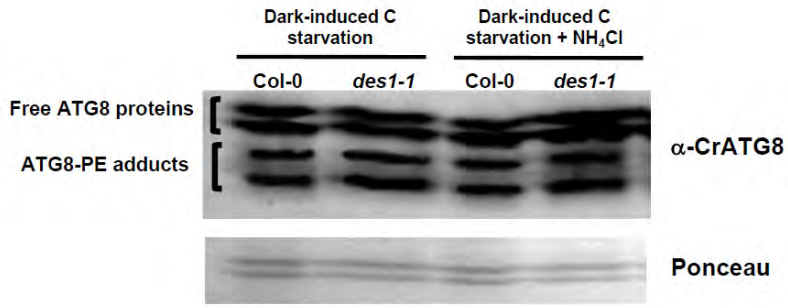

Figure 7. Effect of exogenous ammonium on the induced autophagy phenotype of *Arabidopsis* plants subjected to dark-induced carbon starvation. Twenty-day-old Col-0 and *des1-1* plants grown in soil were subjected to carbon starvation in the absence or presence of 200 μM NH_4Cl by placing them in darkness for 3 days and allowing them to recover for additional 5 days. Total lysates prepared from leaves were centrifuged at low speed, and the supernatants were resolved by 15% SDS-PAGE and subjected to immunoblot analysis with anti-CrATG8 antibodies. The Ponceau staining is shown as the protein loading control. The experiment was repeated at least three times with different batches of plants, and a representative image is shown.

plants under carbon starvation (Fig. 7).

The *DES1* Mutation Significantly Alters the Transcriptional Profile at the Late Growth Stage

Using the Affymetrix ATH1 GeneChip, we performed a comparative transcriptomic analysis on leaves of *des1-1* and Col-0 plants. Total RNA was extracted from the rosette leaves of plants grown for 20 days in soil under identical long-day conditions, with three biological replicates for each genotype. These samples were used to prepare complementary RNA, which was then hybridized to the chips (raw data, Supplemental Dataset 1; Gene Expression Omnibus repository GSE19244).

The normalized data from the replicates revealed differential expression of 24 genes in the *des1-1* mutant, with 16 genes up-regulated and 8 genes down-regulated by more than two-fold, with a False Discovery Rate (FDR) < 0.05 and with an intensity signal restriction of lgSignal > 7 (see Supplemental Tables 1 and 2 online).

The 24 identified genes were classified into 9 functional groups using MapMan categorization (see Supplemental Fig. 3 online). The most abundant group of induced genes corresponded to proteins that respond to abiotic and biotic stimuli. This group included the *PLANT DEFENSIN PROTEIN1.2B* (*PDF1.2B*) and *1.2A* (*PDF1.2A*) genes and *CATION EXCHANGER 3 (CAX3*), a gene encoding the vacuolar H^+/Ca^{2+} antiporter. Two genes closely associated with senescence in rosette leaves, *SAG21* (At4g02380) and the NAC transcription factor *NAP* (At1g69490), were also induced in *des1-1* at this growth stage. The repressed genes in *des1-1* rosette leaves belong to a unique functional group related to protein synthesis within the chloroplast and include 8 genes from the chloroplast genome (see Supplemental Table 2 online). These genes encode components of the 30S and 50S ribosomal subunits. There are 59 plastid ribosomal proteins, with 33 in the 50S subunit, 25 in the 30S subunit and a ribosome recycling factor in the 70S subunit (Yamaguchi and Subramanian, 2000). The repressed genes in *des1-1* therefore represent a reduction in 13% of genes encoding plastid ribosomal proteins.

In another experiment, total RNA was extracted after 30 days of growth under identical conditions, and the *des1-1* transcriptional profile in rosette leaves changed dramatically at this later growth stage (raw data, Supplemental Dataset 2; Gene Expression Omnibus repository GSE32566). The normalized data from the replicates showed differential expression of 1614 genes in the *des1-1* mutant, with 701 genes down-regulated and 913 genes up-regulated by more than two-fold, with a False Discovery Rate (FDR) of < 0.05 and with an intensity signal restriction of lgSignal > 7. These differences between the transcriptional profiles of young and old *des1-1* plants were clearly visible when the scatter plots of normalized transcript values were compared (Figs. 8A and B).

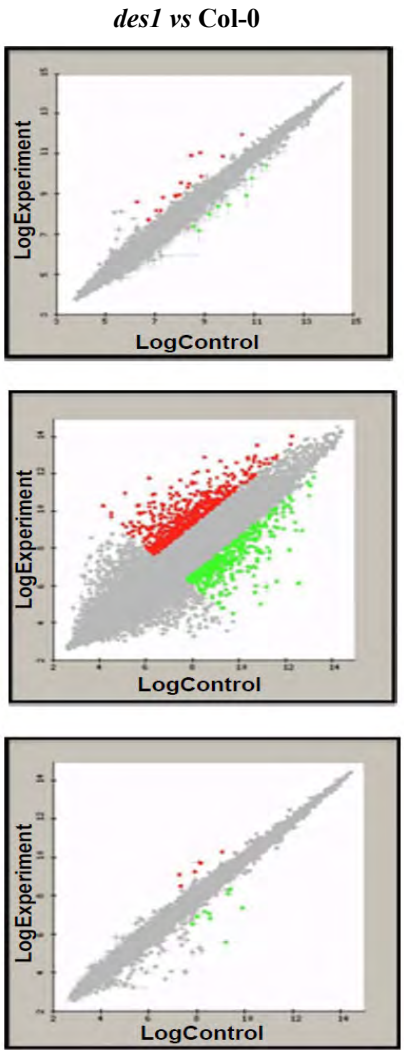

Figure 8. Scatter plots of normalized transcript values. Using the Affymetrix ATH1 GeneChips, we performed a comparative transcriptomic analysis on leaves of *des1-1* and Col-0 plants grown under identical long-day conditions in soil for 20 days **(A)**, 30 days **(B)** or 20 days with an additional 10 days of treatment with 200 µM Na_2S **(C)**. The mean intensity values (log_2 values) of all genes that were differentially regulated in the *des1-1* mutant (Y-axis) were plotted against the wild-type values (X-axis). Red and green dots represent genes up-regulated and down-regulated, respectively, by more than two-fold, with an FDR of < 0.05 and an intensity signal restriction of lgSignal > 7.

The 1614 genes with altered transcript levels were classified into 23 functional groups using MapMan categorization (see Supplemental Fig. 4 online). The most highly induced genes were a cysteine-type endopeptidase with unknown function (At2g27420)

(71-fold induction), *SENESCENCE-ASSOCIATED GENE 29* (*SAG29*) (60-fold induction), which integrates environmental stress responses into the process of senescence, and *LATE ELONGATED HYPOCOTYL* (*LHY*) (50-fold induction). LHY encodes a MYB-transcription factor involved in the circadian rhythm along with the MYB transcription factor encoded by *CCA1*, which was also induced at this stage (10-fold induction). The genes up-regulated at the earlier growth stage were induced at the later growth stage with even higher levels of induction; for example, the vacuolar H^+/Ca^{2+} antiporter gene *CAX3* exhibited a 12-fold induction at the later growth stage.

Excluding genes with no functional assignments, the category PROTEINS was the most important functional group and included 103 up-regulated and 119 down-regulated genes. Of the genes within this functional group, 41 encode proteins associated with ubiquitin- and autophagy-dependent degradation (Fig. 9, see Supplemental Table 3 online). The autophagic recycling of intracellular constituents requires the ubiquitin-fold proteins autophagy-related ATG8 and ATG12 (Chung et al., 2010). The genes encoding the ATG8B and ATG12A isoforms were induced in *des1-1* at the later growth stage, corresponding with the induced autophagy phenotype observed in the mutant at this stage of growth. Furthermore, most of the de-regulated genes in this functional group are related to ubiquitin-dependent degradation. Genes encoding proteins of the ubiquitin-proteasome system de-regulated in *des1-1* include three genes associated with the ubiquitin conjugating E2 enzyme, 23 RING-domain E3 ligase genes and components of the SCF ubiquitin ligase complex (Fig. 9). Among the up-regulated RING domain E3 ligase genes, we identified the gene encoding the XERICO protein, which promotes abscisic acid accumulation and drought tolerance and has been identified as a DELLA target (Daviere et al., 2008). The SCF ubiquitin ligase complex genes include *SKP1* (At5g57900), the protein product of which functions as an adaptor to bind F-box proteins in the SCF complex, and 11 F-box genes including *AFR*, which mediates the turnover of a repressor of *phyA* signaling (see Supplemental Table 3 online). Four functional groups stand out among the repressed genes in *des1-1*: TETRAPYRROLE SYNTHESIS, DNA, CELL WALL and PROTEINS (see Supplemental Fig. 4 online). The group of genes associated with the synthesis of tetrapyrroles comprised 13 genes, including *PROTOCHLOROPHYLLIDE OXIDOREDUCTASE B* (*PORB*), which exhibited the largest change (84-fold reduction); a glutamyl-tRNA(Gln) amidotransferase gene involved in the early steps of the precursor 5-aminolaevulinic acid synthesis pathway; *CHL12*, which encodes one of the two isoforms of the I subunit of magnesium-chelatase; and *FLU*, which encodes a coiled-coil TPR-domain containing protein that is localized to the chloroplast membrane and is involved in chlorophyll biosynthesis. The CELL WALL group comprised 41 genes, including multiple expansins (ATEXP1, 5, 6, 8 and 11), pectinases and pectate lyases. The DNA functional group was mainly composed of histone-related genes, and 50% of the genes included in this functional group encode ribosomal proteins.

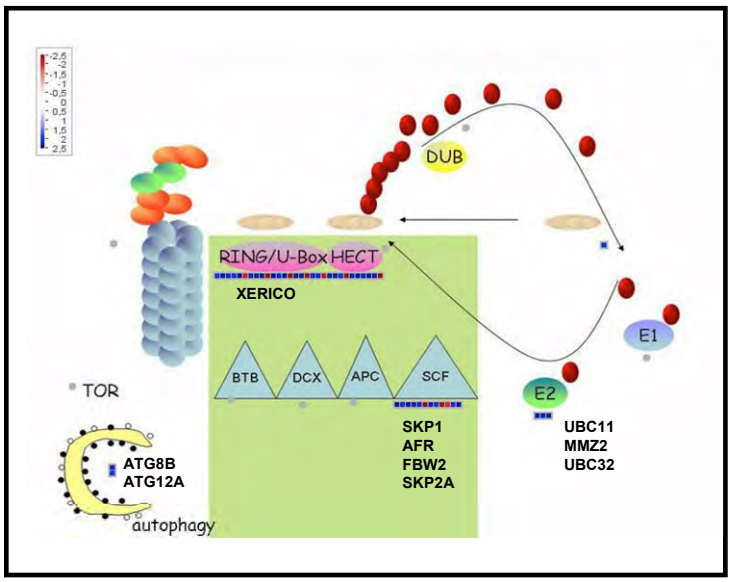

Figure 9. Diagram of ubiquitin- and autophagy-dependent degradation pathways. Small blue and red boxes represent up-regulated and down-regulated genes, respectively, in *des1-1* mutants relative to wild-type Col-0 plants grown for 30 days. Some representative genes are highlighted. The diagram was generated using the MapMan application.

Sulfide Reverses the Transcriptional Profile Changes of the *des1-1* Mutant

The effects of sulfide observed in the *des1* mutants, namely the reversal of the early senescence phenotype evidenced by the presence of SAVs and the increase of ATG8 protein accumulation, were also observable at the transcriptional level. Total RNA was extracted from rosette leaves of *des1-1* and wild-type plants grown for 20 days and treated with sodium sulfide for 10 additional days, with three biological replicates for each genotype (raw data, Supplemental Dataset 3; Gene Expression Omnibus repository GSE32566).

A comparison of the transcriptional profiles of *des1-1*+Na_2S and Col-0+Na_2S revealed that exogenous sulfide reversed differences at the transcript level between the mutant and the wild-type lines, analogous to the reversion of autophagy observed in previous experiments (Figs. 8B and C). The transcriptional profile of the treated plants was similar to the profile at the earlier growth stage, with only 6 genes up-regulated and 9 genes down-regulated by more than two-fold, with a False Discovery Rate (FDR) of < 0.05 and with an intensity signal restriction of lgSignal > 7 (see Supplemental Table 4 online). The induced genes included *PLANT DEFENSIN PROTEIN1.2B* (*PDF1.2B*) and *1.2A* (*PDF1.2A*), which were also induced in untreated plants at the earlier growth stage (Fig. 8A).

We performed real-time RT-PCR on a subset of genes to validate the expression data of the microarray analysis performed in plants at the late growth stage. Genes with altered expression were selected, including those with the largest fold changes in the *des1-1* transcriptional profile of rosette leaves from plants grown for 30 days. The qRT-PCR analysis was performed in both mutant plants, *des1-1* and *des1-2*, and compared with the wild-type Col-0 and No-0, respectively. Overall, there was qualitative agreement between the qRT-PCR results and the microarray analysis. The *des1-1* mutant showed the same changes in gene expression; the *des1-2* mutant also showed the same pattern of regulation, but with some quantitative differences (see Supplemental Fig. 5 online). We also performed qRT-PCR analysis in plants grown for 20 days and treated with sodium sulfide for 10 additional days to validate the results of the transcriptomic analysis. In both mutants, we observed a reversal of the transcriptional changes of the selected genes to the expression levels observed in the wild type. Therefore, the microarray data and the results of the qRT-PCR analysis in the *des1-1* and *des1-2* mutants were strongly correlated (see Supplemental Fig. 5 online).

The effect of the sulfide treatment on the transcriptional regulation of genes in both mutants was reinforced by the qRT-PCR analysis of the senescence-associated genes. In untreated plants, *SEN1* and *SAG21* showed low levels of transcript abundance, with higher levels in *des1-2* than in *des1-1*, while *SAG12*, *NAP* and *PR1* had high transcript levels, and these levels were highest in *des1-1*. Sulfide strongly reduced most of the transcript level changes in both mutants, supporting the role of sulfide as a transcriptional regulator in the *des1* mutants. *SAG12*, *NAP* and *PR1* transcripts levels were significantly reduced by sulfide treatment in both mutant plants, and *SEN1* and *SAG21* transcript levels decreased in *des1-2* and *des1-1*, but only

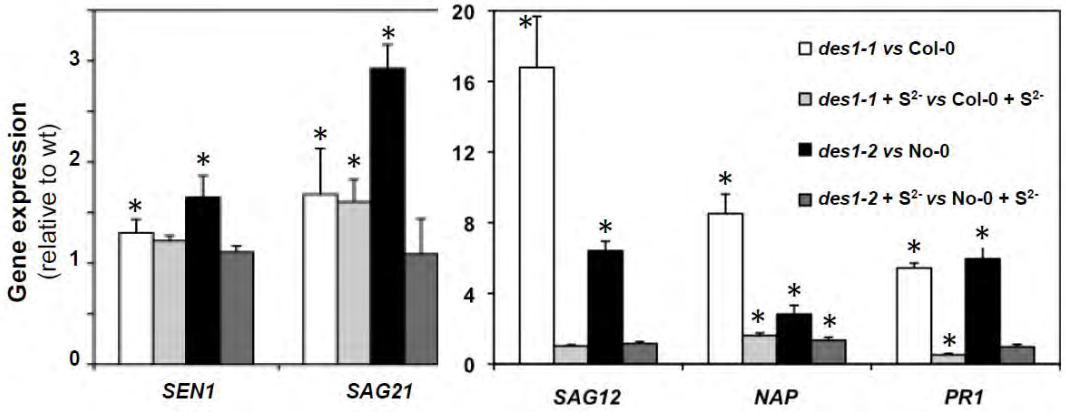

Figure 10. Relative expression levels of senescence-associated genes in *des1* mutant plants. Real-time RT-PCR analysis of *SEN1 (At4g35770), SAG21 (At4g02380), SAG12 (AT5G45890), NAP (At1g69490)* and *PR1 (At2g14610)* was performed in leaves from *des1-1* and *des1-2* mutant plants and their respective wild-types. Mutant and wild-type plants were grown under identical long-day conditions in soil for 30 days or for 20 days with 200 µM Na_2S treatment for 10 additional days, as indicated in the figure. The transcript levels were normalized to the constitutive *UBQ10* gene. Data shown are the means ± SD for three independent analyses and represent the transcript levels of each gene in the *des1* mutant plant relative to the respective wild-type plant. *, P< 0.05.

slightly in the latter (Fig. 10). Furthermore, the observed transcript level accumulation of the SA-responsive defense marker *PR1* in both mutants in the absence of sulfide correlated with an increase of SA. We measured the levels of SA in leaf extracts from *des1* and wild-type plants grown for four weeks under a long-day photoperiod, using HPLC coupled with MS. We found 1.6- and 1.9-fold increases in SA levels in the *des1-1* and *des1-2* mutants, respectively, compared to their corresponding wild-type ecotypes (Fig. 11).

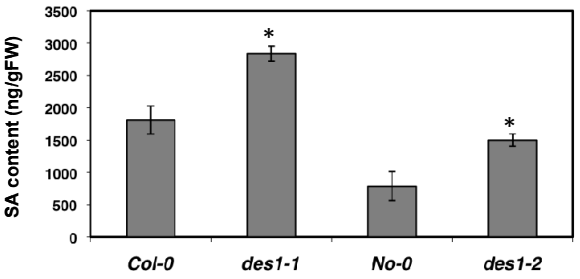

Figure 11. SA accumulation in *des1* mutant plants. Total SA was quantified by HPLC coupled to mass spectrometry in leaf extracts of the *des1* mutant and the respective wild-type plant grown in soil for four weeks under long-day conditions. Data shown are the means ± SD for three independent experiments. *, P< 0.05.

DISCUSSION

Although much attention has been given to the OASTL gene family of *Arabidopsis* in recent years (Heeg et al., 2008; Lopez-Martin et al., 2008a; Watanabe et al., 2008a; Alvarez et al., 2010; Bermudez et al., 2010; Garcia et al., 2010; Wirtz et al., 2010; Alvarez et al., 2011), little importance has been assigned to the minor OASTL-like proteins with different enzyme activities of cysteine biosynthesis, which can strongly affect plant metabolism when their function is disrupted (Gotor et al., 2010). This is the case for DES1, which is the only identified L-cysteine desulfhydrase located in the cytosol and is involved in the degradation of cysteine and the concomitant generation of H_2S in this cellular compartment. The function of DES1 is evidenced by the fact that the T-DNA insertion mutants *des1-1* and *des1-2* exhibit 20% and 25% increases, respectively, in their total Cys content relative to their respective wild-types (Alvarez et al., 2010). Accordingly, the leaf endogenous H_2S concentrations in the null mutants are 30% less than the quantified amount in the respective wild-types (this work).

The T-DNA insertion mutants deficient in DES1 show a characteristic phenotype, whose detailed characterization has been informative about the functions of this protein related to Cys metabolism (Alvarez et al., 2010) and the role of H_2S generated from Cys in the cytosol as a signaling molecule (present work). The recently described responses of *des1* mutants together with other Cys-deficient mutants to biotrophic and necrotrophic pathogens led us to conclude that cytosolic Cys plays a role in the establishment and signaling of the plant response to pathogens and that accurate regulation of cytosolic Cys homeostasis is critical for plant immunity (Alvarez et al., 2011).

Another aspect of plant physiology affected by disrupted DES1 function is leaf senescence. Recent work has shown that a mutation in the DES1 gene leads to premature leaf senescence, as evidenced by the increased expression of senescence-associated genes (Alvarez et al., 2010). In the present study, we provide new experimental evidence at the cellular and transcriptional levels assessing the senescence-induced phenotypes of the *des1-1* and *des1-2* mutants. During leaf senescence, the senescence-associated vacuoles (SAVs) are formed de novo; they are smaller in size than the central vacuole, and they can be detected using specific fluorescent markers due to their acidic pH. We have detected vacuoles with the same characteristics as SAVs in mesophyll protoplasts from *des1* mutants, which exhibit an acidic pH, small size and peripheral localization in the cytoplasms of cells that contain chloroplasts (Otegui et al., 2005). Our transcriptional data corresponds strongly with other feature of SAVs, including their intense Cys-protease activity, and that SAG12, a papain-like Cys-protease, localizes to the SAVs, although other proteases besides SAG12 are targeted to this compartment (Otegui et al., 2005). The transcriptional profile of *des1-1* at a later growth stage and validation by qRT-PCR revealed that one of the most highly induced genes is one that encodes a Cys-type endopeptidase of the family C1A located in the endomembrane system. In addition, the qRT-PCR analysis of senescence-associated genes showed that SAG12 expression is highly induced in both *des1* mutants compared to their respective wild-types.

During leaf senescence, cellular components are recycled for redistribution from senescent leaves to younger leaves and reproductive organs. It has been shown that the ubiquitin-26S proteasome pathway mediates senescence-associated protein degradation (Yoshida, 2003; Lin and Wu, 2004), and our transcriptomic data provide further evidence that mutations in the DES1 gene promote premature senescence. Categorization of the genes with altered expression levels in the *des1-1* mutant at a later growth stage indicated that the most important functional group was PROTEINS, which included a high proportion of genes encoding proteins involved in the ubiquitin-dependent degradation pathway.

Genes encoding ubiquitin conjugating E2 enzymes, RING-domain E3 ubiquitin ligases and components of the SCF ubiquitin ligase complex were de-regulated in the mutant. In addition, the senescence process is also influenced by various environmental and internal factors, with the latter including phytohormones (Lim et al., 2007). SA, the hormone typically involved in the plant response to pathogens, has been implicated in leaf senescence. Higher SA levels have been reported in senescing *Arabidopsis* leaves, and this observation correlates with the up-regulation of several SAG genes, including PR1 or SAG12 (Morris et al., 2000). Once again, our experimental data support the involvement of the SA-signaling pathway in the senescence-associated phenotype of *des1* mutants because higher SA levels in both mutants were observed in addition to the accumulation of the SA-responsive defense markers PR1 and SAG12.

Links between leaf senescence and autophagy have been established (Doelling et al., 2002; Hanaoka et al., 2002; Yoshimoto et al., 2009). Autophagy is a universal mechanism in eukaryotic cells and involves the digestion of cell contents to recycle needed nutrients or to degrade damaged or toxic components. The most important feature of autophagy is the *de novo* synthesis of double membrane-bound structures called autophagosomes, which sequester materials for degradation. The molecular machinery operating during autophagy was elucidated at the cellular level in yeast and mammals before it was described in plants, and the genes involved in this fundamental machinery are referred to as ATG genes. They can be divided into the five following functional groups based on the characterization of their gene products: the ATG1 protein kinase complex; the PI3 kinase complex specific for autophagy; the ATG9 complex; and two ubiquitination-like systems, the ATG8 lipidation system and the ATG12 protein conjugation system (Thompson and Vierstra, 2005; Bassham, 2007; Yoshimoto et al., 2010). The best-studied proteins involved in plant autophagy are those of the ubiquitin-like conjugation systems, both of which are essential for autophagosome formation. The first contributes to the formation of the covalently linked conjugate of ATG5 and ATG12 through ATG12 activation by the E1-type enzyme ATG7 and transfer of the Cys residue of the E2-like enzyme ATG10 before the final conjugation to ATG5. The second system is more unusual because the ATG8 protein is conjugated to a lipid, phosphatidylethanolamine (PE). The C-terminal extension of the newly synthesized ATG8 is cleaved by the Cys-type protease ATG4, and the truncated ATG8 product is activated by the E1-type enzyme ATG7, transferred to a Cys residue of the E2-like enzyme ATG3 and finally conjugated to the head group of PE. The complex ATG8-PE is deconjugated by the protease ATG4, which facilitates the release of ATG8 from membranes.

The vast majority of homologs of the yeast ATG genes have been identified primarily in *Arabidopsis* and other plants. Most of the essential residues are conserved, suggesting that the molecular basis of the core autophagy machinery is essentially the same in plants and yeast (Thompson and Vierstra, 2005; Bassham, 2007; Yoshimoto et al., 2010). *Arabidopsis* contains nine highly conserved ATG8 proteins that are processed at their C-termini and associated with autophagosomes in a comparable manner (Doelling et al., 2002; Yoshimoto et al., 2004; Slavikova et al., 2005; Thompson et al., 2005). Five loci encoding ATG8 proteins have been identified in rice and maize, and maize ATG8 has been shown to be lipidated *in planta* and appears to be functional during autophagy mechanisms in this crop (Chung et al., 2009). The genome of the model alga *Chlamydomonas reinhardtii* contains a single ATG8 gene the product of which displays high identity with plant ATG8 proteins and performs a function similar to that of yeast ATG8 (Perez-Perez and Crespo, 2010; Perez-Perez et al., 2010). Soon after the discovery of autophagy-dependent ATG8 lipidation, ATG8 has become one of the main tools for monitoring autophagy. In SDS-PAGE protein gels, the molecular weight of ATG8 changes from 18 kDa for the free cytosolic form to 16 kDa for the lipidated form, ATG8-PE. Making use of antibodies raised against recombinant ATG8 from *C. reinhardtii* that have a high affinity for *Arabidopsis* ATG8 proteins (Perez-Perez et al., 2010), we have demonstrated that DES1 deficiency induces the ATG8 lipidation typically associated with the autophagy process. In two ecotypic backgrounds, Col-0 and No-0, we observed that a mutation in the DES1 gene strongly promotes ATG8 protein accumulation, particularly in the lipidated form. Similar ATG8 protein accumulation profiles have been reported in *Arabidopsis*, and increased ATG8 lipidation has been correlated with autophagy induction (Yoshimoto et al., 2004; Thompson et al., 2005; Phillips et al., 2008; Chung et al., 2010). In addition, our transcriptomic data confirm an induction of autophagy in the *des1-1* mutant, in which we observed that the ATG8B and ATG12A genes were up-regulated by more than two-fold and that other members of the ATG8 gene family were also up-regulated, including ATG8A (1.97-fold induction) and ATG8G (1.82-fold induction) (GSE32566). Increased expression levels of different ATG8 family members in *Arabidopsis* have been observed when plants exhibit an induced autophagy phenotype. The abundance of ATG8 transcript levels has been shown to increase in both wild-type plants and autophagy mutants in response to sucrose starvation of suspension-cultured cells (Rose et al., 2006), darkness-induced carbon starvation of intact plants (Thompson et al., 2005;

Phillips et al., 2008) and detached leaves (Doelling et al., 2002) and nitrogen starvation in hydroponic media (Yoshimoto et al., 2004). Furthermore, the two ATG12 genes of *Arabidopsis* show distinct expression patterns: ATG12B transcripts are more abundant during early development, while ATG12A expression is higher at later growth stages and is greatly induced during leaf senescence (Chung et al., 2010).

Mutation of the DES1 gene disrupts H_2S generation in the *Arabidopsis* cytosol. Restoring the capacity of H_2S generation, through exogenous sources (Na_2S or NaHS) or by genetic complementation, eliminates the phenotypic differences of the *des1* mutants from wild-type plants. Exogenous sulfide reverses the defects of *des1* mutants not only at the cellular (undetectable SAVs) and protein levels (reduction of ATG8 protein accumulation) but also at the transcriptional level. When autophagy is induced by carbon starvation, sulfide is able to revert the ATG8 protein accumulation even in wild-type plants, suggesting a general effect of sulfide on autophagy. The mutation of the DES1 gene also disrupts the production of ammonium from cysteine in the cytosol; however, exogenous ammonium did not have the same effect on autophagy as exogenous sulfide. Therefore, sulfide in particular was found to negatively regulate autophagy, and this regulation was unrelated to nutrient limitation stress. Evidence indicates that autophagy is a major mechanism for nutrient mobilization under starvation conditions in plants, and deficits of both carbon and nitrogen have been shown to induce autophagy (Doelling et al., 2002; Hanaoka et al., 2002; Yoshimoto et al., 2004; Thompson et al., 2005; Xiong et al., 2005; Rose et al., 2006; Phillips et al., 2008; Chung et al., 2009). In light of the possibility that the observed effects of exogenous sulfide could be due to a compensation of nutrient starvation, we confirmed that our plant system did not exhibit sulfur limitation conditions. The control of sulfur assimilation occurs primarily at the steps of sulfate uptake and APS reduction, where the transcript levels of sulfate transporters and APS reductases are strictly regulated by sulfate availability, and are induced under sulfur limitation conditions (Takahashi et al., 2011). We did not observe significant differences in the transcript levels of four sulfate transporters and three APS reductase genes in wild-type plants in the absence and presence of exogenous sulfide.

Our results suggest that cysteine-generated sulfide in the cytosol acts as a negative regulator of autophagy and a modulator of the transcriptional profile of *Arabidopsis* and that this effect is independent of the sulfur nutrient status of the plant. Recently, subcellular sulfide concentrations were determined, with 125 μM in plastids and 55 μM in the cytosol (Krueger et al., 2009). In plants, sulfate reduction takes place in plastids (Takahashi et al., 2011), which is consistent with the large amount of sulfide in plastids. The presence of sulfide in compartments other than plastids requires for it to be transported across membranes to those compartments. Although it has been proposed that H_2S reaches the cytosol via diffusion through the chloroplast envelope membrane, the chloroplast stroma reaches a pH of 8.5 under illumination (Heldt et al., 1973; Wu and Berkowitz, 1992), conditions at which 95% of sulfide would be present in the charged HS-form. Thus, sulfide transport across the chloroplast envelope membrane may be limited (Lowicka and Beltowski, 2007; Kabil and Banerjee, 2010), and the sulfide in the cytosolic compartment should be metabolically generated to act as a signaling molecule.

Hydrogen sulfide is already recognized as an important signaling molecule in mammalian systems (Lowicka and Beltowski, 2007; Szabo, 2007; Gadalla and Snyder, 2010), and emerging data suggest the same for plants. It has been reported to be involved in the protection against copper, aluminum and boron stress (Zhang et al., 2008; Wang et al., 2010; Zhang et al., 2010) and in the regulation of photosynthesis (Chen et al., 2011). It has also been identified as a component of the ABA signaling pathway in guard cells (Garcia-Mata and Lamattina, 2010; Lisjak et al., 2010). The findings presented in this work support these findings and highlight the role of sulfide as an important regulator of autophagy. In animal systems, the mechanisms of H_2S action and its molecular targets are poorly understood. H_2S appears to signal predominantly through S-sulfhydrating cysteine residues in its target proteins, which is analogous to S-nitrosylation by NO. In order for this post-translation modification to occur, the cysteine residue must exit in an oxidized state (e.g., as sulfenic acid or as a disulfide) and then be attacked by the hydrosulfide anion to yield a persulfide product. Whereas S-nitrosylation typically inhibits enzymes, S-sulfhydration activates them because the latter merely changes an –SH to an –SSH, enhancing the chemical reactivity of enzymes and possibly improving their access to their respective targets (Gadalla and Snyder, 2010; Kabil and Banerjee, 2010).

It has been suggested that the thiol redox state can profoundly influence autophagy, especially during the initiation and completion of the autophagosomes, because of the remarkable number of proteins involved in autophagy that are able to sense alterations of the cellular redox state by means of reactive cysteine residues (Filomeni et al., 2010). In particular, redox mechanisms are involved in the regulation of ubiquitination processes through the reversible S-thiolation of E1 and E2 enzymes

(Jahngen-Hodge et al., 1997). Redox regulation of the cysteine protease ATG4 has been characterized in detail in mammalian systems, and a cysteine residue located near the catalytic site has been found to be critical for this regulation (Scherz-Shouval et al., 2007). Autophagy is a process involved in the proteolytic degradation of cellular macromolecules and requires the activity of proteases. The *A. thaliana* genome has over 550 protease sequences, and recent findings highlight the potential of proteases to participate in many aspects of plant growth and development. One of the most frequently studied but poorly understood plant proteases is the C1A family of papain-like cysteine proteases, which have the characteristic papain protease unit consisting of the catalytic dyad, Cys25 and His159 (papain numbering) (Beers et al., 2004). Some of the members of this family are transcriptionally up-regulated in the *des1* mutants, and it is therefore possible that the cysteine residues of their active centers are the targets of S-sulfhydration.

MATERIALS AND METHODS

Plant Material, Growth Conditions and Treatments

Arabidopsis thaliana wild-type ecotypes Col-0 and No-0 and the SALK_103855 (*des1-1*) and RIKEN RATM13-27151_G (*des1-2*) lines were used in this work (Alonso et al., 2003; Ito et al., 2005; Alvarez et al., 2010).

To generate the *des1-1* complementation line, a 972-bp cDNA fragment containing the full-length coding sequence of DES1 was obtained by RT-PCR amplification using the proof reading Platinum Pfx DNA polymerase (Invitrogen) and the primers DES1F and DES1R (see Supplemental Table 5 online). The fragment was cloned into the pENTR/D-TOPO vector (Invitrogen) and transferred into the pMDC32 vector (Curtis and Grossniklaus, 2003) using the Gateway system (Invitrogen) according to the manufacturer's instructions. The final construct was generated by transformation into *Agrobacterium tumefaciens* and then introduced into *des1-1* null plants using the floral-dip method (Clough and Bent, 1998).

Plants were grown in soil with a photoperiod of 16 h of white light (120 μE m^{-2} s^{-2}) at 20ºC and 8 h of dark at 18ºC. Twenty-day-old plants were irrigated for 10 additional days with 200 μM Na_2S, 50-200 μM NaHS or 200 μM NH_4Cl in water. Dark-induced carbon starvation was performed on 20-day-old plants by placing them in darkness for 3 days and allowing them to recover for an additional 5 days (Thompson et al., 2005).

Preparation of Leaf Protoplasts

Leaves were collected from 4-week-old plants, and the 'Tape-*Arabidopsis* Sandwich' experiment protocol was performed (Wu et al., 2009). The upper epidermal surface was stabilized with a strip of labeling tape (Shamrock), while the lower epidermal surface was affixed to a strip of Magic tape 3 M (Scotch). The Magic tape was carefully pulled away from the labeling tape to peel away the lower epidermal surface cell layer. The peeled leaves (7 to 10 leaves) adhering to the labeling tape were transferred to a Petri dish containing 10 mL of enzyme solution [1% cellulose R10 (Serva), 0.25% macerozyme R10 (Serva), 0.4 M mannitol, 10 mM $CaCl_2$, 20 mM KCl, 0.1% BSA and 20 mM MES, pH 5.7]. The leaves were gently shaken (40 rpm on a platform shaker) with light for 20 to 60 min until the protoplasts were released into the solution. The protoplasts were centrifuged at 100 g for 3 min, washed twice with 25 mL of pre-chilled modified W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose and 2 mM MES, pH 5.7) and incubated on ice for 30 min. The resulting protoplast preparations were used for further analysis.

Detection of Senescence-Associated Vacuoles (SAVs)

Isolated protoplasts were incubated with 5 μM Lysotracker Red DND-99 (Molecular Probes) for 10 min at ambient temperature. After washing off any excess dye, samples were mounted onto a slide, with a spacer between the slide and coverslip, and observed using a TCS SP2 spectral confocal microscope (Leica Microsystems). Lysotracker Red was excited with a 543 nm Helium-Neon laser line, and emitted light was detected after spectral separation in the 560-605 nm range (pseudocoloured in yellow).

Western-Blot Analysis

Plant leaf material (200 mg) was ground in liquid nitrogen with 400 μL of extraction buffer [100 mM Tris-HCl (pH 7.5), 400 mM sucrose, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 mg mL-1 pepstatin A and 4% (v/v) protease inhibitor cocktail (Roche)] using a MM400 mixer mill (Retsch) and centrifuged at 500 g for 10 min to obtain the supernatant fraction. The total amount of protein in the resulting supernatant was determined using a method previously described (Bradford, 1976). For immunoblot analyses, 60 μg of leaf protein extracts waselectrophoresed on 15% acrylamide gels before transfer to polyvinylidene fluoride membranes (Bio-Rad) according to the manufacturer's instructions. Anti- CrATG8 (Perez-Perez et al., 2010) and

secondary antibodies were diluted 1:2,000 and 1:10,000, respectively, in phosphate-buffered saline containing 0.1% Tween20 (Sigma-Aldrich) and 5% milk powder. The ECL-Advance immunoblotting detection system (GE Healthcare) was used to detect the proteins with horseradish peroxidase-conjugated anti-rabbit secondary antibodies.

Real-Time RT-PCR

Quantitative real-time RT-PCR was used to analyze the expression of several sulfur-responsive genes, senescence-associated genes and other genes for the validation of the microarray data. Total RNA was extracted from *Arabidopsis* leaves using the RNeasy Plant Mini Kit (Qiagen) and reverse transcribed using an oligo(dT) primer and the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. Gene-specific primers for each gene were designed using the Vector NTI Advance 10 software (Invitrogen; see Supplemental Table 5 online), and the PCR efficiency of all primer pairs was confirmed to be close to 100%. Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad), and the signals were detected on an iCYCLER (Bio-Rad) according to the manufacturer's instructions. The cycling profile consisted of 95ºC for 10 min followed by 45 cycles of 95ºC for 15 s and 60ºC for 1 min. A melting curve from 60ºC to 90ºC was run following the PCR cycling. The expression levels of the genes of interest were normalized to the constitutive UBQ10 gene by subtracting the cycle threshold (CT) value of UBQ10 from the CT value of the gene (ΔCT). The fold change was calculated as $2^{-(\Delta CT \text{ mutant} - \Delta CT \text{ wild-type})}$. The results shown are the means ± SD for at least three independent RNA samples.

Microarray Hybridization

Total RNA was isolated with Trizol reagent (Invitrogen) and purified using the RNeasy Plant Mini Kit (Qiagen). The 3' Amplification One-Cycle Target Labeling Kit and the purified RNA were used to synthesize biotinylated cRNA for hybridization to the *Arabidopsis* ATH1 arrays (Affymetrix). Briefly, 4μg of RNA was reverse transcribed to produce first-strand cDNA using a $(dT)_{24}$ primer with a T7 RNA polymerase promoter site added to the 3' end. After second-strand synthesis, in vitro transcription was performed using T7 RNA polymerase and biotinylated nucleotides to produce biotin-labeled cRNA. The cRNA preparations (15 μg) were fragmented into 35 bp to 200 bp lengths at 95°C for 35 min. The fragmented cRNAs were hybridized to the *Arabidopsis* ATH1 microarrays at 45ºC for 16 h. Each microarray was washed and stained in the Affymetrix Fluidics Station 400 following standard protocols. Microarrays were scanned using an Affymetrix GeneChip® Scanner 3000.

Microarray Data Analysis

Microarray analysis was performed using the affylmGUI R package (Wettenhall et al., 2006). The Robust Multi-array Analysis (RMA) algorithm was used for background correction, normalization and summarizing expression levels (Irizarry et al., 2003). Differential expression analysis was performed using Bayes t-statistics using linear models for microarray data (Limma), which is included in the affylmGUI package. P-values were corrected for multiple-testing using Benjamini-Hochberg's method (False Discovery Rate) (Benjamini and Hochberg, 1995; Reiner et al., 2003). A two-fold cutoff with a False Discovery Rate (FDR) of < 0.05 and an intensity signal restriction of lgSignal > 7 were adopted to identify genes that were differentially expressed. Gene classification into functional groups was obtained from TAIR (http://www.arabidopsis.org) and MapMan (http://gabi.rzpd.de/projects/MapMan/). The microarray data were meta-analyzed using the Bio-Array Resource for *Arabidopsis* Functional Genomics (http://www.bar.utoronto.ca) (Toufighi et al., 2005).

Measurements of Endogenous H_2S

Leaves (200 mg) were ground in liquid nitrogen to a fine powder and suspended in 150 μL of antioxidant buffer (62.5 g L^{-1} of sodium salicylate, 16.25 g L^{-1} of ascorbic acid and 21.25 g L^{-1} sodium ascorbate). After vortexing for 1 min and centrifugation at 15,000 g for 15 min at 4ºC, H_2S was measured for 20 min at 25ºC in the resulting supernatant using a Micro Sulfide Ion Electrode (LIS-146AGSCM; Lazar Research Lab. Inc.). Concentrations of H_2S were determined from a calibration curve made with increasing concentrations of NaSH in antioxidant buffer. Each measurement was repeated twice, and data are indicated as the means ± SD for at least three independent biological experiments.

Measurement of Total SA Content

The quantification of total SA content was performed using a previously described method (Alvarez et al., 2011). Leaves (100 mg) were ground in liquid nitrogen to a fine powder and suspended in 0.5 mL of 90% methanol. This mixture was vortexed for 1 min, sonicated for 5 min and centrifuged for 5 min at maximum speed. The supernatant was collected, and the pellet was re-suspended in 0.25 mL of 100% methanol. The sonication and centrifugation

steps were repeated. The supernatants were combined and centrifuged again, and the methanol:water mixtures were evaporated in a SpeedVac concentrator. A volume of 250 µL trichloroacetic acid (TCA; 5% solution in water) was added to the residue, and the mixture was vortexed. Partitioning with 800 µL of ethyl acetate:cyclohexane (1:1, v/v) resulted in the separation of two phases and was performed twice. The aqueous phase was subjected to acid hydrolysis by adding approximately 300 µL of 8 M hydrochloric acid to the remaining TCA fraction and heating the sample at 80°C for 1 h. The residue was dissolved in 200 µL of sodium acetate (pH 5.5) and methanol (9:1) and analyzed by HPLC-MS. SA analyses were performed with a linear ion trap mass spectrometer equipped with an electrospray ionization source (Bruker Daltonics) coupled to a liquid chromatograph (Ultimate 3000; Dionex). A zwitterionic ZIC®-HILIC stationary phase column (Meck SeQuant 200 Å, 3.5 µm particle size, 100 x 2.1 mm) was used at 25ºC. The elution gradient was performed with a binary solvent system consisting of 5 mM ammonium acetate (solvent A) and 0.1% acetonitrile/formic acid (solvent B) at a constant flow-rate of 0.2 mL min-1. The following gradient profile was used: a linear gradient from 90 to 10% B (0 to 16 min), 10% B (16 to 17 min), a linear gradient from 10 to 90% B (17 to 18 min) and 90% B (18 to 28 min). SA was detected in negative mode, giving the spectra of the deprotonated molecule $[M - H]^-$ (*m/z* 137) and the isotopic form *m/z* 138 with a retention time of 1.6 min. The system was controlled with the software package HyStar (version 3.2; Bruker Daltonic). A standard solution of SA was used for the calibration curve.

Statistical Analysis

ANOVA statistical analysis of the data was performed using the program OriginPro7.5 (OriginLab Corporation).

Supplemental Data

Supplemental Figure 1 online. Relative expression levels of sulfur-responsive genes in Col-0 wild-type plants treated with exogenous sulfide.
Supplemental Figure 2 online. Effect of exogenous sulfide and ammonium on the phenotype of *Arabidopsi*s plants under dark-induced carbon starvation.
Supplemental Figure 3 online. Gene categorization according to biological processes for the genes with altered transcript levels in the *des1-1* mutant compared to Col-0 plants.
Supplemental Figure 4 online. Gene categorization according to biological processes for the genes with altered transcript levels in the *des1-1* mutant compared to Col-0 plants.
Supplemental Figure 5 online. Relative expression levels of selected genes in the *des1-1* and *des1-2* mutant plants.
Supplemental Table 1 online. List of differentially induced genes in *des1-1* mutant plants grown for 20 days in soil under long-day conditions.
Supplemental Table 2 online. List of differentially repressed genes in *des1-1* mutant plants grown for 20 days in soil under long-day conditions.
Supplemental Table 3 online. List of differentially regulated genes in *des1-1* mutant plants categorized within the ubiquitin- and autophagy-dependent degradation functional groups.
Supplemental Table 4 online. List of differentially regulated genes in *des1-1* mutant plants grown for 20 days in soil under long-day conditions and treated with 200 µM Na_2S for 10 additional days.

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REFERENCES

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, Gadrinab C, Heller C, Jeske A, Koesema E, Meyers CC, Parker H, Prednis L, Ansari Y, Choy N, Deen H, Geralt M, Hazari N, Hom E, Karnes M, Mulholland C, Ndubaku R, Schmidt I, Guzman P, Aguilar-Henonin L, Schmid M, Weigel D, Carter DE, Marchand T, Risseeuw E, Brogden D, Zeko A, Crosby WL, Berry CC, Ecker JR (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science **301:** 653-657
Alvarez C, Angeles Bermudez M, Romero LC, Gotor C, Garcia I (2011) Cysteine homeostasis plays an essential role in plant immunity. New Phytol
Alvarez C, Calo L, Romero LC, Garcia I, Gotor C (2010) An O-acetylserine(thiol)lyase homolog with homeostasis in Arabidopsis. Plant Physiol **152:** 656-669

Alvarez C, Lozano-Juste J, Romero LC, Garcia I, Gotor C, Leon J (2011) Inhibition of *Arabidopsis* O-acetylserine(thiol)lyase A1 by tyrosine nitration. J Biol Chem **286:** 578-586

Bassham DC (2007) Plant autophagy--more than a starvation response. Curr Opin Plant Biol **10:** 587-593

Beers EP, Jones AM, Dickerman AW (2004) The S8 serine, C1A cysteine and A1 aspartic protease families in Arabidopsis. Phytochemistry **65:** 43-58

Benjamini Y, Hochberg Y (1995) Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society Series B-Methodological **57:** 289-300

Bermudez MA, Paez-Ochoa MA, Gotor C, Romero LC (2010) *Arabidopsis* S-sulfocysteine synthase activity is essential for chloroplast function and long-day light-dependent redox control. Plant Cell **22:** 403-416

Bloem E, Riemenschneider A, Volker J, Papenbrock J, Schmidt A, Salac I, Haneklaus S, Schnug E (2004) Sulphur supply and infection with Pyrenopeziza brassicae influence L-cysteine desulphydrase activity in Brassica napus L. J Exp Bot **55:** 2305-2312

Bogdanova N, Hell R (1997) Cysteine synthesis in plants: protein-protein interactions of serine acetyltransferase from *Arabidopsis thaliana*. Plant J **11:** 251-262

Bonner ER, Cahoon RE, Knapke SM, Jez JM (2005) Molecular basis of cysteine biosynthesis in plants: structural and functional analysis of O-acetylserine sulfhydrylase from *Arabidopsis* thaliana. J Biol Chem **280:** 38803-38813

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248-254

Chen J, Wu FH, Wang WH, Zheng CJ, Lin GH, Dong XJ, He JX, Pei ZM, Zheng HL (2011) Hydrogen sulphide enhances photosynthesis through promoting chloroplast biogenesis, photosynthetic enzyme expression, and thiol redox modification in Spinacia oleracea seedlings. J Exp Bot

Chung T, Phillips AR, Vierstra RD (2010) ATG8 lipidation and ATG8-mediated autophagy in *Arabidopsis* require ATG12 expressed from the differentially controlled ATG12A AND ATG12B loci. Plant J **62:** 483-493

Chung T, Suttangkakul A, Vierstra RD (2009) The ATG autophagic conjugation system in maize: ATG transcripts and abundance of the ATG8-lipid adduct are regulated by development and nutrient availability. Plant Physiol **149:** 220-234

Contento AL, Kim SJ, Bassham DC (2004) Transcriptome profiling of the response of *Arabidopsis* suspension culture cells to Suc starvation. Plant Physiol **135:** 2330-2347

Curtis MD, Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiol **133:** 462-469

Daviere JM, de Lucas M, Prat S (2008) Transcriptional factor interaction: a central step in DELLA function. Curr Opin Genet Dev **18:** 295-303

Doelling JH, Walker JM, Friedman EM, Thompson AR, Vierstra RD (2002) The APG8/12-activating enzyme APG7 is required for proper nutrient recycling and senescence in *Arabidopsis thaliana.* J Biol Chem **277:** 33105-33114

Droux M (2004) Sulfur assimilation and the role of sulfur in plant metabolism: a survey. Photosynth Res **79:** 331-348

Droux M, Ruffet ML, Douce R, Job D (1998) Interactions between serine acetyltransferase and O-acetylserine (thiol) lyase in higher plants--structural and kinetic properties of the free and bound enzymes. Eur J Biochem **255:** 235-245

Filomeni G, Desideri E, Cardaci S, Rotilio G, Ciriolo MR (2010) Under the ROS...thiol network is the principal suspect for autophagy commitment. Autophagy **6:** 999-1005

Gadalla MM, Snyder SH (2010) Hydrogen sulfide as a gasotransmitter. J Neurochem **113:** 14-26

Garcia I, Castellano JM, Vioque B, Solano R, Gotor C, Romero LC (2010) Mitochondrial {beta}-Cyanoalanine Synthase Is Essential for Root Hair Formation in *Arabidopsis* thaliana. Plant Cell **22:** 3268-3279

Garcia-Mata C, Lamattina L (2010) Hydrogen sulphide, a novel gasotransmitter involved in guard cell signalling. New Phytol **188:** 977-984

Gotor C, Alvarez C, Bermudez MA, Moreno I, Garcia I, Romero LC (2010) Low abundance does not mean less importance in cysteine metabolism. Plant Signal Behav **5**

Hanaoka H, Noda T, Shirano Y, Kato T, Hayashi H, Shibata D, Tabata S, Ohsumi Y (2002) Leaf senescence and starvation-induced chlorosis are accelerated by the disruption of an *Arabidopsis* autophagy gene. Plant Physiol **129:** 1181-1193

Hatzfeld Y, Maruyama A, Schmidt A, Noji M, Ishizawa K, Saito K (2000) beta-Cyanoalanine synthase is a mitochondrial cysteine synthase-like protein in spinach and Arabidopsis. Plant Physiol **123:** 1163-1171

Heeg C, Kruse C, Jost R, Gutensohn M, Ruppert T, Wirtz M, Hell R (2008) Analysis of the *Arabidopsis* O-acetylserine(thiol)lyase gene family demonstrates compartment-specific differences in the regulation of cysteine synthesis. Plant Cell **20:** 168-185

Heldt HW, Werdan K, Milovancev M, Geller G

(1973) Alkalization of the chloroplast stroma caused by light-dependent proton flux into the thylakoid space. Biochim Biophys Acta **314:** 224-241

Howarth JR, Dominguez-Solis JR, Gutierrez-Alcala G, Wray JL, Romero LC, Gotor C (2003) The serine acetyltransferase gene family in *Arabidopsis* thaliana and the regulation of its expression by cadmium. Plant Mol Biol **51:** 589-598

Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics **4:** 249-264

Ito T, Motohashi R, Kuromori T, Noutoshi Y, Seki M, Kamiya A, Mizukado S, Sakurai T, Shinozaki K (2005) A resource of 5,814 dissociation transposon-tagged and sequence-indexed lines of *Arabidopsis* transposed from start loci on chromosome 5. Plant Cell Physiol **46:** 1149-1153

Jahngen-Hodge J, Obin MS, Gong X, Shang F, Nowell TR, Jr., Gong J, Abasi H, Blumberg J, Taylor A (1997) Regulation of ubiquitin-conjugating enzymes by glutathione following oxidative stress. J Biol Chem **272:** 28218-28226

Kabil O, Banerjee R (2010) Redox biochemistry of hydrogen sulfide. J Biol Chem **285:** 21903-21907

Krueger S, Niehl A, Lopez Martin MC, Steinhauser D, Donath A, Hildebrandt T, Romero LC, Hoefgen R, Gotor C, Hesse H (2009) Analysis of cytosolic and plastidic serine acetyltransferase mutants and subcellular metabolite distributions suggests interplay of the cellular compartments for cysteine biosynthesis in Arabidopsis. Plant Cell Environ **32:** 349-367

Li L, Moore PK (2008) Putative biological roles of hydrogen sulfide in health and disease: a breath of not so fresh air? Trends Pharmacol Sci **29:** 84-90

Lim PO, Kim HJ, Nam HG (2007) Leaf senescence. Annual Review of Plant Biology **58:** 115-136

Lin JF, Wu SH (2004) Molecular events in senescing *Arabidopsis* leaves. Plant J **39:** 612-628

Lisjak M, Srivastava N, Teklic T, Civale L, Lewandowski K, Wilson I, Wood ME, Whiteman M, Hancock JT (2010) A novel hydrogen sulfide donor causes stomatal opening and reduces nitric oxide accumulation. Plant Physiol Biochem **48:** 931-935

Lopez-Martin MC, Becana M, Romero LC, Gotor C (2008a) Knocking out cytosolic cysteine synthesis compromises the antioxidant capacity of the cytosol to maintain discrete concentrations of hydrogen peroxide in Arabidopsis. Plant Physiol **147:** 562-572

Lopez-Martin MC, Romero LC, Gotor C (2008b) Cytosolic cysteine in redox signaling. Plant Signaling & Behavior **3:** 880-881

Lowicka E, Beltowski J (2007) Hydrogen sulfide (H2S) - the third gas of interest for pharmacologists. Pharmacol Rep **59:** 4-24

Meyer AJ, Hell R (2005) Glutathione homeostasis and redox-regulation by sulfhydryl groups. Photosynth Res **86:** 435-457

Morris K, MacKerness SA, Page T, John CF, Murphy AM, Carr JP, Buchanan-Wollaston V (2000) Salicylic acid has a role in regulating gene expression during leaf senescence. Plant J **23:** 677-685

Mullineaux PM, Rausch T (2005) Glutathione, photosynthesis and the redox regulation of stress-responsive gene expression. Photosynth Res **86:** 459-474

Nakatogawa H, Suzuki K, Kamada Y, Ohsumi Y (2009) Dynamics and diversity in autophagy mechanisms: lessons from yeast. Nat Rev Mol Cell Biol **10:** 458-467

Petroff MG, Staehelin LA, Amasino RM, Guiamet JJ (2005) Senescence-associated vacuoles with intense proteolytic activity develop in leaves of *Arabidopsis* and soybean. Plant J **41:** 831-844

Perez-Perez ME, Crespo JL (2010) Autophagy in the model alga Chlamydomonas reinhardtii. Autophagy **6**

Perez-Perez ME, Florencio FJ, Crespo JL (2010) Inhibition of Target of Rapamycin Signaling and Stress Activate Autophagy in Chlamydomonas reinhardtii. Plant Physiol **152:** 1874-1888

Phillips AR, Suttangkakul A, Vierstra RD (2008) The ATG12-conjugating enzyme ATG10 Is essential for autophagic vesicle formation in *Arabidopsis thaliana.* Genetics **178:** 1339-1353

Rausch T, Wachter A (2005) Sulfur metabolism: a versatile platform for launching defence operations. Trends Plant Sci **10:** 503-509

Reiner A, Yekutieli D, Benjamini Y (2003) Identifying differentially expressed genes using false discovery rate controlling procedures. Bioinformatics **19:** 368-375

Rose TL, Bonneau L, Der C, Marty-Mazars D, Marty F (2006) Starvation-induced expression of autophagy-related genes in Arabidopsis. Biol Cell **98:** 53-67

Scherz-Shouval R, Shvets E, Fass E, Shorer H, Gil L, Elazar Z (2007) Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. Embo J **26:** 1749-1760

Slavikova S, Shy G, Yao Y, Glozman R, Levanony H, Pietrokovski S, Elazar Z, Galili G (2005) The autophagy-associated Atg8 gene family operates both under favourable growth conditions and under starvation stresses in *Arabidopsis* plants. J Exp Bot **56:** 2839-2849

Szabo C (2007) Hydrogen sulphide and its therapeutic potential. Nat Rev Drug Discov **6:** 917-935

Takahashi H, Kopriva S, Giordano M, Saito K, Hell R (2011) Sulfur assimilation in photosynthetic

organisms: molecular functions and regulations of transporters and assimilatory enzymes. Annu Rev Plant Biol **62:** 157-184

Thompson AR, Doelling JH, Suttangkakul A, Vierstra RD (2005) Autophagic nutrient recycling in *Arabidopsis* directed by the ATG8 and ATG12 conjugation pathways. Plant Physiol **138:** 2097-2110

Thompson AR, Vierstra RD (2005) Autophagic recycling: lessons from yeast help define the process in plants. Curr Opin Plant Biol **8:** 165-173

Toufighi K, Brady SM, Austin R, Ly E, Provart NJ (2005) The Botany Array Resource: e-Northerns, Expression Angling, and promoter analyses. Plant J **43:** 153-163

Van Hoewyk D, Pilon M, Pilon-Smits EAH (2008) The functions of NifS-like proteins in plant sulfur and selenium metabolism. Plant Science **174:** 117-123

Wang BL, Shi L, Li YX, Zhang WH (2010) Boron toxicity is alleviated by hydrogen sulfide in cucumber (Cucumis sativus L.) seedlings. Planta **231:** 1301-1309

Watanabe M, Kusano M, Oikawa A, Fukushima A, Noji M, Saito K (2008a) Physiological roles of the beta-substituted alanine synthase gene family in Arabidopsis. Plant Physiol **146:** 310-320

Wettenhall JM, Simpson KM, Satterley K, Smyth GK (2006) affylmGUI: a graphical user interface for linear modeling of single channel microarray data. Bioinformatics **22:** 897-899

Wirtz M, Berkowitz O, Droux M, Hell R (2001) The cysteine synthase complex from plants. Mitochondrial serine acetyltransferase from *Arabidopsis thaliana* carries a bifunctional domain for catalysis and protein-protein interaction. Eur J Biochem **268:** 686-693

Wirtz M, Birke H, Heeg C, Muller C, Hosp F, Throm C, Konig S, Feldman-Salit A, Rippe K, Petersen G, Wade RC, Rybin V, Scheffzek K, Hell R (2010) Structure and function of the hetero-oligomeric cysteine synthase complex in plants. J Biol Chem **285:** 32810-32817

Wirtz M, Droux M (2005) Synthesis of the sulfur amino acids: cysteine and methionine. Photosynth Res **86:** 345-362

Wirtz M, Droux M, Hell R (2004) O-acetylserine (thiol) lyase: an enigmatic enzyme of plant cysteine biosynthesis revisited in *Arabidopsis thaliana.* J Exp Bot **55:** 1785-1798

Wirtz M, Hell R (2006) Functional analysis of the cysteine synthase protein complex from plants: structural, biochemical and regulatory properties. J Plant Physiol **163:** 273-286

Wu FH, Shen SC, Lee LY, Lee SH, Chan MT, Lin CS (2009) Tape-*Arabidopsis* Sandwich - a simpler *Arabidopsis* protoplast isolation method. Plant Methods **5:** 16

Wu W, Berkowitz GA (1992) Stromal pH and Photosynthesis Are Affected by Electroneutral K and H Exchange through Chloroplast Envelope Ion Channels. Plant Physiol **98:** 666-672

Xiong Y, Contento AL, Bassham DC (2005) AtATG18a is required for the formation of autophagosomes during nutrient stress and senescence in *Arabidopsis thaliana*. Plant J **42:** 535-546

Yamaguchi K, Subramanian AR (2000) The plastid ribosomal proteins. Identification of all the proteins in the 50 S subunit of an organelle ribosome (chloroplast). J Biol Chem **275:** 28466-28482

Yamaguchi Y, Nakamura T, Kusano T, Sano H (2000) Three *Arabidopsis* genes encoding proteins with differential activities for cysteine synthase and beta-cyanoalanine synthase. Plant Cell Physiol **41:** 465-476

Yoshida S (2003) Molecular regulation of leaf senescence. Curr Opin Plant Biol **6:** 79-84

Yoshimoto K, Hanaoka H, Sato S, Kato T, Tabata S, Noda T, Ohsumi Y (2004) Processing of ATG8s, ubiquitin-like proteins, and their deconjugation by ATG4s are essential for plant autophagy. Plant Cell **16:** 2967-2983

Yoshimoto K, Jikumaru Y, Kamiya Y, Kusano M, Consonni C, Panstruga R, Ohsumi Y, Shirasu K (2009) Autophagy negatively regulates cell death by controlling NPR1-dependent salicylic acid signaling during senescence and the innate immune response in Arabidopsis. Plant Cell **21:** 2914-2927

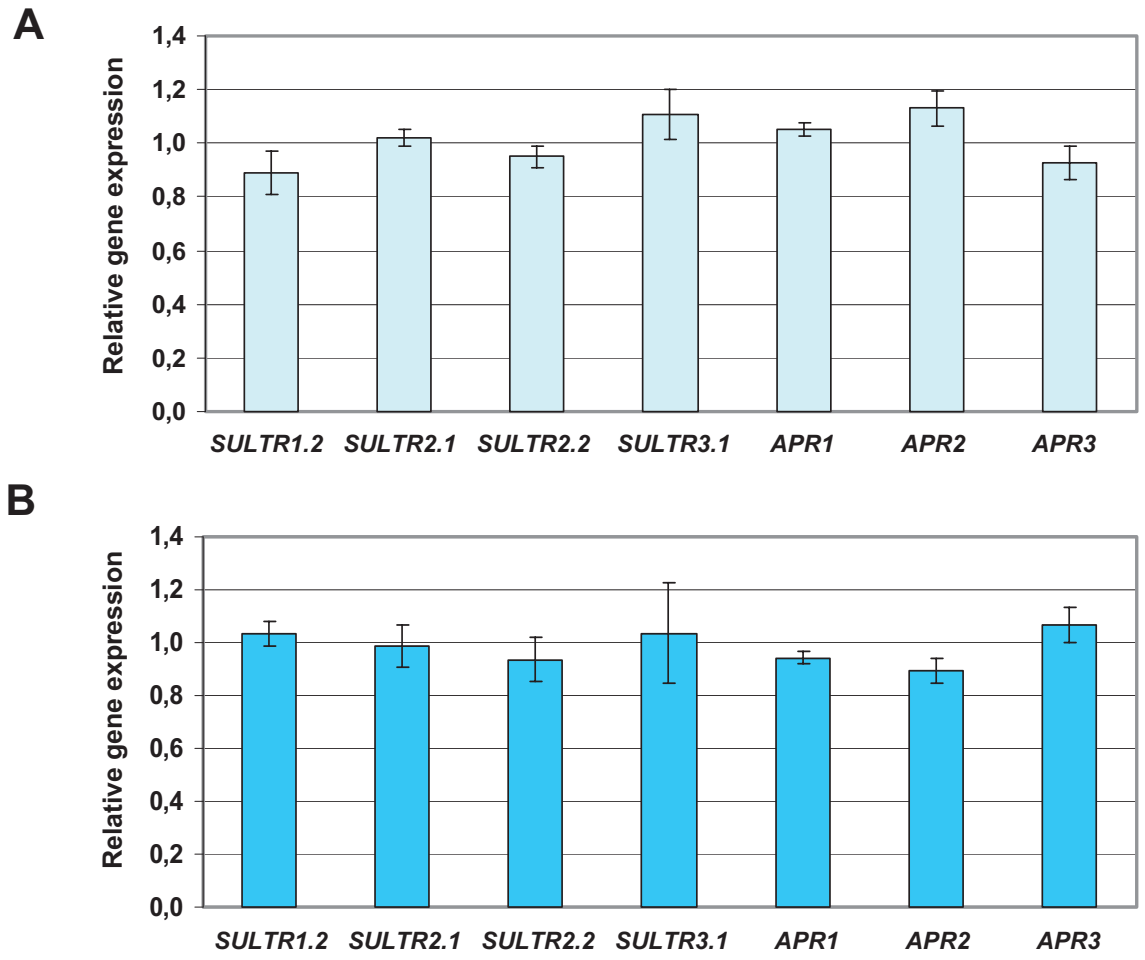
Yoshimoto K, Takano Y, Sakai Y (2010) Autophagy in plants and phytopathogens. FEBS Lett **584:** 1350-1358

Zhang H, Hu LY, Hu KD, He YD, Wang SH, Luo JP (2008) Hydrogen sulfide promotes wheat seed germination and alleviates oxidative damage against copper stress. J Integr Plant Biol **50:** 1518-1529

Zhang H, Tan ZQ, Hu LY, Wang SH, Luo JP, Jones RL (2010) Hydrogen sulfide alleviates aluminum toxicity in germinating wheat seedlings. J Integr Plant Biol **52:** 556-567

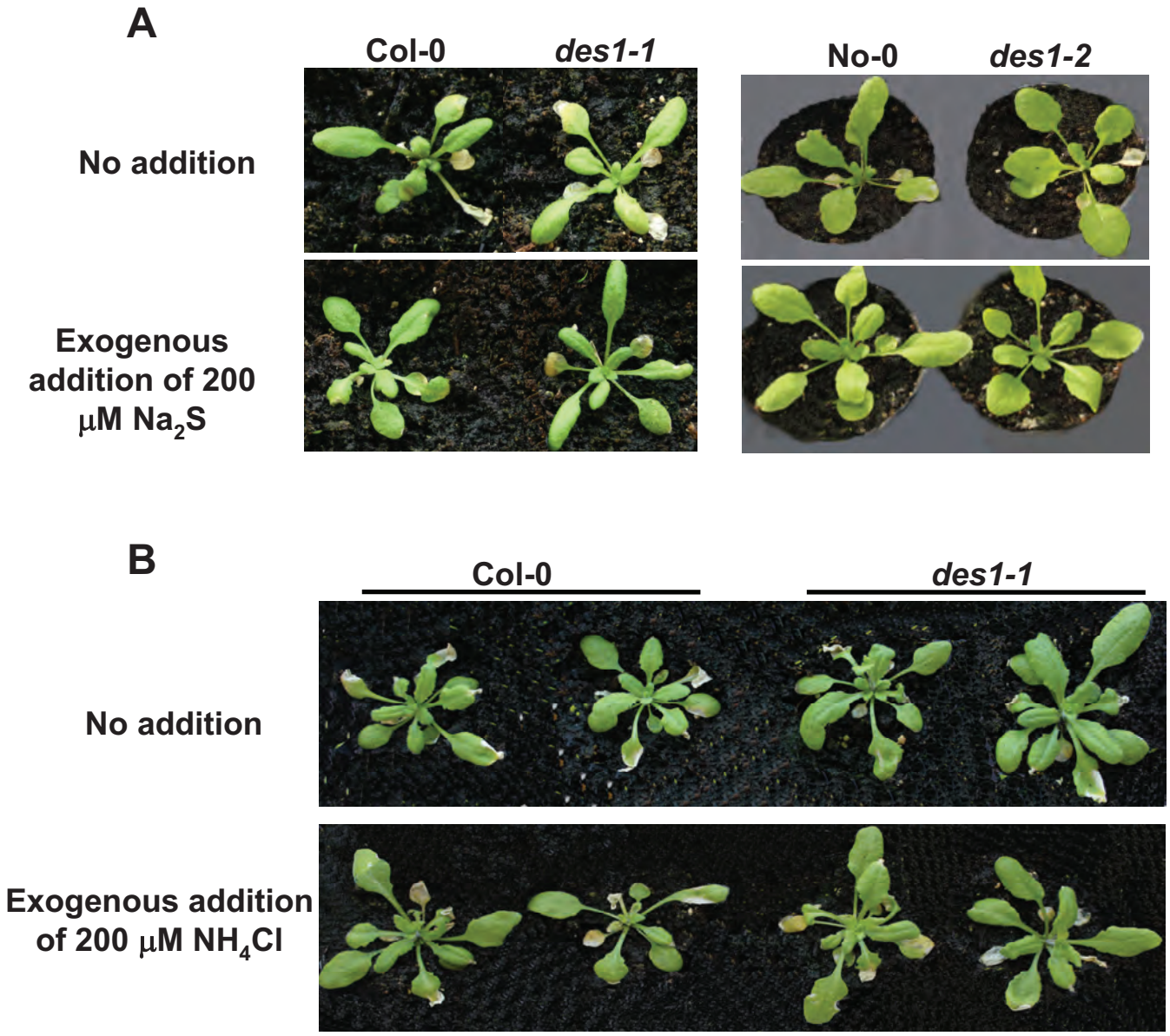
Zouhar J, Rojo E (2009) Plant vacuoles: where did they come from and where are they heading? Curr Opin Plant Biol **12:** 677-684

Supplemental Data



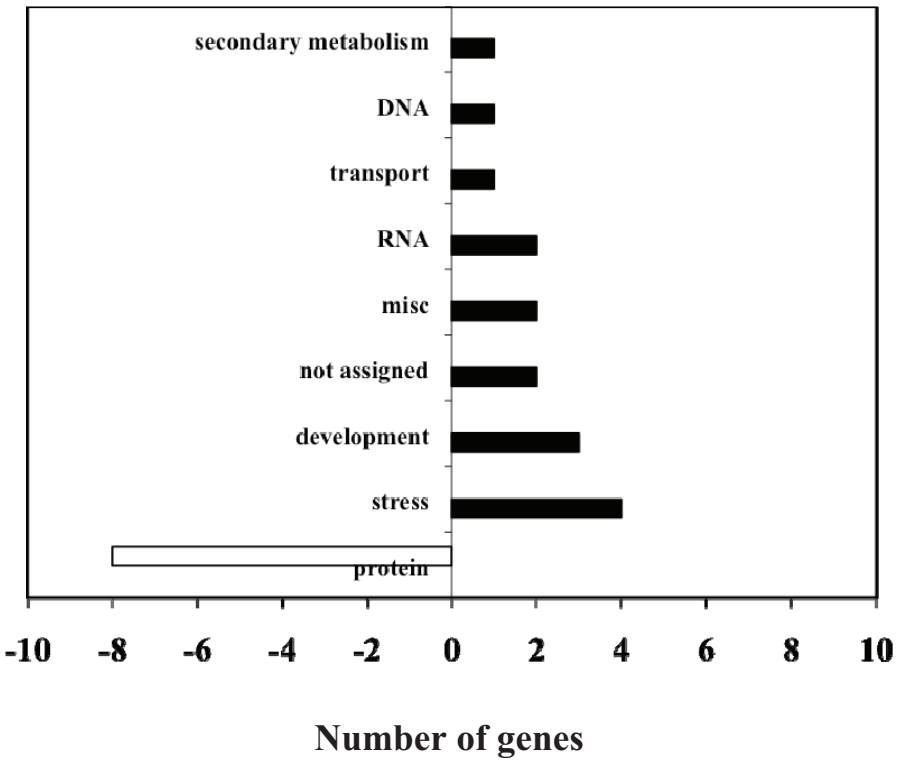
Supplemental Figure 1 online. Relative expression levels of sulfur-responsive genes in Col-0 wild-type plants treated with exogenous sulfide.

Real-time RT-PCR analysis of expression of the *SULTR1.2 (At1g78000), SULTR2.1 (At5g10180), SULTR2.2 (At1g77990), SULTR3.1 (At3g51895), APR1 (At4g04610), APR2 (At1g62180), and APR3 (At4g21990)* was performed in leaves from 20-day-old wild-type plants grown in soil and irrigated with water or 200 μM Na_2S in water for 10 days **(A)** and in leaves from 20-day-old wild-type Col-0 plants subjected to carbon starvation in the absence or presence of 200 μM Na_2S **(B)**. Carbon starvation was induced by placing the plants in darkness for 3 days and allowing them to recover for an additional 5 days before leaves were collected. The transcript levels were normalized to the internal constitutive *UBQ10* gene. Data shown are the means $\pm$ SD for three independent analyses and represent the transcript levels of each gene in the sulfide-treated Col-0 plants relative to the transcript levels in the untreated plants.

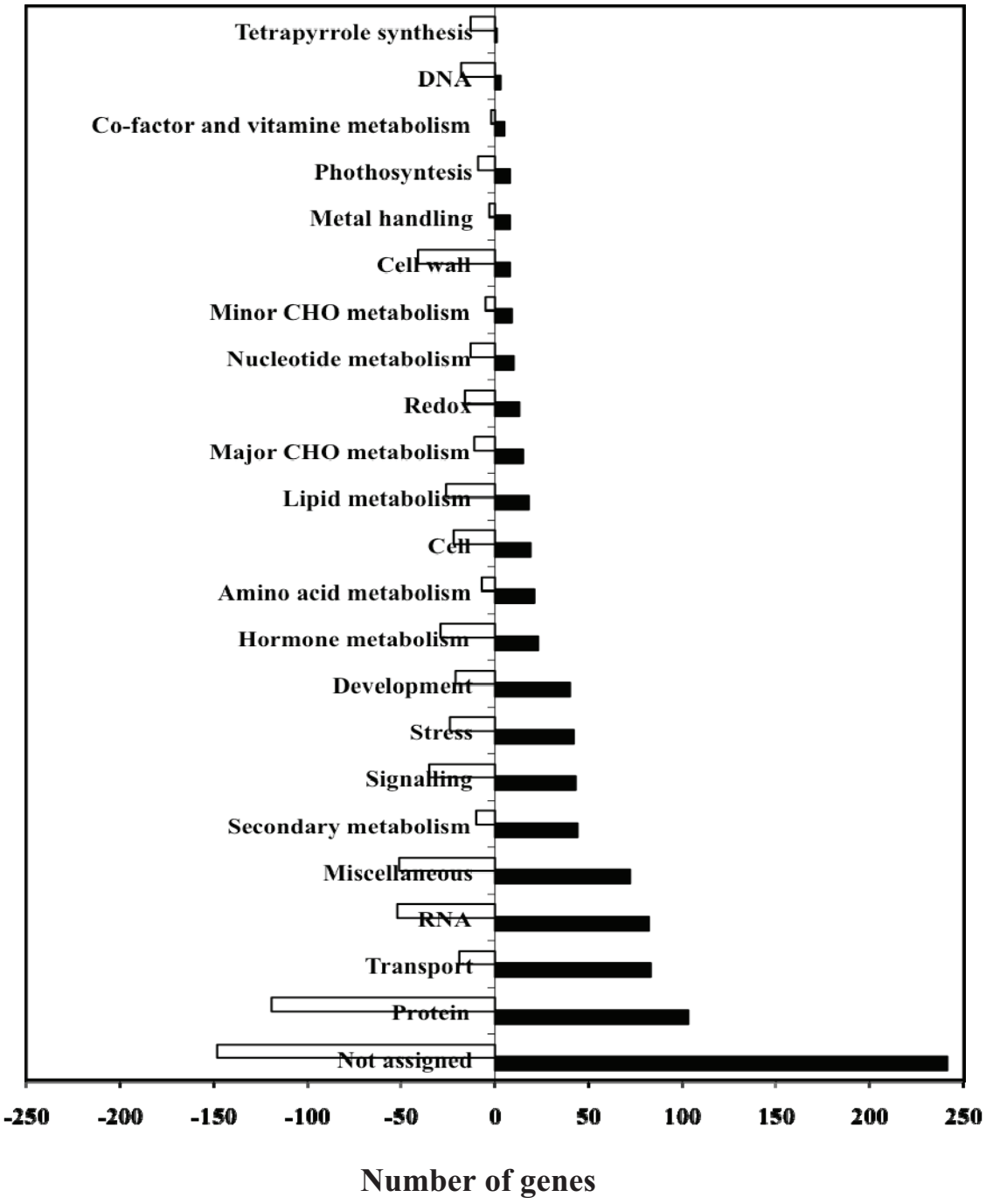


Supplemental Figure 2 online. Effect of exogenous sulfide and ammonium on the phenotype of *Arabidopsi*s plants under dark-induced carbon starvation.

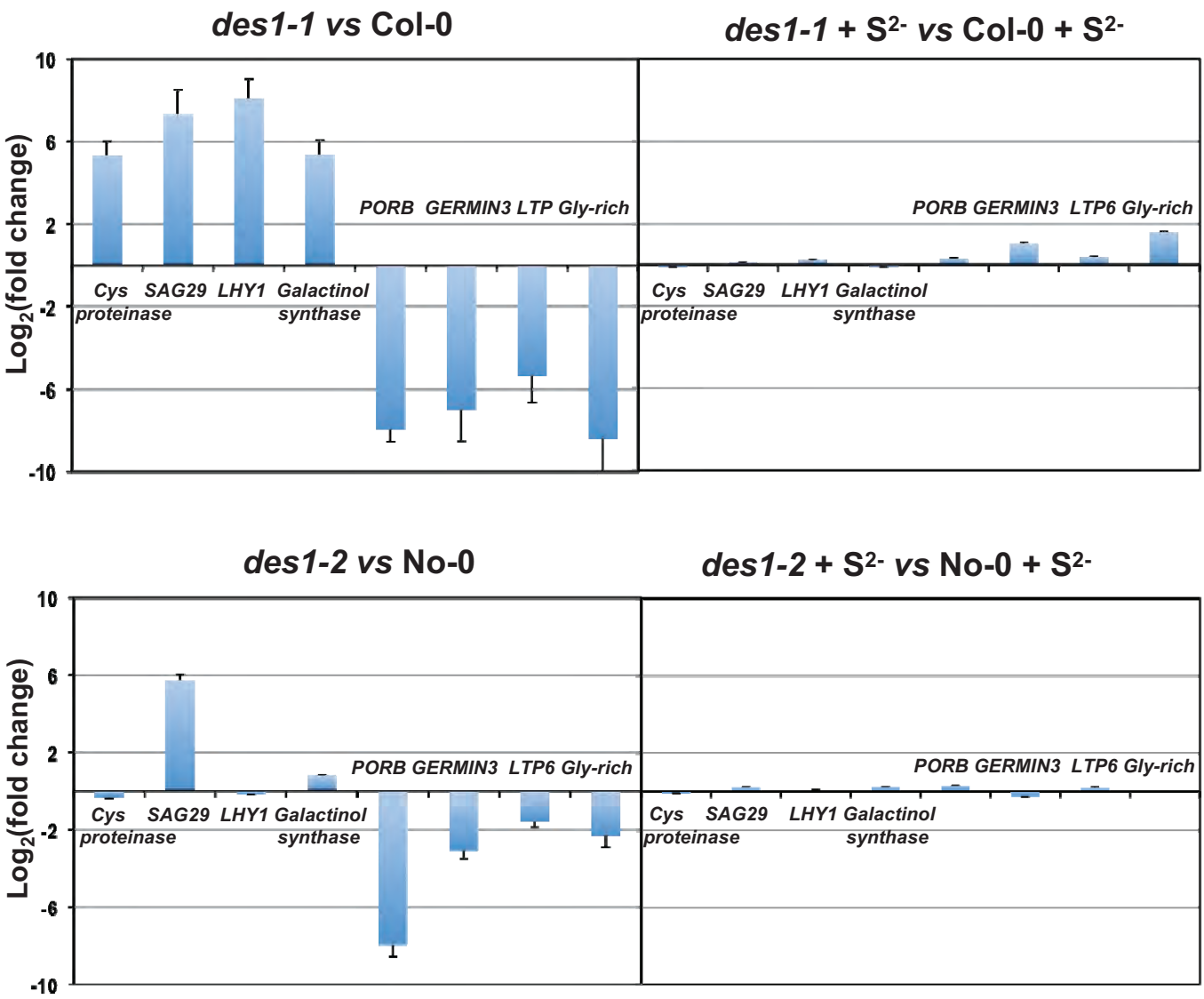
Twenty-day-old wild-type Col-0 and No-0 ecotypes and *des1-1* and *des1-2* mutant plants grown in soil were subjected to carbon starvation in the absence or presence of 200 μM Na_2S **(A)** and in the absence or presence of 200 μM NH_4Cl **(B)**. Carbon starvation was induced by placing the plants in darkness for 3 days and allowing them to recover for an additional 5 days. The plants were photographed, and a representative image is shown. The experiment was repeated at least three times, and the results are similar across all replicates.



Supplemental Figure 3 online. Gene categorization according to biological processes for the genes with altered transcript levels (induced, black bars; repressed, white bar) in the *des1-1* mutant compared to Col-0 plants grown for 20 days in soil under long-day conditions.



Supplemental Figure 4 online. Gene categorization according to biological processes for the genes with altered transcript levels (induced, black bars; repressed, white bar) in the *des1-1* mutant compared to Col-0 plants grown for 30 days in soil under long-day conditions.



Supplemental Figure 5 online. Relative expression levels of selected genes in the *des1-1* and *des1-2* mutant plants.

Real-time RT-PCR analysis was performed for *Cys proteinase (At2g27420), SAG29 (At5g13170), LHY1 (At1g01060), Galactinol synthase (At1g56600), PORB (At4g27440), GERMIN3 (At5g20630), LTP6 (At3g08770) and Gly-rich protein (At4g29020)* in leaves from the *des1-1* and *des1-2* mutants and their respective wild-type ecotypes. The plants were grown under identical long-day conditions in soil for 30 days (left panels) or for 20 days followed by treatment with 200 μM Na_2S for 10 additional days (right panels). The transcript levels were normalized to the constitutive *UBQ10* gene. Data shown are the means $\pm$ SD for three independent analyses and represent the transcript levels in the *des1* mutant plants relative to the transcript levels in the respective wild-type ecotypes. *, $P<0.05$.

Supplemental Table 1 online. List of differentially induced genes in *des1-1* mutant plants grown for 20 days in soil under long-day conditions.

Locus	Fold Change	logRatio (M)	FDR	Gene Symbol | Gene Title
AT2G26020	+5.63	+2.49	0.00125322	Plant defensin protein (PDF1.2b)
AT3G51860	+5.19	+2.38	0.00250694	Cation exchanger 3 (CAX3)
AT5G44420	+4.80	+2.26	0.00005174	Plant defensin protein (PDF1.2a)
AT5G24770	+2.95	+1.56	0.01218093	Vegetative storage protein 2 (VSP2)
AT2G05540	+2.94	+1.55	0.00626105	Glycine-rich protein (GRP3S)
AT3G04720	+2.79	+1.48	0.00052794	Hevein-like protein (HEL)
AT1G49500	+2.33	+1.22	0.00125322	Expressed protein
AT2G18050	+2.33	+1.22	0.00066110	Histone H1-3 (HIS1-3)
AT3G16770	+2.32	+1.21	0.00125322	Encodes a member of the ERF/AP2 transcription factor family (RAP2.3)
AT2G43510	+2.19	+1.13	0.02435701	Trypsin inhibitor, putative
AT1G69490	+2.11	+1.08	0.03301533	Encodes a member of the NAC transcription factor gene family, NAP
AT1G03940	+2.10	+1.07	0.00678828	HXXXD-type acyl-transferase family protein
AT4G02380	+2.07	+1.05	0.01165748	Senescence-associated gene 21 (SAG21)
AT5G17220	+2.05	+1.04	0.00761956	Glutathione S-transferase 16
AT4G38540	+2.05	+1.04	0.00508650	Monooxygenase, putative (MO2)
AT1G66390	+2.05	+1.03	0.01226802	Myb family transcription factor/ production of anthocyanin pigment 2 protein (PAP2)

Supplemental Table 2 online. List of differentially repressed genes in *des1-1* mutant plants grown for 20 days in soil under long-day conditions.

Locus	Fold Change	logRatio (M)	FDR	Gene Symbol | Gene Title
ATCG00790	-3.35	-1.75	0.013657964	Chloroplast gene encoding a ribosomal protein L16, a constituent of 50S large ribosomal subunit
ATCG00780	-2.90	-1.53	0.014789662	Encodes a chloroplast ribosomal protein L14, a constituent of 50S large ribosomal subunit
ATCG00810	-2.75	-1.46	0.015175542	Encodes a chloroplast ribosomal protein L22, a constituent of 50S large ribosomal subunit
ATCG00065	-2.36	-1.24	0.011979825	Chloroplast gene encoding ribosomal protein S12, a constituent of 30S ribosomal subunit. The gene is located in three distinct loci on the chloroplast.
ATCG00760	-2.22	-1.15	0.013223655	Encodes a chloroplast ribosomal protein L36, a constituent of 50S large ribosomal subunit
ATCG00770	-2.14	-1.10	0.014074880	Encodes a chloroplast ribosomal protein S8, a constituent of 30S ribosomal subunit
ATCG00800	-2.13	-1.09	0.016882221	Encodes a chloroplast ribosomal protein S3, a constituent of 30S ribosomal subunit.
ATCG01230	-2.08	-1.06	0.015837838	Chloroplast gene encoding ribosomal protein S12, a constituent of 30S ribosomal subunit.. The gene is located in three distinct loci on the chloroplast.

Supplemental Table 3 online. List of differentially regulated genes in *des1-1* mutant plants grown for 30 days in soil under long-day conditions categorized within the ubiquitin and autophagy dependent degradation functional groups.

Protein Function	Locus	Gene Symbol / Gene Title	Fold Change
Autophagy			
protein.degradation.autophagy	At4g04620	ATG8B	2.37
protein.degradation.autophagy	At1g54210	ATG12A	2.08
E2			
protein.degradation.ubiquitin.E2	At3g08690	UBC11; ubiquitin-conjugating enzyme 11	2.28
protein.degradation.ubiquitin.E2	At1g70660	UEV1B; ubiquitin E2 variant 1B	2.33
protein.degradation.ubiquitin.E2	At3g17000	UBC32; ubiquiting-conjugating enzyme 32	3,33
SCF			
protein.degradation.ubiquitin.E3.SCF.SKP	At5g57900	SKP1 INTERACTING PARTNER 1	2.6
protein.degradation.ubiquitin.E3.SCF.FBOX	At3g12350	F-box family protein	2.09
protein.degradation.ubiquitin.E3.SCF.FBOX	At2g24540	AFR (ATTENUATED FAR-RED RESPONSE)	7.33
protein.degradation.ubiquitin.E3.SCF.FBOX	At3g07870	F-box family protein	4.02
protein.degradation.ubiquitin.E3.SCF.FBOX	At1g78100	F-box family protein	2.14
protein.degradation.ubiquitin.E3.SCF.FBOX	At1g80440	kelch repeat-containing F-box family protein	-2.68
protein.degradation.ubiquitin.E3.SCF.FBOX	At1g70590	F-box family protein	2.28
protein.degradation.ubiquitin.E3.SCF.FBOX	At1g67480	kelch repeat-containing F-box family protein	2.09
protein.degradation.ubiquitin.E3.SCF.FBOX	At1g23390	kelch repeat-containing F-box family protein	-3.65
protein.degradation.ubiquitin.E3.SCF.FBOX	At3g59940	kelch repeat-containing F-box family protein	-2.06
protein.degradation.ubiquitin.E3.SCF.FBOX	At4g08980	F-box family protein (FBW2)	2.1
protein.degradation.ubiquitin.E3.SCF.FBOX	At1g21410	SKP2A	4.59
Ring/ U-box Hect			
protein.degradation.ubiquitin.E3.RING	At4g28270	zinc finger (C3HC4-type RING finger) family protein	5.16
protein.degradation.ubiquitin.E3.RING	At5g47050	protein binding / zinc ion binding	3.86
protein.degradation.ubiquitin.E3.RING	At2g17450	RHA3A; protein binding / zinc ion binding	-3.25
protein.degradation.ubiquitin.E3.RING	At5g01520	zinc finger (C3HC4-type RING finger) family protein	2.66
protein.degradation.ubiquitin.E3.RING	At1g18660	zinc finger (C3HC4-type RING finger) family protein	2.12
protein.degradation.ubiquitin.E3.RING	At1g49200	zinc finger (C3HC4-type RING finger) family protein	4.69
protein.degradation.ubiquitin.E3.RING	At1g73760	zinc finger (C3HC4-type RING finger) family protein	-3.74
protein.degradation.ubiquitin.E3.RING	At5g41400	zinc finger (C3HC4-type RING finger) family protein	5.15
protein.degradation.ubiquitin.E3.RING	At1g79110	protein binding / zinc ion binding	3.0
protein.degradation.ubiquitin.E3.RING	At3g47160	protein binding / zinc ion binding	-16.12
protein.degradation.ubiquitin.E3.RING	At5g10380	RING1; protein binding / ubiquitin-protein ligase	2.25
protein.degradation.ubiquitin.E3.RING	At4g19670	zinc finger (C3HC4-type RING finger) family protein	2.17
protein.degradation.ubiquitin.E3.RING	At3g46620	zinc finger (C3HC4-type RING finger) family protein	-2.23
protein.degradation.ubiquitin.E3.RING	At1g10650	protein binding / zinc ion binding	3.11
protein.degradation.ubiquitin.E3.RING	At1g15100	RHA2A; protein binding / ubiquitin-protein ligase	5.92
protein.degradation.ubiquitin.E3.RING	At2g40830	RHC1A; protein binding / zinc ion binding	2.05
protein.degradation.ubiquitin.E3.RING	At5g22920	zinc finger (C3HC4-type RING finger) family protein	-11.19
protein.degradation.ubiquitin.E3.RING	At2g17730	zinc finger (C3HC4-type RING finger) family protein	2.32
protein.degradation.ubiquitin.E3.RING	At5g47610	zinc finger (C3HC4-type RING finger) family protein	10.66
protein.degradation.ubiquitin.E3.RING	At1g55530	zinc finger (C3HC4-type RING finger) family protein	2.1
protein.degradation.ubiquitin.E3.RING	At5g17600	zinc finger (C3HC4-type RING finger) family protein	3.89
protein.degradation.ubiquitin.E3.RING	At2g04240	XERICO; protein binding / zinc ion binding	2.37
protein.degradation.ubiquitin.E3.RING	At1g19310	zinc finger (C3HC4-type RING finger) family protein	-2.91
protein.degradation.ubiquitin.ubiquitin	At4g05050	UBQ11 (UBIQUITIN 11)	2.01

Supplemental Table 4 online. List of differentially regulated genes in *des1-1* mutant plants grown for 20 days in soil under long-day conditions and treated with 200 μM Na_2S for 10 additional days.

Locus	Fold Change	logRatio (M)	FDR	Gene Symbol | Gene Title
AT4G37610	+3.70	+1.89	0.00001352	TAZ zinc finger family protein / BTB/POZ domain-containing protein
AT2G26020	+3.22	+1.69	0.00222678	Plant defensin-fusion protein, putative (PDF1.2b)
AT5G44420	+2.97	+1.57	0.00007942	Plant defensin protein, putative (PDF1.2a)
AT5G61590	+2.66	+1.41	0.00084213	AP2 domain-containing transcription factor family protein
AT5G19120	+2.41	+1.27	0.00007929	Expressed protein
AT5G57560	+2.41	+1.27	0.00013501	TCH4 / xyloglucan:xyloglucosyl transferase / xyloglucan endotransglycosylase
AT2G20870	-11.70	-3.55	0.00000079	Cell wall protein precursor, putative
AT3G08770	-5.47	-2.45	0.00000131	LTP6 / lipid transfer protein 6 (LTP6)
AT2G42840	-3.17	-1.67	0.00002669	Protodermal factor 1 (PDF1)
AT3G28500	-2.64	-1.40	0.00013273	60S acidic ribosomal protein P2 (RPP2C)
AT5G08000	-2.38	-1.25	0.00189281	Glycosyl hydrolase family protein 17
AT4G23600	-2.18	-1.12	0.00092288	Coronatine-responsive tyrosine aminotransferase / tyrosine transaminase
AT1G13990	-2.09	-1.07	0.00067246	Expressed protein
AT2G14610	-2.05	-1.04	0.02475385	Pathogenesis-related protein 1 (PR-1)
AT2G02100	-2.04	-1.03	0.00097258	Plant defensin-fusion protein, putative (PDF2.2)

Supplemental Table 5 online. Oligonucleotides used in this work.

DES1 *(At5g28030)*	DES1F	CACCATGGAAGACCGCGTCTTGAT
	DES1R	TCATTCAACTGGCAAATTCTCAGCTT
UBQ10 *(At4g05320)*	UBQ10F	GGCCTTGTATAATCCCTGATGAATAAG
	UBQ10R	AAAGAGATAACAGGAACGGAAACATAGT
SULTR1.2 *(At1g78000)*	SULTR1.2F	GGATCCAGAGATGGCTACATGA
	SULTR1.2R	TCGATGTCCGTAACAGGTGAC
SULTR2.1 *(At5g10180)*	SULTR2.1F	ATTGTTGCTCTAACCGAGGCGATT
	SULTR2.1R	TGTACCCTTTTATTCCGGCGAACG
SULTR2.2 *(At1g77990)*	SULTR2.2F	CGACATGTCTTGCGTGATGGGCG
	SULTR2.2R	GCTCGCTTCAATTTGTGAAGTACCCT
SULTR3.1 *(At3g51895)*	SULTR3.1F	CCAGATCCAAATTCATCGGTGA
	SULTR3.1R	CGGTTCGGTTTTAAACGTGTG
APR1 *(At4g04610)*	APR1F	CATTGGAGCCAAAAGTTTCGC
	APR1R	TCCTCAATCTCAACCACATCAAC
APR2 *(At1g62180)*	APR2F	GAGCTCCACGGGCTATTAAGTA
	APR2R	TTCTCTTTCATTCTTCATCACCC
APR3 *(At4g21990)*	APR3F	GGAAGAGATCCTCCGTGAAAGC
	APR3R	CTGTAACCTCAGAAGCAACAATGGA
Cys Proteinase *(At2g27420)*	CysProtF	TGGCCATTCT TGCTTTCTAT
	CysProtR	TCCATTTGCACGAAAACAT
SAG29 *(At5g13170)*	SAG29F	GTGGAGGAGCCG TCAA
	SAG29R	GCCCTGACGAGCCGAGAG
LHY1 *(At1g01060)*	LHY1F	TCGATTGGAAGGAGAAGCTT
	LHY1R	AAAGAAATGTTCGGAAGGAA
Galactinol synthase *(At1g56600)*	GalactinolsynF	CGAG CTCGTTGCTG CATAC
	GalactinolsynR	GTGGAAATTTAAAAAATCCTCC
PORB *(At4g27440)*	PORBF	AGGTGGTGAG TGATCCAAGC
	PORBR	AGTGAGAAGCTCGTGGGC

GERMIN3 *(At5g20630)*	GERMIN3F GERMIN3R	CGTTCCTTAG CGATGCAGAA TCGAAATAACAAAACGCACA
LTP6 *(At3g08770)*	LTP6F LTP6R	CCTTCCAC TGACTGCGAC GTATAGGGTGGAAGCATGGA
Gly-rich protein *(At4g29020)*	Gly-richF Gly-richR	GCGGTGCAGGTGGTCTA CGACCGATCTTTGGTTTATAG
SEN1 *(At4g35770)*	SEN1F SEN1R	AACATGTGGATCTTTCAAGTGCC GTCGTTGCTTTCCTCCATCG
SAG21 *(At4g02380)*	SAG21F SAG21R	ATTACAGACCCGAAACCG GCACCAGATCATTACCCA
SAG12 *(AT5G45890)*	SAG12F SAG12R	CGTCTGGTGTGTTCACTGGA TCATATATCCACTTTCTCCCCA
NAP *(At1g69490)*	NAPF NAPR	CGTCGATGAAACTTCCAAGG CCGAACCAACTAGACTCCGAAT
PR1 *(At2g14610)*	PR1F PR1R	TCAGTGAGACTCGGATGTG CCTGCATATGATGCTCCTT

Capítulo III

La homeostasis de cisteína juega un papel esencial en la respuesta inmune de las plantas

New Phytologist

Cysteine homeostasis plays an essential role in plant immunity

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Summary

- Cysteine is the metabolic precursor of essential biomolecules such as vitamins, cofactors, antioxidants and many defense compounds. The last step of cysteine metabolism is catalysed by *O*-acetylserine(thiol)lyase (OASTL), which incorporates reduced sulfur into *O*-acetylserine to produce cysteine. In *Arabidopsis thaliana*, the main OASTL isoform OAS-A1 and the cytosolic desulfhydrase DES1, which degrades cysteine, contribute to the cytosolic cysteine homeostasis.
- Meta-analysis of the transcriptomes of knockout plants for *OAS-A1* and for *DES1* show a high correlation with the biotic stress series in both cases.
- The study of the response of knockout mutants to plant pathogens shows that *des1* mutants behave as constitutive systemic acquired resistance mutants, with high resistance to biotrophic and necrotrophic pathogens, salicylic acid accumulation and *WRKY54* and *PR1* induction, while *oas-a1* knockout mutants are more sensitive to biotrophic and necrotrophic pathogens. However, *oas-a1* knockout mutants lack the hypersensitive response associated with the effector-triggered immunity elicited by *Pseudomonas syringae pv. tomato DC3000 avrRpm1*.
- Our results highlight the role of cysteine as a crucial metabolite in the plant immune response.

Introduction

Plants are continuously subjected to attack by a plethora of pathogens and herbivores. Only a small proportion of them are able to invade the plant and exploit it as a source of energy because the plants are resistant to most of them. In addition to nonhost resistance, plants are able to recognize pathogen-associated molecular patterns (PAMPs) and induce a response leading to a basal or PAMP-triggered immunity (PTI). However, pathogens have evolved to avoid recognition by the host and delay the defense responses until they are no longer effective, resulting in a compatible plant–pathogen interaction. For their defense, plants have developed specific resistance mechanisms based on gene-for-gene interaction (Staskawicz *et al.*, 1995; Jones & Dangl, 2006). During the so-called effector-triggered immunity (ETI), an avirulence protein from the pathogen is recognized by a resistance protein from the plant, and this interaction rapidly starts the plant's defense program strongly enough to halt pathogen spreading (Whalen *et al.*, 1991; Hammond-Kosack & Jones, 1997; Dangl & Jones, 2001). Interestingly, most of the defense response is activated in both PTI and ETI, but the timing makes the difference (Tao *et al.*, 2003).

One of the earliest responses to pathogen infection is the production of reactive oxygen species (ROS, reviewed in Lamb & Dixon, 1997). The production of ROS is weakly induced in both PTI and ETI at the very early stages of the infection, but only in the ETI is there a second more prolonged and extensive induction of the oxidative burst. This second phase of ROS induction is therefore characteristic of the ETI response and is accompanied by an accumulation of nitric oxide (NO) and salicylic acid (SA), which, together with ROS, promote the hypersensitive response (HR) (Delledonne *et al.*, 1998; Alvarez, 2000). The HR is therefore typical for the ETI, and it causes a rapid death of the infected cells (Heath, 2000). In addition to limiting the spread of biotrophic pathogens, HR contributes to the activation of defense in adjacent cells and to the activation of systemic acquired resistance (SAR), a broad-spectrum form of disease resistance mediated by the action of SA, which is accompanied by the systemic activation of some defense responses (Vlot *et al.*, 2008).

In addition to its structural role in proteins, cysteine functions as a precursor for essential biomolecules, such as vitamins and cofactors (Droux, 2004; Wirtz & Droux, 2005), antioxidants, such as glutathione (Meyer & Hell, 2005; Mullineaux & Rausch, 2005), and many defense compounds, such as glucosinolates, thionins or phytoalexins (Rausch & Wachter, 2005). Cysteine is synthesized in plants in the cytosol, plastids and mitochondria by the sequential action of the enzymes serine acetyltransferase (SAT, EC 2.3.1.30), which synthesizes the intermediary product *O*-acetylserine (OAS), and *O*-acetylserine(thiol)lyase (OASTL, EC 2.5.1.47), which combines a sulfide molecule with an OAS molecule to produce cysteine. In *A. thaliana* plants, several OASTLs and SAT encoding genes have been identified by sequence

homology, and the functionality of some of them has been demonstrated by several studies (Haas *et al.*, 2008; Heeg *et al.*, 2008; Lopez-Martin *et al.*, 2008a; Watanabe *et al.*, 2008a,b; Alvarez *et al.*, 2010; Bermudez *et al.*, 2010). Most of the cysteine is formed and accumulated in the cytosol (Krueger *et al.*, 2009, 2010) by the action of the major cytosolic OASTL, encoded by *OAS-A1* (Barroso *et al.*, 1995; Lopez-Martin *et al.*, 2008a). It has been demonstrated that OAS-A1 is involved in the defense responses against abiotic stresses (Barroso *et al.*, 1999; Dominguez-Solis *et al.*, 2001, 2004) and is essential for maintaining the antioxidant capacity of the cytosol (Lopez-Martin *et al.*, 2008a,b). In knockout *oas-a1* plants, intracellular cysteine and glutathione levels are significantly reduced, and the glutathione redox state is shifted toward its oxidized form. Moreover, *oas-a1* knockout mutants accumulate ROS in the absence of external stress, and show spontaneous cell death lesions in the leaves. In addition, cysteine can be degraded in the cytosol by the action of L-cysteine desulfhydrase (DES, EC 4.4.1.1), which catalyses the formation of sulfide, ammonia and pyruvate from cysteine in a stoichiometric ratio of 1 :1 :1. In *A. thaliana*, the only cytosolic desulfhydrase described to date is encoded by *DES1* (previously known as *CS-LIKE*, At5g28030). Knockout *des1* plants show enhanced antioxidant defenses and tolerance to conditions promoting oxidative stress (Alvarez *et al.*, 2010). Thus, *des1* and *oas-a1* knockout mutants show opposite phenotypes, suggesting that they exert opposing functions by regulating cytosolic cysteine homeostasis.

To investigate the role of cysteine in response to stress further, we have explored its role in the plant–pathogen response by analysing the responses of the different *oas-a1* and *des1* alleles under pathogen attack.

Materials and Methods

Plant material and growth conditions

Arabidopsis (*A. thaliana*) wild-type ecotypes Col-0 and No-0, the SALK_103855 and RATM13-2715-1_G (*des1-1 and des1-2*, Alvarez *et al.*, 2010), the SALK_072213 and SAIL_94_E12 (*oas-a1.1 and oas-a1.2*, Lopez-Martin *et al.*, 2008a) and *cad2-1* (Cobbett *et al.*, 1998) mutants were used in this work. The plants were grown in soil under a photoperiod of 8 h of white light (120 μE m^{-2} s^{-1}) at 20°C/16 h dark at 18°C. Plants were cultivated for 6- to 7-wk. Under these conditions, size, developmental stage, leaf size and thickness of mutant plants were undistinguishable of their respective wild type.

RNA extraction and microarray hybridization

For microarray of the *des1-1* mutant, plants were grown in soil under a photoperiod of 16 h of white light (120 μE m^{-2} s^{-1}) at 20°C/8 h dark at 18°C. Rosette leaves of 20-d-old plants were used for total RNA isolation with Trizol reagent (Invitrogen) and cleaning with the RNeasy Plant Mini Kit (Qiagen). This was used to synthesize biotinylated cRNA for hybridization to *Arabidopsis* ATH1 arrays (Affymetrix, Santa Clara, CA, USA), using the 3′ Amplification One-Cycle Target Labeling Kit. Briefly, 4 mg of RNA was reverse transcribed to produce first-strand cDNA using a $(dT)_{24}$ primer with a T7 RNA polymerase promoter site added to the 3′ end. After second-strand synthesis, *in vitro* transcription was performed using T7 RNA polymerase and biotinylated nucleotides to produce biotin-labeled cRNA. The cRNA preparations (15 μg) were fragmented into fragments of 35–200 bp at 95°C for 35 min. The fragmented cRNAs were hybridized to the *Arabidopsis* ATH1 microarrays at 45°C for 16 h. Each microarray was washed and stained in the Affymetrix Fluidics Station 400 following standard protocols. Microarrays were scanned using an Affymetrix GeneChip Scanner 3000.

Microarray data analysis

Microarray analysis was performed using the affylmGUI R package (Wettenhall *et al.*, 2006). The Robust Multi-array Analysis (RMA) algorithm was used for background correction, normalization and for summarizing expression levels (Irizarry *et al.*, 2003). Differential expression analysis was performed using Bayes *t*-statistics using the linear models for microarray data (Limma), which is included in the affylmGUI package. The *P*-values were corrected for multiple testing using Benjamini–Hochberg's method (False Discovery Rate) (Benjamini & Hochberg, 1995; Reiner *et al.*, 2003). A cutoff value of a 1.5-fold change and P value of < 0.05 were adopted to discriminate expression of genes that were differentially expressed in the mutant plant with respect to the wild type. Gene classification into functional groups was obtained from the Bio-Array Resource for *Arabidopsis* Functional Genomics (http://www.bar.utoronto.ca) and the MAPMAN software (http://gabi.rzpd.de/projects/MapMan/). The microarray data for the *oas-a1.1* previously published (Lopez-Martin *et al.*, 2008a) and *des1-1* mutants were meta-analysed using the Bio-Array Resource for *Arabidopsis* Functional Genomics (http://www.bar.utoronto.ca, Toufighi *et al.*, 2005).

Bacterial pathogen infections

The bacterial strains used in this study were *Pseudomonas syringae pv. tomato* (*Pst*) *DC3000* and *Pst DC3000* bearing a plasmid containing the *avrRpm1* avirulence gene (Grant *et al.*, 1995). For treatment of the plants, bacterial cultures were collected from plates in 10 mM $MgCl_2$ and their concentrations were adjusted to 5×10^6 bacteria ml^{-1} (DO_{600} = 0.01, *Pst DC3000 avrRpm1*) or 2.5×10^6 bacteria ml^{-1} (DO_{600} = 0.005, *Pst DC3000*). Sterile 10 mM $MgCl_2$ was used as a mock solution. The bacterial suspension or the mock solution was then pressure infiltrated into the abaxial side of the leaves of 6- to 7-wk-old plants, both wild types and mutants grown at the same time and conditions, using a syringe without a needle (Swanson *et al.*, 1988).

Bacteria growth tests

Pst DC3000 avrRpm1 bacteria were collected from Luria–Bertani (LB) plates supplemented with rifampicin (50 μg ml^{-1}) in 10 mM $MgCl_2$, and their concentration was adjusted to

5×10^6 bacteria ml^{-1} (DO_{600} = 0.01). Six series of 1 : 10 dilutions were done and 10 µl of the resulting suspensions were plated in parallel in LB supplemented with rifampicin and LB supplemented with rifampicin and 0.5 mM L-cysteine. Plates were grown for 48 h at 28°C and photographed.

In planta growth of virulent or avirulent *P. syringae DC3000*

The protocol for measuring the growth of bacteria was adapted from (Tornero & Dangl, 2001). Wild-type and mutant plants were grown for 6–7 wk at the same time and conditions and inoculated with bacterial pathogens as described earlier. One hour after the inoculation, the samples for day zero were taken. To determine bacterial growth, 100 mg of leaves were ground in 500 µl of 10 mM $MgCl_2$ and gently vortexed. From each sample, 20 µl was added to the wells of a microtiter plate containing 180 µl of 10 mM $MgCl_2$, and serial 10-fold dilutions were plated on Petri dishes containing 50 µg ml^{-1} rifampicin. The plates were incubated at 30°C and after 30 h the number of colonies was counted. The number of colony forming units (CFU) mg^{-1} fresh weight was determined by the formula:

$$\text{cfu mg}^{-1}\text{FW} = k(N \times 10^{d-1})/(\text{weight of the tissue}),$$

where N is the number of colonies counted in the dilution number d, and the constant k (500 in our case) represents the number of CFU present in the sample per colony appearing in the first dilution (Tornero & Dangl, 2001).

Fungal infections

The *Botrytis cinerea* strain ME4 was grown in a solid strawberry broth for 12 d, and spore suspensions were prepared at a concentration of 5×10^5 spores ml^{-1} in 12 g l^{-1} potato dextrose broth (PDB). Six- to seven-week-old wild-type and mutant plants grown at the same time and conditions were pulverized with a Preval sprayer (Coal City, IL, USA) with spore suspension. Approximately 2 ml of spore suspension per plant was used. The plants were covered with a transparent film to keep 100% humidity. The plants were collected for PCR analysis after 5 d.

Quantification of *B. cinerea* DNA accumulation in infected plants

DNA from infected plants was quantified by real-time PCR according to a previous study (Calo *et al.*, 2006). DNA from the *B. cinerea creA* gene (Tudzynski *et al.*, 2000) was amplified using the oligonucleotides *creABOT-F* and *creABOT-R* (see the Supporting Information, Table S1). As an internal standard to normalize the real-time PCR, *A. thaliana UBQ10* DNA was amplified using the oligonucleotides *UBQ10F* and *UBQ10R* (Table S1). Relative quantifications were performed by subtracting the cycle threshold (CT) value of *UBQ10* from the CT value of *creA* (ΔCT). The percentage of *B. cinerea* DNA was calculated as $(2^{-\Delta \text{CT mutant}} \times 100)/2^{-\Delta \text{CT wild type}}$.

Measurement of the total SA content

The method for total SA extraction was adapted from a previous study (Verberne *et al.*, 2002). A 100 mg sample of 6- to 7-wk-old plant leaf material was collected and pulverized in liquid nitrogen. After this, 0.5 ml of 90% methanol was added to the powder, which was vortexed for 1 min, sonicated for 5 min and centrifuged for 5 min at maximum speed. The supernatant was collected and the pellet resuspended in 0.25 ml of 100% methanol, and the sonication and centrifugation steps were repeated. The supernatants were combined, centrifuged again and the methanol–water mixtures evaporated in a SpeedVac concentrator (Eppendorf, Hamburg, Germany). To the residue, 250 µl trichloroacetic acid (TCA; 5% solution in water) was added and the mixture was vortex mixed. Partitioning with 800 ml ethyl acetate–cyclohexane (1 : 1, v : v) resulted in the separation of two phases. This partitioning was carried out twice. The aqueous phase was subjected to acid hydrolysis by adding *c.* 300 µl of 8 M hydrochloric acid to the remaining TCA fraction and heating the sample at 80°C for 1 h (Meuwly & Metraux, 1993). The residue was dissolved in 200 µl of sodium acetate pH 5.5/methanol (9 : 1) and analysed by high-pressure liquid chromatography–mass spectrometry (HPLC-MS)s. The analyses of SA were performed with a linear ion trap mass spectrometer equipped with an electrospray ionization source (Bruker Daltonics, Bremen, Germany) coupled to a liquid chromatograph (Ultimate 3000; Dionex, Waltham, MA, USA). A zwitterionic ZIC-HILIC stationary phase column (200 Å, 3.5 µm particle size, 100 × 2.1 mm; Merck SeQuant, Umea, Sweden) was used at 25°C. The elution gradient was carried out with a binary solvent system consisting of 5 mM ammonium acetate (solvent A) and 0.1% acetonitrile/formic acid (solvent B) at a constant flow rate of 0.2 ml min^{-1}. We used the following gradient profile: a linear gradient from 90% to 10% B (0–16 min), 10% B (16–17 min), a linear gradient from 10% to 90% B (17–18 min) and 90% B (18–28 min). Salicylic acid was detected in negative mode giving the spectra of the deprotonated molecule $[M - H]^-$ (m/z 137) and the isotopic form m/z 138 with a retention time of 1.6 min (Fig. S1). The system was controlled with the software package HYSTAR (version 3.2; Bruker Daltonic). A standard solution of SA was used to carry out a calibration curve.

Quantification of thiol compounds

To quantify the total cysteine and glutathione contents, thiols were extracted, reduced with $NaBH_4$, and quantified by reverse-phase HPLC after derivatization with monobromobimane (Molecular Probes, Invitrogen, Paisley, UK) as described previously (Dominguez-Solis *et al.*, 2001). To quantify the reduced cysteine and glutathione, thiols were extracted and directly quantified by reverse-phase HPLC (Lopez-Martin *et al.*, 2008a).

RNA isolation and RT reaction

Total RNA was extracted from *Arabidopsis* leaves using the RNeasy Plant Mini Kit (Qiagen) and reverse transcribed using an

oligo(dT) primer and the SuperScript First-Strand Synthesis System for reverse-transcription (RT)-PCR (Invitrogen) following manufacturer's instructions.

Real-Time RT-PCR

Quantitative real-time RT-PCR was used to analyse the expression of the *OAS-A1* and *DES1* genes implicated in the synthesis or degradation of cysteine, respectively, and the *RPM1*, *PR1*, *WRKY54* and *WRKY70* genes implicated in plant immunity. First-strand cDNA was synthesized as described earlier. Gene-specific primers for each gene were designed using the Vector NTI Advance 10 software (Invitrogen; Table S1). The PCR efficiency of all primer pairs was determined to be 100%. Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad), and the signals were detected on an iCYCLER (Bio-Rad) according to the manufacturer's instructions. The cycling profile consisted of 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. A melt curve from 60 to 90°C was run following the PCR cycling. The expression levels of the genes of interest were normalized to that of the constitutive *UBQ10* gene by subtracting the CT value of *UBQ10* from the CT value of the gene (ΔCT). The fold change was calculated as $2^{-(\Delta CT\,mutant - \Delta CT\,wild\,type)}$.

Cell death measurements

The protocol for measuring the electrolyte leakage was adapted from (Dellagi *et al.*, 1998). Six-week-old wild-type and mutant plants grown at the same time and conditions were infiltrated with bacteria in 10 mM $MgCl_2$ as described earlier. At different times, 10 5-mm diameter leaf discs collected from the injected area were placed in a plate with 3 ml of water and incubated for 2 h. Conductivity measurements (four replicates for each treatment) were taken from the plate over time using an Eco Scan CON6 (Eutech, Nijkerk, the Netherlands) conductivity-meter. The units of this measurement are $\mu S\ cm^{-1}$, where cm refers to the distance between electrodes. No increase in the conductivity was observed when plants were injected with the mock solution.

Statistical analysis

For all the experiments shown, at least three independent samples were analysed (for details, see the legend of the respective figure). ANOVA statistical analysis of data was performed using the program ORIGINPRO 7.5 (OriginLab Corporation, Northampton, MA, USA).

Results

oas-a1.1 and *des1-1* mutants transcriptomes show a high correlation with biotic stresses

Microarrays of mutant *Arabidopsis* plants lacking the cytosolic OASTL *OAS-A1* have been published (Microarray Gene Expression Omnibus database accession number: GSE19245, Lopez-Martin *et al.*, 2008a). Microarrays of plants lacking the cytosolic *DES1* gene were also performed on *des1-1* mutant and wild-type leaves grown on soil under standard long-day conditions for 20 d. Total RNA was prepared and analysed using the Affymetrix-*Arabidopsis* ATH1GeneChip array. Three biological replicates were performed for each sample. Restricting the analysis to the genes whose expression was changed at least 1.5-fold as a threshold and a significance level of $P < 0.05$, we identified 179 genes that exhibited alterations in their transcription level. Among them, 68 genes were upregulated in the *des1-1* mutant plant compared with the wild-type plant and 111 were downregulated (Microarray Gene Expression Omnibus database accession number GSE19244). To detect physiologically relevant patterns, the genes with altered expression were assigned to functional categories based on the Classification SuperView tool available in the Bio-Array Resource for *Arabidopsis* Functional Genomics (BAR, Toufighi *et al.*, 2005, http://bar.utoronto.ca). The resulting group lists revealed that a high proportion of genes with altered expression levels in the *des1-1* mutant were associated with the plant's responses to biotic and abiotic stress (Fig. S2), similar to that described for the *oas-a1.1* mutant plant microarray analysis (Lopez-Martin *et al.*, 2008a).

A meta-analysis of the *oas-a1.1* and *des1-1* transcript profiles data was made by comparing with all databases available in the BAR Expression Browser tools (Toufighi *et al.*, 2005, http://bar.utoronto.ca). These analyses showed that both sets of data had an elevated percentage of co-regulated genes with datasets obtained from *Botrytis*- and *Pst DC3000*-infected *Arabidopsis* plants, as well as with data obtained from other pathogen-infected plants (Figs S3–S6). Moreover, data were analysed using the MAPMAN software. Fig. 1 shows that many genes differentially regulated in both *des1-1* and *oas-a1.1* mutant compared with wild-type plants fit into the Biotic Stress pathway. We observe that these genes are putatively involved in a variety of responses to pathogen attack. These results indicate that the imbalance of cytosolic cysteine alters the expression of groups of genes involved in the plant response to pathogens, suggesting that this thiol could play a role in the plant immunity network. We aimed then to investigate this hypothesis further.

Cysteine content is correlated with plant resistance to pathogens

To investigate the possible role of cytosolic cysteine in plant defense against pathogens, the two alleles defective in the cytosolic L-cysteine desulfhydrase DES1, *des1-1* and *des1-2* (Alvarez *et al.*, 2010), and the two alleles defective in the cytosolic OASTL OAS-A1, *oas-a1.1* and *oas-a1.2* (Lopez-Martin *et al.*, 2008a) were challenged by a necrotrophic (*B. cinerea*) and a hemibiotrophic (*P. syringae pv. tomato* DC3000 – *Pst DC3000*) compatible pathogen. When challenged with the fungus, *des1-1* accumulated 36% less *B. cinerea* DNA than wild-type plants of the Col-0 ecotype, whereas *des1-2* accumulated up to 56% less *B. cinerea* DNA than its corresponding No-0 ecotype wild type (Fig. 2a). Conversely, *oas-a1.1* and *oas-a1.2* accumulated, respectively, 86% and 130% more *B. cinerea* DNA than their

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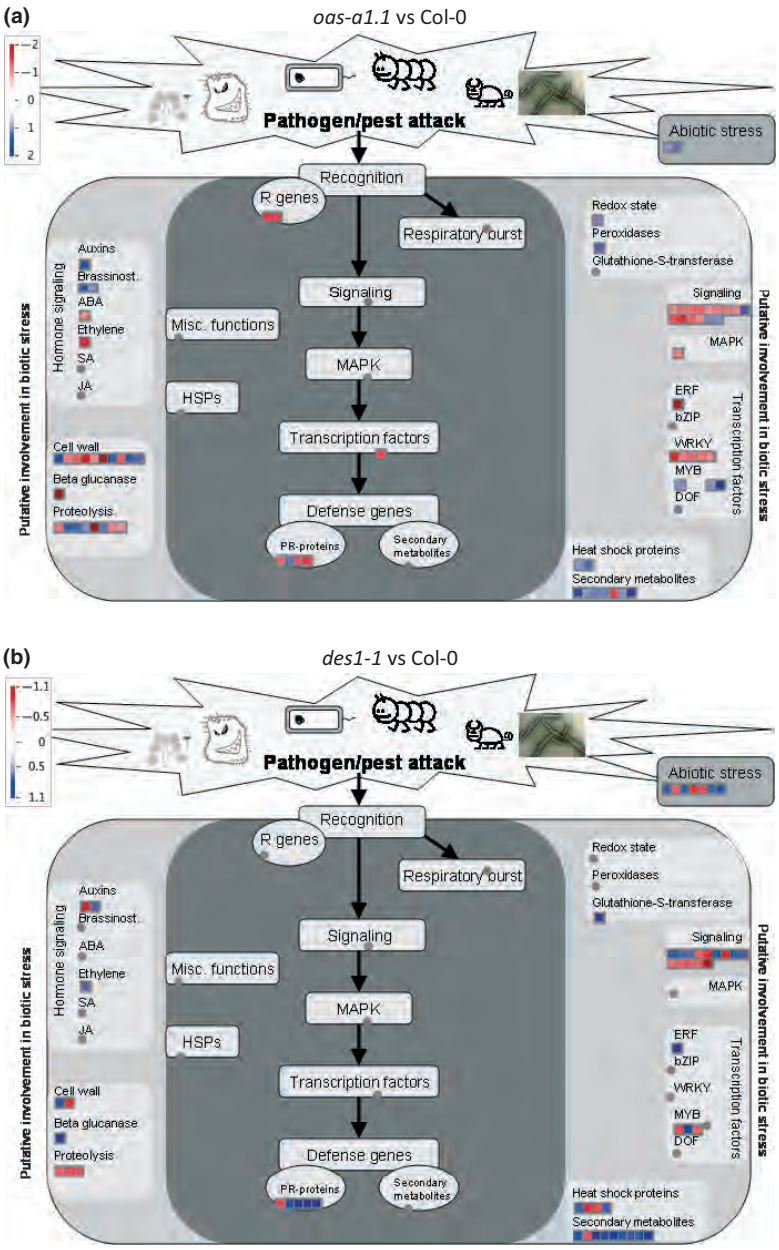

Fig. 1 Analysis of the *Arabidopsis thaliana oas-a1.1* and *des1-1* transcriptomes. Data were analysed using the MAPMAN software. The plant's reaction to biotic stress involves an initial input from the pathogen, which is recognized by the related receptors (R genes), triggering a signaling cascade leading to the production of defense molecules (inside the cell). On the left and right sides are genes and pathways putatively involved in plant response to pathogens. In both cases, the signal is expressed as a ratio relative to the signal in the wild-type plants, converted to a $\log_2$ scale, and displayed. The scale is shown in the figures.

corresponding wild type of the Col-0 ecotype (Fig. 2a). Similarly, both *oas-a1.1* and *oas-a1.2* showed an earlier infection by *Pst DC3000* than the Col-0 wild type, as 2.5 times more *Pst DC3000* CFU mg^{-1} FW was found at 1 d after infection (dpi) than Col-0 and the difference was 1.5 times at 3 dpi (Fig. 2b). The *des1-1* mutant was more resistant to *Pst DC3000* infection than Col-0 at both 1 dpi (eight times less CFU mg^{-1} than Col-0) and 3 dpi (2.5 less infected than Col-0). In addition, the *des1-2* mutant showed a behavior very similar to the *des1-1* mutant; at 1 dpi, it showed five times fewer CFU mg^{-1} than its corresponding No-0 wild type and at 3 dpi it showed two times fewer CFU mg^{-1} than No-0. In summary, both kinds of mutants presented opposite phenotypes: whereas *des1-1* and *des1-2* were more resistant to either *B. cinerea* or *Pst DC3000* infection, *oas-a1.1* and *oas-a1.2* were more sensitive to both pathogens.

An increase in SA elicits a SAR phenotype in many instances (Vlot *et al.*, 2008). Because *des1-1* and *des1-2* were more resistant to both a biotrophic and a necrotrophic pathogen, we measured the total SA content in wild-type and *des1* mutant plants. As expected, the total SA content in *des1-1* mutant was significantly higher (1.5 times) than in wild-type Col-0 plants (Fig. 3a). However, the SA content in *des1-2* plants was not statistically different from its corresponding wild type (No-0), although the difference was 1.3 times the No-0 levels (Fig. 3a). We also measured the total amount of SA in the both *oas-a1* mutants and no differences were observed in comparison with wild-type Col-0 plants (Fig. 3a). When we further measured thiol content of wild type and mutants, we found that *des1* mutants showed increases of 20–30% in Cys content and 14–15% in glutathione content when compared with the respective wild-type plants, whereas

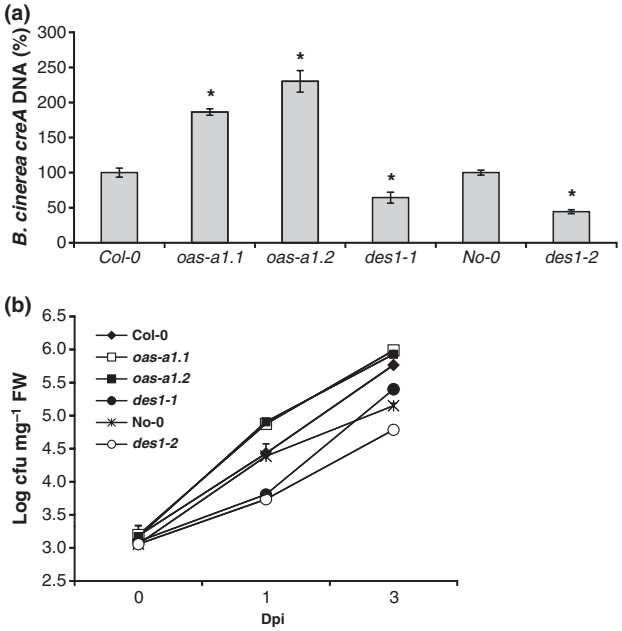

Fig. 2 Responses of the *Arabidopsis thaliana oas-a1* and *des1* mutants to biotic stress. (a) Susceptibility of wild-type (Col-0 and No-0 ecotypes) and *oas-a1.1*, *oas-a1.2*, *des1-1* and *des1-2* mutant lines to *Botrytis cinerea* infection. Quantification of fungus growth was performed by real-time reverse-transcription polymerase chain reaction amplification of the *B. cinerea creA* gene, normalized against the *Arabidopsis UBQ10* gene and represented as the percentage of *Botrytis creA* DNA present in wild-type plants. DNA was isolated from leaves 5 d after spore inoculation of 6- to 7-wk-old wild-type and mutant plants grown in parallel. The data correspond to the mean ± SD of three independent analysis made from material grown in different batches at different times. For each analysis, 20 infected plants were pooled and six independent DNA extractions were made from the pooled material. Moreover, two experimental replicates were done from each sample. (b) Susceptibility of wild-type and mutant lines to virulent *Pseudomonas syringae pv. tomato DC3000* bacteria infection. Colony-forming units (CFU) were counted at 0, 1 and 3 d post-infection (dpi) of 6- to 7-wk-old wild-type and mutant plants grown in parallel. Twelve to 14 leaves were pooled for each analysis, in which three independent counts were made from the pooled material and two experimental replicates were made from each sample. The data correspond to the mean ± SD of one representative experiment. *, $P < 0.05$. The experiment was done three times with material grown in different batches at different times with similar results.

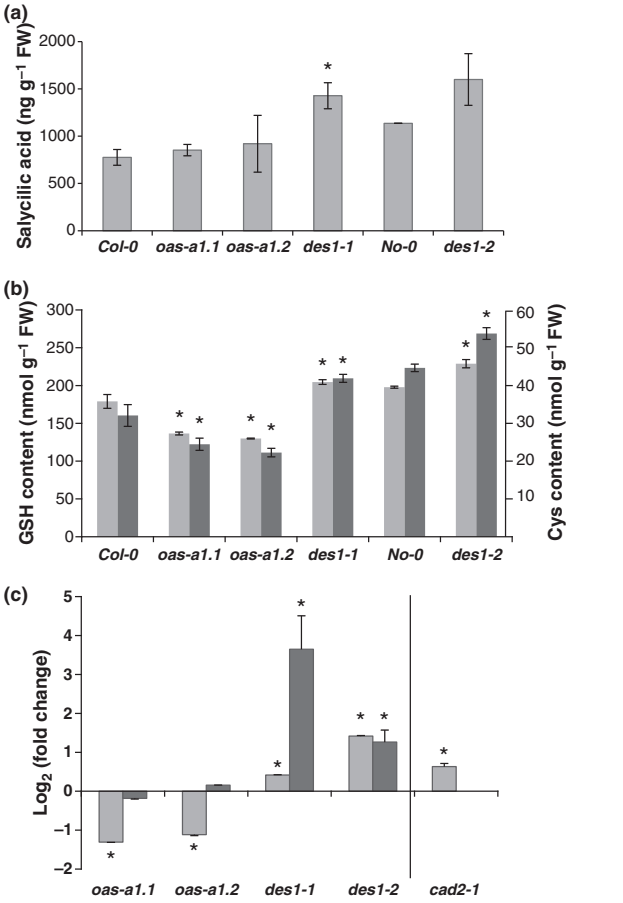

Fig. 3 The accumulation of cytosolic cysteine triggers the accumulation of salicylic acid and pathogenesis-induced transcripts. (a) Salicylic acid accumulation in noninfected *Arabidopsis thaliana* wild type (Col-0 and No-0 ecotypes), *oas-a1.1*, *oas-a1.2*, *des1-1* and *des1-2* plants was measured in leaf extracts from 6- to 7-wk-old plants grown in parallel and quantified by mass spectrometry after high-pressure liquid chromatography separation. Data presented here correspond to the mean ± SD of three independent analysis made from material grown in different batches at different times. For each analysis, five plants were pooled and three independent extractions were made from the pooled material. *, $P < 0.05$. (b) Total glutathione (GSH, light tinted bars) and cysteine (Cys, dark tinted bars) content in and in Col-0, No-0, *oas-a1.1*, *oas-a1.2*, *des1-1* and *des1-2* 6- to 7-wk-old plants under normal growth conditions. Data presented here correspond to the mean ± SD of three independent analysis made from material grown in different batches at different times. For each analysis, five or six plants were pooled and three independent extractions were made from the pooled material. Moreover, two experimental replicates were made for each sample. (c) Relative expression of *WRKY54* (light tinted bars) and *PR1* (dark tinted bars) in 6- to 7-wk-old noninfected *oas-a1.1*, *oas-a1.2*, *des1-1*, *des1-2* and (only for *WRKY54* expression) *cad2-1* mutant plants (right panel). The data refer to *WRKY54* and *PR1* levels in the respective wild-type plants grown in parallel with the mutant plants. The results shown are means ± SD of three independent analysis using material grown in different batches at different times. For each analysis, five or six plants were pooled and three independent RNA extractions were made from the pooled material. Two experimental replicates were made for each sample. *, $P < 0.05$.

oas-a1 mutants showed 24–31% less Cys and 24–28% less glutathione that wild-type plants (Fig. 3b), similar to previously published data (Lopez-Martin *et al.*, 2008a; Alvarez *et al.*, 2010). Furthermore, we calculated the degree of oxidation of each thiol in the different plant lines (Table S2). The values for the degree of glutathione oxidation calculated for both ecotypes of wild type *Arabidopsis* plants are in accord with those previously reported (Meyer *et al.*, 2007). While the degree of glutathione oxidation in the *des1* mutant alleles was slightly lower, it was significantly greater in the *oas-a1* mutant alleles, as reported previously (Lopez-Martin *et al.*, 2008a; Alvarez *et al.*, 2010). With respect to the degree of cysteine oxidation, to our knowledge there is no published report of this, and when compared with the wild-type plants we observed similar behavior in the mutants as for glutathione. While the *des1* mutants showed a lower degree of oxidation, the *oas-a1* mutants showed a greater degree, but this increase was not as strong as that observed for glutathione. Collectively, these data suggest that a 20–30% increase in the

cytosolic cysteine or 14–15% glutathione contents, mainly in their reduced forms (as reflected by their lower degree of oxidation), could elicit basal resistance to pathogens via SA signaling.

One of the effects of SA accumulation is the nuclear localization of NPR1 (Tada *et al.*, 2008), which directly induces the expression of several *WRKY* transcription factors (Wang *et al.*, 2006). Most of these are activators of PR genes, such as *PR1*, a gene widely used as a SAR reporter. According to the broad-spectrum disease resistance phenotype shown by *des1* mutant plants, the expression of *WRKY54* and *PR1* were induced in both *des1-1* and *des1-2* mutants in the absence of infection (Fig. 3c). However, the SA content in *oas-a1* mutant plants was indistinguishable from the wild-type concentrations, although they were more sensitive to pathogens. By contrast, the *WRKY54* but not the *PR1*, transcription level was reduced in the *oas-a1* mutants compared with wild-type plants (Fig. 3c). Transcriptomic data of *oas-a1.1* leaves show that five different WRKY transcription factors (including WRKY54) and three PR proteins are repressed in the mutant compared with wild-type leaves (Lopez-Martin *et al.*, 2008a), suggesting that some components of the immune response are compromised in the *oas-a1.1* plants.

Because the *oas-a1.1* and *oas-a1.2* mutants present not only a 24–31% reduction in cysteine but also a 24–28% reduction in glutathione levels (Fig. 3b), we analysed, in parallel, the behavior of the *cad2-1* mutant, which has a 70% reduction in glutathione compared with the wild-type, but the amounts of cysteine are not reduced (Ball *et al.*, 2004). *WRKY54* was not repressed in the *cad2-1* mutants (Fig. 3c, right panel), demonstrating that the repression of this transcription factor is specifically caused by the decrease in cytosolic cysteine content shown by the *oas-a1.1* and *oas-a1.2* mutants.

In conclusion, the *des1* mutants, which have increased cysteine levels, behave as constitutive SAR mutants, whereas *oas-a1* mutant plants, which have reduced cysteine levels, are more sensitive to pathogens.

Cytosolic cysteine is required for the HR in an incompatible interaction

In addition to the basal resistance triggered by PAMPs, *Pst DC3000* can elicit an ETI in *Arabidopsis* when expressing either of two sequence-unrelated type III effectors, AvrRpm1 or AvrB (Bent *et al.*, 1994; Mindrinos *et al.*, 1994; Grant *et al.*, 1995). As the HR is one of the responses associated with ETI, we measured the ion leakage of leaf discs infected with the avirulent bacterium *Pst DC3000* expressing *avrRpm1* (*Pst DC3000 avrRpm1*). *Pst DC3000 avrRpm1* did not induce ion leakage in *oas-a1.1* or *oas-a1.2* plants (Fig. 4a), although their basal conductivity is higher than that displayed by the wild-type plants (Table S3) as a result of spontaneous cell death lesions in the *oas-a1* mutants (Lopez-Martin *et al.*, 2008a). This indicates that *oas-a1* mutants have an impaired ETI response elicited by the avrRpm1–RPM1 interaction. Conversely, *des1-1* mutant plants, which have an increased cysteine level of 30% (Fig. 3b), elicited ion leakage similar to that of wild-type plants after 9 h of *Pst DC3000 avrRpm1* infection (Fig. 4a). The *cad2-1* mutant plants showed

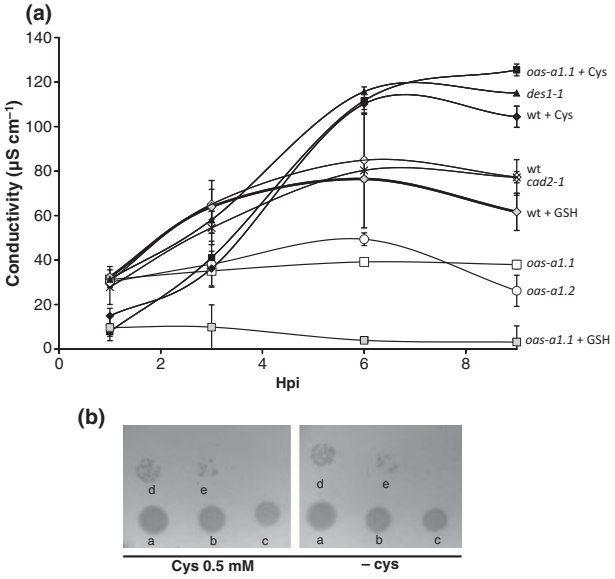

Fig. 4 The hypersensitive response is dependent on the cytosolic cysteine. (a) *Arabidopsis thaliana* wild-type and mutant plants grown in parallel were infected with *Pseudomonas syringae pv. Tomato* (*Pst*) *DC3000 avrRpm1*, or co-infiltrated when indicated, with 0.5 mM of cysteine (+Cys) or 5 mM of glutathione (+GSH). The conductivity was measured in leaf discs from 6- to 7-wk-old plants infiltrated with a bacteria suspension. Results shown are mean ± SD of three independent analysis using material grown in different batches at different times. For each treatment, 12–14 leaves of three plants were infected and four experimental replicates were made; hpi, hours post-infection. (b) Growth tests of *Pst DC avrRpm1* bacteria grown in Luria–Bertani (LB) medium supplemented with rifampicin and cysteine 0.5 mM (Cys 0.5 mM) or with rifampicin and without added cysteine (–Cys); a–e, 10 µl of serial 10-fold dilutions of a 5×10^6 bacteria ml^{-1} *Pst DC3000 avrRpm1* suspension.

an increase in conductivity during the infection with the avirulent *Pst DC3000 avrRpm1* strain, which was indistinguishable from the increase in wild-type plants under the same conditions. This response demonstrates that the lack of a HR response during an incompatible interaction in the *oas-a1.1* and *oas-a1.2* mutants was caused by its cysteine deficit and its decreased glutathione content.

To demonstrate that cysteine is a molecule mediating the HR response in *A. thaliana*, we carried out experiments of reversion of the phenotype *in vivo* with cysteine. The addition of 500 µM of cysteine at the site of infection reverted the phenotype shown by the *oas-a1.1* mutant, whereas the co-infiltration of *Pst DC3000 avrRpm1* with 5 mM glutathione did not produce any effect on the HR response in the wild-type or *oas-a1.1* mutant plants (Fig. 4a). Furthermore, the addition of cysteine, but not of glutathione, seemed to increase the intensity of the total ion leakage reached by both wild-type and *oas-a1.1* mutant plants (Fig. 4a). In order to exclude that the cysteine directly affected pathogen growth instead of functioning through the plant response to the pathogen, we performed growth tests of *Pst DC3000 avrRpm1* in solid culture LB media in the absence and in the presence of 500 µM of cysteine. No differences were observed in any of two conditions (Fig. 4b), therefore excluding the possibility of a direct effect of cysteine in the pathogen's growth rather than an effect in rescuing the *oas-a1.1* phenotype.

Cytosolic cysteine is required for the transcriptional regulation of R proteins

One of the earliest steps during the ETI is the effector recognition, that is, a direct or indirect recognition of microbe avirulence effectors by plant resistance proteins (reviewed in Panstruga *et al.*, 2009). In the *A. thaliana–Pst DC3000 avrRpm1* system, the interaction between the effector *avrRpm1* and the RPM1 CC-NB-LRR resistance protein initiates the plant response, driving an incompatible interaction (Grant *et al.*, 1995). In wild-type plants, the *RPM1* transcript is transiently induced during the *Arabidopsis–Pst DC3000 avrRpm1* interaction (Fig. 5a). In *oas-a1.1* and *oas-a1.2* plants, the *RPM1* transcription was reduced by 50% with respect to the wild type in control conditions without infection (Fig. 5b). *RPM1* expression in noninfected plants was significantly unaltered in the *des1-1* and *cad2-1* mutants (Fig. 5b) and was slightly increased in the *des1-2* mutant. These data suggest that the repression of *RPM1* transcription observed in *oas-a1.1* plants was a result of their decreased cytosolic cysteine content. A reduction in the *RPM1* transcription would be reflected in reduced effector recognition and poor plant response when infected with *Pst DC3000 avrRpm1*, which was exactly the effect observed. To test whether this poor ETI response affected the development of bacteria *in planta*, we quantified the evolution of the infection by counting the number of bacteria developing in infected wild type and *oas-a1.1* mutant plants at 1 dpi and 3 dpi. As already established (Tao *et al.*, 2003), bacteria growth in wild-type plants is arrested 1 d after infection. As we expected, we observed a poor plant response to infection in the *oas-a1.1* mutant, thus the incompatible *Pst DC3000 avrRpm1* bacteria were able to grow as a compatible *Pst DC3000* strain does (Fig. 5c) while *des1-1*, *des1-2* and *cad2-1* mutants showed a phenotype indistinguishable from wild-type plants.

Cysteine and glutathione accumulation patterns and transcript regulation during ETI

The results presented here indicate that cytosolic cysteine is essential to induce the HR response associated with the ETI triggered by the *Arabidopsis–Pst DC3000 avrRpm1* interaction, so we determined the kinetics of accumulation of cysteine and glutathione during infection with an avirulent *Pst DC3000 avrRpm1* strain. Samples were taken at 1, 3, 6, 9 and 24 h post-infection (hpi). Fig. 6 shows the accumulation of cysteine (Fig. 6a) and glutathione (Fig. 6b) during an incompatible *Arabidopsis–Pst* interaction. Interestingly, cysteine content decreased transiently, and at 1–3 hpi, its content was reduced to 50% of the level of cysteine in the absence of infection. After 3 hpi, cysteine content started increasing drastically reaching 150% of the noninoculated plant content at 9 hpi and 200% at 24 hpi. Glutathione concentrations, in turn, increased drastically during an incompatible *Arabidopsis–Pst DC3000 avrRpm1* interaction, even in the earlier stages of infection, reaching up to 300% of the noninoculated plant level at 6 hpi.

Therefore, cysteine content showed a 'down and up' regulation during an incompatible *Arabidopsis–Pst DC3000 avrRpm1*

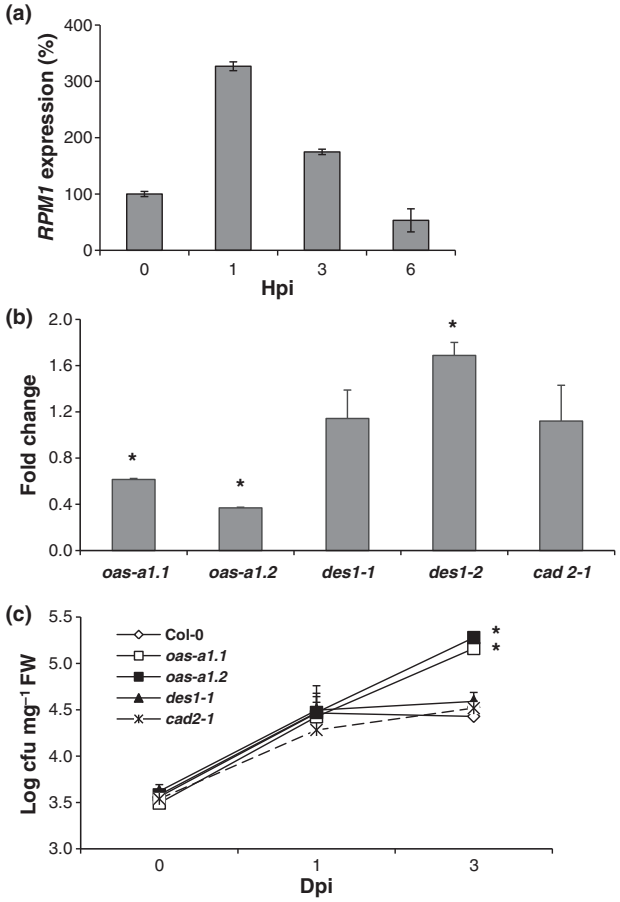

Fig. 5 *RPM1* transcription is dependent on cytosolic cysteine. (a) Time-course of *RPM1* transcription during an infection of 6- to 7-wk-old *Arabidopsis thaliana* wild type with *Pseudomonas syringae pv. Tomato* (*Pst*) *DC3000 avrRpm1*, measured by real-time reverse-transcription polymerase chain reaction (RT-PCR). The transcript level was normalized using the constitutive *UBQ10* gene as an internal control. (b) *RPM1* steady-state transcription in 6- to 7-wk-old noninfected *oas-a1.1*, *oas-a1.2*, *des1-1*, *des1-2* and *cad 2-1* plants, measured by real-time RT-PCR, compared with the *RPM1* steady-state level in noninfected wild-type plants grown in parallel with the mutant plants. In (a) and (b), the results shown are means ± SD of three independent analysis using material grown in different batches at different times. For each analysis, five or six plants were pooled and three independent RNA extractions were made from the pooled material. Two experimental replicates were made for each sample. *, $P < 0.05$. (c) Growth of avirulent *Pst DC3000 avrRpm1* bacteria in wild-type, *oas-a1.1*, *oas-a1.2*, *des1-1* and *cad 2-1* plants grown in parallel. The colony forming units (CFU) were counted at 0, 1 and 3 d post-infection (dpi) of 6- to 7-wk-old plants. Twelve to 14 leaves were pooled for each analysis, in which three independent counts were made from the pooled material, and two experimental replicates were made from each sample. The data correspond to the mean ± SD. *, P value < 0.05. The experiment was done three times with material grown in different batches at different times with similar results.

interaction, which decreased at the beginning of the interaction and increased later. To determine whether the cysteine content increase is caused by protein degradation during the HR response or *de novo* cysteine synthesis, we analysed regulation of the transcripts of genes involved in cytosolic cysteine synthesis and degradation during the infection of *A. thaliana* with *Pst DC3000 avrRpm1*. *OAS-A1*- and *DES1*-coding genes were regulated in an

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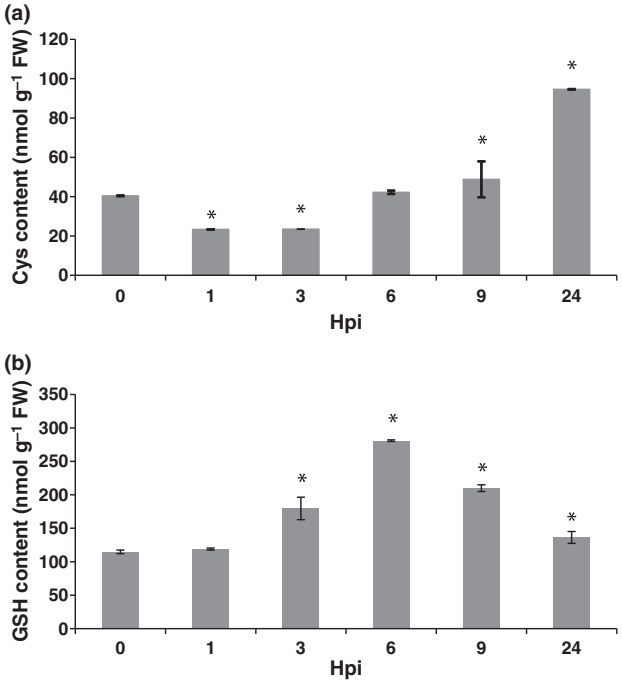

Fig. 6 Time-course of thiol accumulation during *Arabidopsis thaliana–Pseudomonas syringae pv. Tomato DC3000 avrRpm1* interaction. Total (a) cysteine (Cys) and (b) glutathione (GSH) content was measured in leaf extracts of wild-type plants grown for 6- to 7 wk and infected with a bacterial suspension. The values were normalized against the data obtained from plants treated with a mock solution. Data presented here correspond to the mean ± SD of four independent analysis made from material grown in different batches at different times. For each analysis, 12–14 leaves from infected plants were pooled and three independent extractions were made from the pooled material. Two experimental replicates were made for each sample; hpi, hours post-infection. *, $P < 0.05$.

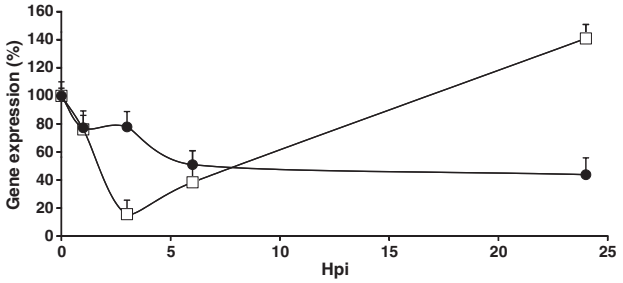

Fig. 7 *OAS-A1* (squares) and *DES1* (circles) expression levels during an incompatible *Arabidopsis thaliana–Pseudomonas syringae pv. Tomato DC3000 avrRpm1* interaction analysed by real-time reverse-transcription polymerase chain reaction and referred to the *UBQ10* internal control; hpi, hours post-infection. The data correspond to means ± SD of three independent analysis using material grown in different batches at different times. For each analysis, five or six plants were pooled and three independent RNA extractions were made from the pooled material. Two experimental replicates were made for each sample.

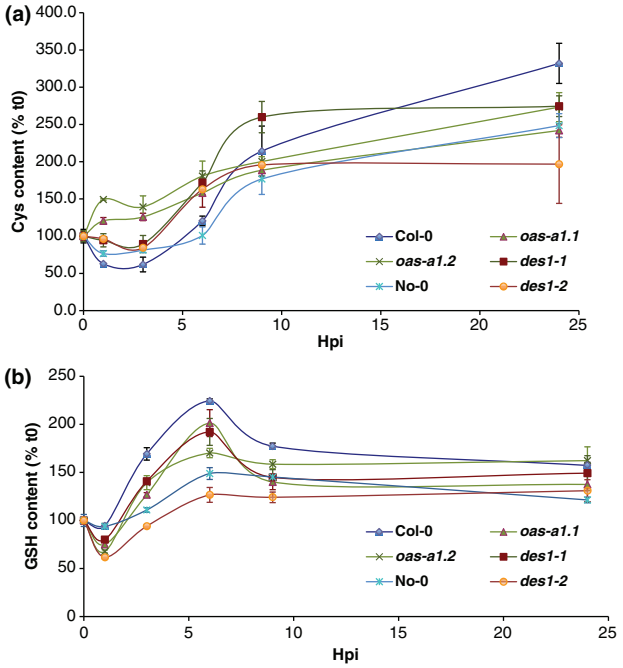

Fig. 8 Evolution of the total thiol content during the *Arabidopsis thaliana–Pseudomonas syringae pv. Tomato DC3000 avrRpm1* interaction with *oas-a1* and *des1* mutants. Total (a) cysteine (Cys) and (b) glutathione (GSH) content was measured in leaf extracts of wild-type and mutant plants grown for 6–7 wk and infected with a bacterial suspension. Each point was calculated as a percentage of the basal thiol content of each plant. Col-0, *oas-a1.1* and *des1-1* data correspond to the mean ± SD of three independent analysis made from material grown in different batches at different times. For each analysis, 12–14 leaves from infected plants were pooled and three independent extractions were made from the pooled material. Two experimental replicates were made for each sample. No-0, *oas-a1.2* and *des1-2* data correspond to the mean ± SD of three different extracts from 12 to 14 pooled infected leaves from plants grown in the same batch at the same time. Two experimental replicates from each extract were done; hpi, hours post-infection.

opposite way in response to the infection with an avirulent strain of *Pst DC3000* (Fig. 7). While the *OAS-A1* transcript transiently decreased, with a minimum of 20% of the uninfected level at 3 hpi, the *DES1* transcript showed significantly different kinetics of expression, remaining high in the early hours of infection and declining after 3 hpi. These results were consistent with the pattern of cysteine accumulation observed during an incompatible interaction. Therefore, the variation in cysteine content observed during an incompatible interaction would be produced by modulation of the enzymatic activities related to the synthesis and degradation of this thiol, rather than from protein degradation associated with the HR.

Cysteine and glutathione accumulation and degree of oxidation during ETI in *oas-a1* and *des1* mutants

The accumulation of cysteine and glutathione, as well as their degree of oxidation, was also measured in *oas-a1* and *des1* mutants in order to further distinguish the roles of these two thiols in the establishment of an incompatible interaction. As the basal cysteine and glutathione content (at time 0) of each plant is different (Fig. 3b), Fig. 8 shows the evolution of the glutathione and cysteine content of both Col-0 and No-0 wild types and *des1* and *oas-a1* mutants relative to the basal content of each plant. Glutathione content showed, in all cases, a very similar curve, as observed in Fig. 6(b), with a slight decrease in glutathione

content at 1 hpi, an increase after 3 hpi and a maximum at 6 hpi, decreasing after this time to reach at 24 hpi a content of *c.* 120–160% of the plant at time 0. By contrast, cysteine content is evolved in a different way in each plant line, as wild-type plants and *des1* mutants showed an early decrease, followed by a drastic increase at 6, 9 and 24 hpi, as shown in Fig. 8(a), whereas the *oas-a1* mutants clearly differed in cysteine content evolution from wild types during ETI, showing a rapid and maintained increase in cysteine content from 1 hpi.

The basal oxidation degree of cysteine and glutathione is also different for every plant line used in this work (Lopez-Martin *et al.*, 2008a; Table S2). Therefore, the progression of the degree of oxidation during ETI was calculated as percentage of the basal oxidation degree of each plant (Fig. 9). In wild types (Col-0 and No-0), the degree of cysteine and glutathione oxidation decreased early (1 hpi) and showed an increase at 3–6 hpi, diminishing slowly to reach values similar to the basal values at 24 hpi (100%). The *des1* mutants did not show an early diminution in the degree of glutathione oxidation and the increase in both thiol oxidation degrees at 1–3 hpi was much greater than their respective wild types, although the shapes of the curves displayed by *des1* mutants were similar to those of the respective wild types. In the case of *oas-a1* mutants, the degree of glutathione and cysteine oxidation remained constant and was unchanged during infection by *PstDC3000 avrRpm1*, which is consistent with the absence of HR during their interaction with the bacteria (Fig. 5a).

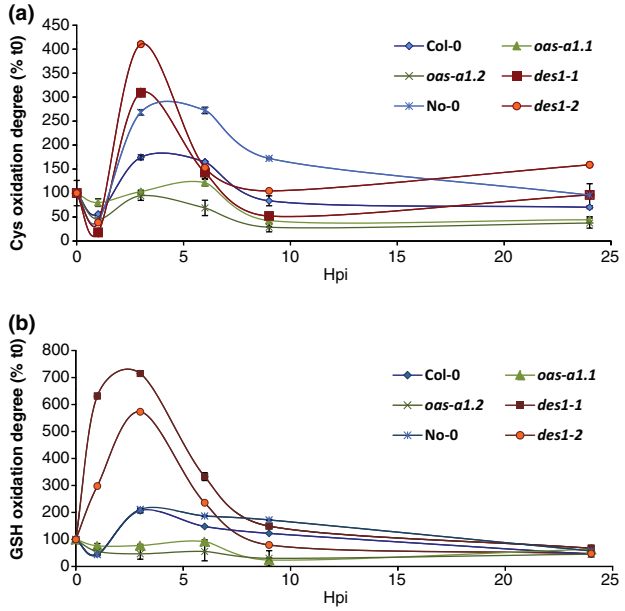

Fig. 9 Progression of the degree of (a) cysteine (Cys) and (b) glutathione (GSH) oxidation during the *Arabidopsis thaliana–Pseudomonas syringae pv. Tomato DC3000 avrRpm1* interaction. Each point was calculated as a percentage of the basal degree of oxidation of each plant. Wild-type and mutant plants were grown for 6–7 wk and infected with a bacterial suspension. Col-0, *oas-a1.1* and *des1-1* data correspond to the mean ± standard deviation (SD) of three independent analyses made from material grown in different batches at different times. For each analysis, 12–14 leaves from infected plants were pooled and three independent extractions were made from the pooled material. Two experimental replicates were made for each sample. No-0, *oas-a1.2* and *des1-2* data correspond to the mean ± SD of three different extracts from 12–14 pooled infected leaves from plants grown in the same batch at the same time. Two experimental replicates from each extract were done; hpi, hours post-infection.

Discussion

In this article, we present evidence consistent with a regulatory role for cysteine during plant–pathogen interaction. Previous work using plant mutants with increased (*des1-1* and *des1-2*) or decreased (*oas-a1.1* and *oas-a1.2*) cytosolic cysteine content has demonstrated that cysteine is very precisely regulated to maintain cellular homeostasis (Lopez-Martin *et al.*, 2008b; Alvarez *et al.*, 2010). Despite this maintenance, the transcriptomes of the *oas-a1.1* and *des1-1* mutants and the transcriptomes of plants challenged with pathogens show a high co-regulation. In fact, these mutants show altered basal resistance to pathogens, so that increased cytosolic cysteine content is associated with enhanced resistance to pathogens, whereas decreased cytosolic cysteine content is associated with decreased resistance to pathogens. The expression of genes associated with the plant immune response, as shown by analysis of the transcriptome of the *oas-a1.1* and *des1-1* mutants or by quantitative real-time RT-PCR, agrees to the phenotype observed in both kinds of mutants, particularly in the case of the *des1* mutants, where the levels of SA are increased, as well as the transcription of *WRKY54* and *PR1*. Moreover, the transcriptomic analysis of the *des1-1* mutant shows that four PR proteins are induced in the mutant, the defensins *PDF1-2a* and *PDF1-2b* among them. The *oas-a1.1* and *oas-a1.2* mutants have SA levels indistinguishable of those of wild-type plants, as well as *PR1* transcription. However, the *WRKY54* transcription is decreased in the *oas-a1.1* and *oas-a1.2* mutants and transcriptomic analysis of the *oas-a1.1* mutant shows that *WRKY54*, *WRKY38*, *WRKY46*, *WRKY33* and *WRKY26* are also repressed in the mutant more than twofold (Lopez-Martin *et al.*, 2008a). Moreover, several *PR* genes and other signaling components of the plant immune response are altered in the *oas-a1.1* mutant (Lopez-Martin *et al.*, 2008a; see Fig. 1). In addition to their altered cysteine levels, *oas-a1* and *des1* mutants showed respectively a 24–28% decrease and a 14–15% increase in glutathione content. Pathogen attack and application of SA trigger an increase in total glutathione content (Fodor *et al.*, 1997; Vanacker *et al.*, 2001; Mou *et al.*, 2003; Mateo *et al.*, 2006). Other glutathione mutants deficient in the γ-glutamylcysteine synthetase gene (*GSH1*) and displaying a 70–80% decrease in glutathione content and no cysteine decrease are more susceptible to several pathogens (Ball *et al.*, 2004; Parisy *et al.*, 2007; Schlaeppi *et al.*, 2008). Therefore, we cannot exclude the possibility that glutathione may have a function in basal resistance, and, in fact, it has been proposed that adequate concentrations of glutathione are important in *Arabidopsis* for limiting the spread of virulent *P. syringae*, probably by regulating the accumulation of resistance-related compounds (Glazebrook & Ausubel, 1994; Roetschi *et al.*, 2001). However, the results in this report demonstrate a specific function for cytosolic cysteine in plant–pathogen interactions. This function could arise from cysteine *per se*, or from its role as a generator of elemental sulfur involved in fungal pathogen defense (Cooper *et al.*, 1996; Williams *et al.*, 2002).

Another possible role of cysteine is as a determinant of the oxidative capacity of the cytosol (Lopez-Martin *et al.*, 2008a,b; Alvarez *et al.*, 2010). Apart from decreased cysteine and glutathione levels, the *oas-a1.1* and *oas-a1.2* mutants showed an increased degree of cysteine and glutathione oxidation, which was decreased in *des1-1* and *des1-2* plants (Lopez-Martin *et al.*, 2008a; Alvarez *et al.*, 2010) (Table S2). Thus, it is also possible that maintaining an adequate thiol redox state is essential for a proper basal response to plant pathogens. In accord with this line of reasoning, *des1-1* and *des1-2* plants possess a nonoxidizing cytosol and displayed enhanced resistance to plant pathogens, whereas the *oas-a1.1* and *oas-a1.2* plants have an oxidizing cytosol and showed enhanced sensitivity to plant pathogens.

Plants induce the ETI when they recognize a pathogen effector (or avirulence factor, avr) through resistance (R) proteins. The avr–R interaction initiates a rapid response including an oxidative burst, a HR, SA accumulation and *PR* gene induction (Panstruga *et al.*, 2009). In *A. thaliana*, variations in cysteine concentrations during the ETI induced by *Pst DC3000 avrRpm1* result from *de novo* synthesis of this thiol because it is accompanied by specific regulation of genes involved in cytosolic cysteine synthesis and degradation. In the *Arabidopsis–Pst DC3000 avrRpm1* system, the RPM1 resistance protein is essential for the initiation of the HR response after challenge by *Pst DC3000 avrRpm1* because it mediates the AvrRpm1 recognition (Holt *et al.*, 2000). In the *oas-a1.1* mutant, *RPM1* is 50% repressed in noninfected plants and it does not reach maximum levels upon infection. Thus, this level of RPM1 seems to be insufficient to initiate the HR in response to *Pst DC3000 avrRpm1*, and the infection is successful, although *oas-a1* mutants displayed spontaneous cell-death lesions (Lopez-Martin *et al.*, 2008a) and basal ion leakage was greater in these mutants than in wild-type plants. In this case, we have shown that the initiation of the HR is independent of glutathione levels in the cell because the lack of an HR response is rescued in the *oas-a1.1* knockout mutants by cysteine, but not by reduced glutathione, and the *cad2-1* mutant is unaltered in the HR response to *Pst avrRpm1*. Therefore, in the *oas-a1.1* mutant, either the reduced amount of cytosolic cysteine or the oxidizing cytosol, or both, seem to be responsible for the poor transcription of *RPM1* and the lack of an HR response after *Pst avrRpm1* infection. Under sulfate deprivation, *Arabidopsis* plants transcribe 0.76 times the *RPM1* gene (Genevestigator, https://www.genevestigator.com/), which suggests that sulfur-containing compounds, like cysteine, could be essential for full *RPM1* transcription. Observations that supplementary sulfur-fertilization reduced the incidence of fungal pathogens on crops have been reported, and the terms 'Sulfur-Induced Resistance' (SIR) or 'S-enhanced defense' (SED) have been proposed to explain this phenomenon (Rausch & Wachter, 2005). It has been recently reported that SIR/SED is induced during a compatible plant–virus interaction in tobacco plants, linked to the activation of cysteine and glutathione metabolism (Holler *et al.*, 2010).

NPR1 is a key regulatory protein involved in SA signaling, which has a redox-regulated cellular localization. When NPR1 is oxidized it is an oligomer and is located in the cytosol. The reduction of thiol groups on NPR1 leads to the monomerization of the protein and its localization in the nucleus, where it promotes the transcriptional activation of *PR* genes (Tada *et al.*, 2008). The oxidized state of the cytosol of the *oas-a1.1* mutants may prevent the pathogen-induced NPR1 nuclear localization and, consequently, NPR1 would not induce the cellular response to pathogens, like *PR* or *WRKY* induction. This lack of induction would lead to an enhanced sensibility to pathogens because of the lack of PR induction. Therefore, a deficiency in cysteine-mediated redox homeostasis could impair redox signaling such as the NPR1 redox modifications.

During the early steps of the interaction with *Pst DC avrRpm1*, the evolution of cysteine content in *oas-a1* and *des1* mutants is completely different, while the evolution of glutathione content is very similar in all plants. This is consistent with a regulatory role of cysteine, and not of glutathione, in the establishment of the ETI. Also, the oxidation state of cysteine and glutathione does not change considerably during the challenge of *oas-a1* mutants with *Pst DC avrRpm1* whether it is transiently augmented with either wild type or *des1* mutants in the early steps of interaction with the avirulent bacteria. This suggests that a correct cytosolic cysteine content is essential for the initiation of the ETI, probably by regulating the redox state of the cell.

The SA content in the *des1-1* and *des1-2* mutants (with elevated cysteine levels) is higher than in wild-type plants and they act like SAR mutants because they accumulate SA and are more resistant to biotrophic and necrotrophic pathogens than wild-type plants. Consistent with this, *PR-1* is constitutively expressed in these mutants.

In conclusion, we have demonstrated that cytosolic cysteine plays a (direct or indirect) role in the establishment and signaling of the plant response to pathogens. First, in light of our observations, we propose that cysteine could have a protective role during PTI, which cannot be separated from the already proposed protective role of glutathione. In addition, cytosolic cysteine is essential to the initiation of the HR response during ETI. Our work suggests that accurate regulation of cytosolic cysteine homeostasis is critical for orchestrating the plant response to pathogens.

Acknowledgements

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References

Alvarez C, Calo L, Romero LC, Garcia I, Gotor C. 2010. An o-acetylserine(thiol)lyase homolog with l-cysteine desulfhydrase activity regulates cysteine homeostasis in *Arabidopsis*. *Plant Physiology* **152**: 656–669.

Alvarez ME. 2000. Salicylic acid in the machinery of hypersensitive cell death and disease resistance. *Plant Molecular Biology* **44**: 429–442.

Ball L, Accotto GP, Bechtold U, Creissen G, Funck D, Jimenez A, Kular B, Leyland N, Mejia-Carranza J, Reynolds H *et al.* 2004. Evidence for a direct link between glutathione biosynthesis and stress defense gene expression in *Arabidopsis*. *Plant Cell* **16**: 2448–2462.

Barroso C, Romero LC, Cejudo FJ, Vega JM, Gotor C. 1999. Salt-specific regulation of the cytosolic o-acetylserine(thiol)lyase gene from *Arabidopsis thaliana* is dependent on abscisic acid. *Plant Molecular Biology* **40**: 729–736.

Barroso C, Vega JM, Gotor C. 1995. A new member of the cytosolic o-acetylserine(thiol)lyase gene family in *Arabidopsis thaliana*. *FEBS Letters* **363**: 1–5.

Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate – a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B – Methodological* **57**: 289–300.

Bent AF, Kunkel BN, Dahlbeck D, Brown KL, Schmidt R, Giraudat J, Leung J, Staskawicz BJ. 1994. Rps2 of *Arabidopsis thaliana*: a leucine-rich repeat class of plant disease resistance genes. *Science* **265**: 1856–1860.

Bermudez MA, Paez-Ochoa MA, Gotor C, Romero LC. 2010. *Arabidopsis* s-sulfocysteine synthase activity is essential for chloroplast function and long-day light-dependent redox control. *Plant Cell* **22**: 403–416.

Calo L, Garcia I, Gotor C, Romero LC. 2006. Leaf hairs influence phytopathogenic fungus infection and confer an increased resistance when expressing a trichoderma alpha-1,3-glucanase. *Journal of Experimental Botany* **57**: 3911–3920.

Cobbett CS, May MJ, Howden R, Rolls B. 1998. The glutathione-deficient, cadmium-sensitive mutant, cad2-1, of *Arabidopsis thaliana* is deficient in gamma-glutamylcysteine synthetase. *Plant Journal* **16**: 73–78.

Cooper RM, Resende MLV, Flood J, Rowan MG, Beale MH, Potter U. 1996. Detection and cellular localization of elemental sulphur in disease-resistant genotypes of *Theobroma cacao*. *Nature* **379**: 159–162.

Dangl JL, Jones JD. 2001. Plant pathogens and integrated defence responses to infection. *Nature* **411**: 826–833.

Dellagi A, Brisset MN, Paulin JP, Expert D. 1998. Dual role of desferrioxamine in erwinia amylovora pathogenicity. *Molecular Plant–Microbe Interactions* **11**: 734–742.

Delledonne M, Xia Y, Dixon RA, Lamb C. 1998. Nitric oxide functions as a signal in plant disease resistance. *Nature* **394**: 585–588.

Dominguez-Solis JR, Gutierrez-Alcala G, Vega JM, Romero LC, Gotor C. 2001. The cytosolic o-acetylserine(thiol)lyase gene is regulated by heavy metals and can function in cadmium tolerance. *Journal of Biological Chemistry* **276**: 9297–9302.

Dominguez-Solis JR, Lopez-Martin MC, Ager FJ, Ynsa MD, Romero LC, Gotor C. 2004. Increased cysteine availability is essential for cadmium tolerance and accumulation in *Arabidopsis thaliana*. *Plant Biotechnology Journal* **2**: 469–476.

Droux M. 2004. Sulfur assimilation and the role of sulfur in plant metabolism: a survey. *Photosynthesis Research* **79**: 331–348.

Fodor J, Gullner G, Adam AL, Barna B, Komives T, Kiraly Z. 1997. Local and systemic responses of antioxidants to tobacco mosaic virus infection and to salicylic acid in tobacco (role in systemic acquired resistance). *Plant Physiology* **114**: 1443–1451.

Glazebrook J, Ausubel FM. 1994. Isolation of phytoalexin-deficient mutants of *Arabidopsis thaliana* and characterization of their interactions with bacterial pathogens. *Proceedings of the National Academy of Sciences, USA* **91**: 8955–8959.

Grant MR, Godiard L, Straube E, Ashfield T, Lewald J, Sattler A, Innes RW, Dangl JL. 1995. Structure of the *Arabidopsis* rpm1 gene enabling dual specificity disease resistance. *Science* **269**: 843–846.

Haas FH, Heeg C, Queiroz R, Bauer A, Wirtz M, Hell R. 2008. Mitochondrial serine acetyltransferase functions as a pacemaker of cysteine synthesis in plant cells. *Plant Physiology* **148**: 1055–1067.

Hammond-Kosack KE, Jones JD. 1997. Plant disease resistance genes. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**: 575–607.

Heath MC. 2000. Hypersensitive response-related death. *Plant Molecular Biology* **44**: 321–334.

Heeg C, Kruse C, Jost R, Gutensohn M, Ruppert T, Wirtz M, Hell R. 2008. Analysis of the *Arabidopsis* o-acetylserine(thiol)lyase gene family demonstrates compartment-specific differences in the regulation of cysteine synthesis. *Plant Cell* **20**: 168–185.

Holler K, Kiraly L, Kunstler A, Muller M, Gullner G, Fattinger M, Zechmann B. 2010. Enhanced glutathione metabolism is correlated with sulfur-induced resistance in tobacco mosaic virus-infected genetically susceptible *Nicotiana tabacum* plants. *Molecular Plant–Microbe Interactions* **23**: 1448–1459.

Holt BF 3rd, Mackey D, Dangl JL. 2000. Recognition of pathogens by plants. *Current Biology* **10**: R5–R7.

Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* **4**: 249–264.

Jones JD, Dangl JL. 2006. The plant immune system. *Nature* **444**: 323–329.

Krueger S, Donath A, Lopez-Martin MC, Hoefgen R, Gotor C, Hesse H. 2010. Impact of sulfur starvation on cysteine biosynthesis in t-DNA mutants deficient for compartment-specific serine-acetyltransferase. *Amino Acids* **39**: 1029–1042.

Krueger S, Niehl A, Lopez Martin MC, Steinhauser D, Donath A, Hildebrandt T, Romero LC, Hoefgen R, Gotor C, Hesse H. 2009. Analysis of cytosolic and plastidic serine acetyltransferase mutants and subcellular metabolite distributions suggests interplay of the cellular compartments for cysteine biosynthesis in *Arabidopsis*. *Plant, Cell & Environment* **32**: 349–367.

Lamb C, Dixon RA. 1997. The oxidative burst in plant disease resistance. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**: 251–275.

Lopez-Martin MC, Becana M, Romero LC, Gotor C. 2008a. Knocking out cytosolic cysteine synthesis compromises the antioxidant capacity of the cytosol to maintain discrete concentrations of hydrogen peroxide in *Arabidopsis*. *Plant Physiology* **147**: 562–572.

Lopez-Martin MC, Romero LC, Gotor C. 2008b. Cytosolic cysteine in redox signaling. *Plant Signaling & Behavior* **3**: 880–881.

Mateo A, Funck D, Muhlenbock P, Kular B, Mullineaux PM, Karpinski S. 2006. Controlled levels of salicylic acid are required for optimal photosynthesis and redox homeostasis. *Journal of Experimental Botany* **57**: 1795–1807.

Meuwly P, Metraux JP. 1993. Ortho-anisic acid as internal standard for the simultaneous quantitation of salicylic acid and its putative biosynthetic precursors in cucumber leaves. *Analytical Biochemistry* **214**: 500–505.

Meyer AJ, Brach T, Marty L, Kreye S, Rouhier N, Jacquot JP, Hell R. 2007. Redox-sensitive gfp in *Arabidopsis thaliana* is a quantitative biosensor for the redox potential of the cellular glutathione redox buffer. *Plant Journal* **52**: 973–986.

Meyer AJ, Hell R. 2005. Glutathione homeostasis and redox-regulation by sulfhydryl groups. *Photosynthesis Research* **86**: 435–457.

Mindrinos M, Katagiri F, Yu GL, Ausubel FM. 1994. The *A. thaliana* disease resistance gene *rps2* encodes a protein containing a nucleotide-binding site and leucine-rich repeats. *Cell* **78**: 1089–1099.

Mou Z, Fan W, Dong X. 2003. Inducers of plant systemic acquired resistance regulate npr1 function through redox changes. *Cell* **113**: 935–944.

Mullineaux PM, Rausch T. 2005. Glutathione, photosynthesis and the redox regulation of stress-responsive gene expression. *Photosynthesis Research* **86**: 459–474.

Panstruga R, Parker JE, Schulze-Lefert P. 2009. Snapshot: plant immune response pathways. *Cell* **136**: 978.e971–978.e973.

Parisy V, Poinssot B, Owsianowski L, Buchala A, Glazebrook J, Mauch F. 2007. Identification of pad2 as a gamma-glutamylcysteine synthetase highlights the importance of glutathione in disease resistance of *Arabidopsis*. *Plant Journal* **49**: 159–172.

Rausch T, Wachter A. 2005. Sulfur metabolism: a versatile platform for launching defence operations. *Trends in Plant Science* **10**: 503–509.

Reiner A, Yekutieli D, Benjamini Y. 2003. Identifying differentially expressed genes using false discovery rate controlling procedures. *Bioinformatics* **19**: 368–375.

Roetschi A, Si-Ammour A, Belbahri L, Mauch F, Mauch-Mani B. 2001. Characterization of an *Arabidopsis–Phytophthora* pathosystem: resistance requires a functional pad2 gene and is independent of salicylic acid, ethylene and jasmonic acid signalling. *Plant Journal* **28**: 293–305.

Schlaeppi K, Bodenhausen N, Buchala A, Mauch F, Reymond P. 2008. The glutathione-deficient mutant pad2-1 accumulates lower amounts of glucosinolates and is more susceptible to the insect herbivore *Spodoptera littoralis*. *Plant Journal* **55**: 774–786.

Staskawicz BJ, Ausubel FM, Baker BJ, Ellis JG, Jones JD. 1995. Molecular genetics of plant disease resistance. *Science* **268**: 661–667.

Swanson J, Kearney B, Dahlbeck D, Staskawicz BJ. 1988. Cloned avirulence gene of *Xanthomonas campestris pv. vesicatoria* complements spontaneous race change mutant. *Molecular Plant–Microbe Interactions* **1**: 5–9.
Tada Y, Spoel SH, Pajerowska-Mukhtar K, Mou Z, Song J, Wang C, Zuo J, Dong X. 2008. Plant immunity requires conformational changes [corrected] of npr1 via s-nitrosylation and thioredoxins. *Science* **321**: 952–956.
Tao Y, Xie Z, Chen W, Glazebrook J, Chang HS, Han B, Zhu T, Zou G, Katagiri F. 2003. Quantitative nature of *Arabidopsis* responses during compatible and incompatible interactions with the bacterial pathogen *Pseudomonas syringae*. *Plant Cell* **15**: 317–330.
Tornero P, Dangl JL. 2001. A high-throughput method for quantifying growth of phytopathogenic bacteria in *Arabidopsis thaliana*. *Plant Journal* **28**: 475–481.
Toufighi K, Brady SM, Austin R, Ly E, Provart NJ. 2005. The botany array resource: e-northerns, expression angling, and promoter analyses. *Plant Journal* **43**: 153–163.
Tudzynski B, Liu S, Kelly JM. 2000. Carbon catabolite repression in plant pathogenic fungi: isolation and characterization of the *Gibberella fujikuroi* and *Botrytis cinerea* crea genes. *FEMS Microbiology Letters* **184**: 9–15.
Vanacker H, Lu H, Rate DN, Greenberg JT. 2001. A role for salicylic acid and npr1 in regulating cell growth in *Arabidopsis*. *Plant Journal* **28**: 209–216.
Verberne MC, Brouwer N, Delbianco F, Linthorst HJ, Bol JF, Verpoorte R. 2002. Method for the extraction of the volatile compound salicylic acid from tobacco leaf material. *Phytochemical Analysis* **13**: 45–50.
Vlot AC, Klessig DF, Park SW. 2008. Systemic acquired resistance: the elusive signal(s). *Current Opinion in Plant Biology* **11**: 436–442.
Wang D, Amornsiripanitch N, Dong X. 2006. A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. *PLoS Pathogens* **2**: e123.
Watanabe M, Kusano M, Oikawa A, Fukushima A, Noji M, Saito K. 2008a. Physiological roles of the beta-substituted alanine synthase gene family in *Arabidopsis*. *Plant Physiology* **146**: 310–320.
Watanabe M, Mochida K, Kato T, Tabata S, Yoshimoto N, Noji M, Saito K. 2008b. Comparative genomics and reverse genetics analysis reveal indispensable functions of the serine acetyltransferase gene family in *Arabidopsis*. *Plant Cell* **20**: 2484–2496.
Wettenhall JM, Simpson KM, Satterley K, Smyth GK. 2006. Affylmgui: a graphical user interface for linear modeling of single channel microarray data. *Bioinformatics* **22**: 897–899.
Whalen MC, Innes RW, Bent AF, Staskawicz BJ. 1991. Identification of *Pseudomonas syringae* pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. *Plant Cell* **3**: 49–59.
Williams JS, Hall SA, Hawkesford MJ, Beale MH, Cooper RM. 2002. Elemental sulfur and thiol accumulation in tomato and defense against a fungal vascular pathogen. *Plant Physiology* **128**: 150–159.
Wirtz M, Droux M. 2005. Synthesis of the sulfur amino acids: cysteine and methionine. *Photosynthesis Research* **86**: 345–362.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Detection of salicylic acid in a standard solution by liquid chromatography–mass spectrometry (LC-MS) in negative ionization mode.

Fig. S2 Functional classification of *des1-1* upregulated and downregulated genes performed by the Classification SuperView tool, represented as the normed frequency.

Fig. S3 Graphic display of hierarchical cluster analysis of *des1-1* upregulated or downregulated genes in response to *Pseudomonas* half-leaf injection.

Fig. S4 Graphic display of hierarchical cluster analysis of *des1-1* upregulated or downregulated genes in response to *Botrytis cinerea*.

Fig. S5 Graphic display of hierarchical cluster analysis of *oas-a1.1* upregulated or downregulated genes in response to *Pseudomonas* half-leaf injection.

Fig. S6 Graphic display of hierarchical cluster analysis of *oas-a1.1* upregulated or downregulated genes in response to *Botrytis cinerea*.

Table S1 Oligonucleotides used in this work

Table S2 Degree of cysteine and glutathione oxidation in Col-0, No-0, *oas-a1.1*, *oas-a1.2*, *des1-1* and *des1-2* plants under control conditions

Table S3 Conductivity measurements of Col-0, No-0, *oas-a1.1*, *oas-a1.2*, *des1-1* and *des1-2* 6- to 7-wk-old plants under normal growth conditions

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Supporting Information

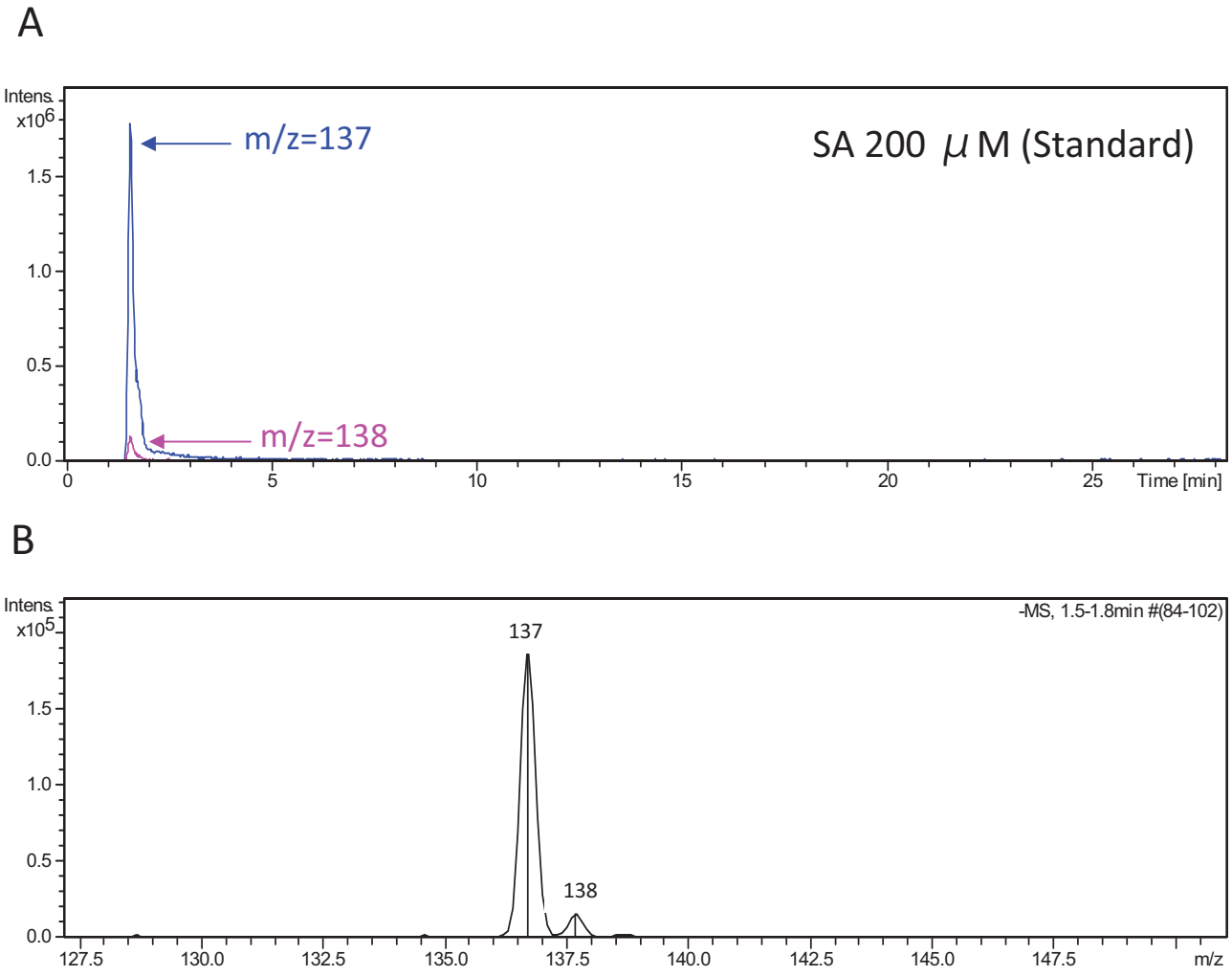

Fig. S1. Detection of Salycilic Acid in a standard solution by LC-MS in negative ionization mode. A) HPLC Chromatogram. The peaks of both the deprotonated molecule [M − H]− (*m/z* 137) and the isotopic form *m/z* 138 are shown. B) Electrospray Ionization Mass Spectra.

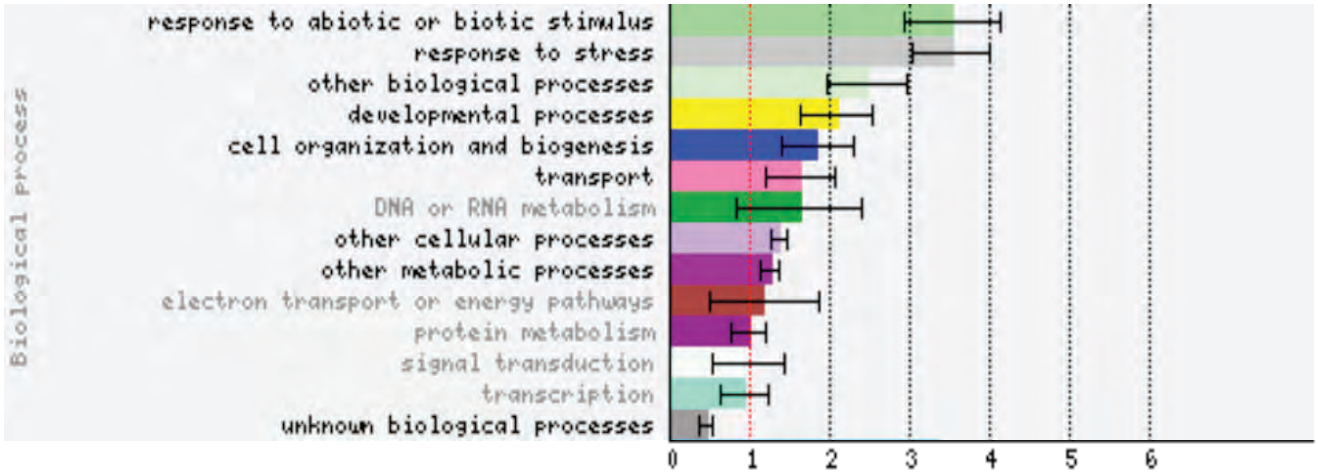

Fig. S2. Functional classification of *des1-1* up- and down-regulated genes performed by the Classification SuperView tool, represented as the normed frequency (Bio-Array Resource for Arabidopsis Functional Genomics, Toufighi *et al*., 2005; Provart and Zhu, 2003; http://bar.utoronto.ca).

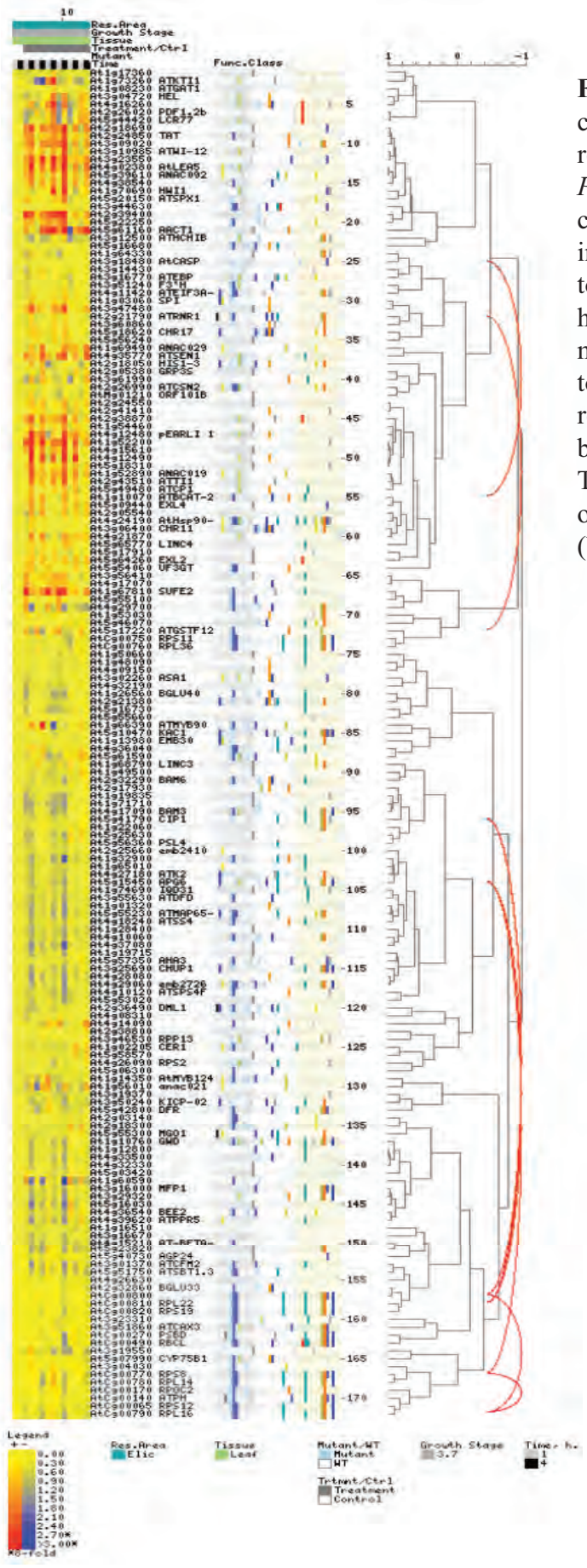

Fig. S3. Graphic display of hierarchical cluster analysis of *des1-1* up- or down-regulated genes in response to *Pseudomonas* half leaf injection. Each column represents the treatment indicated at its top, and each row refers to a gene. A dendrogram representing hierarchical relationships between mutants is shown and the scale to the top marks the correlation coefficient represented by the length of the branches that connect pairs of nodes. The color scale indicates the log2 level of expression above (red) or below (blue) the median.

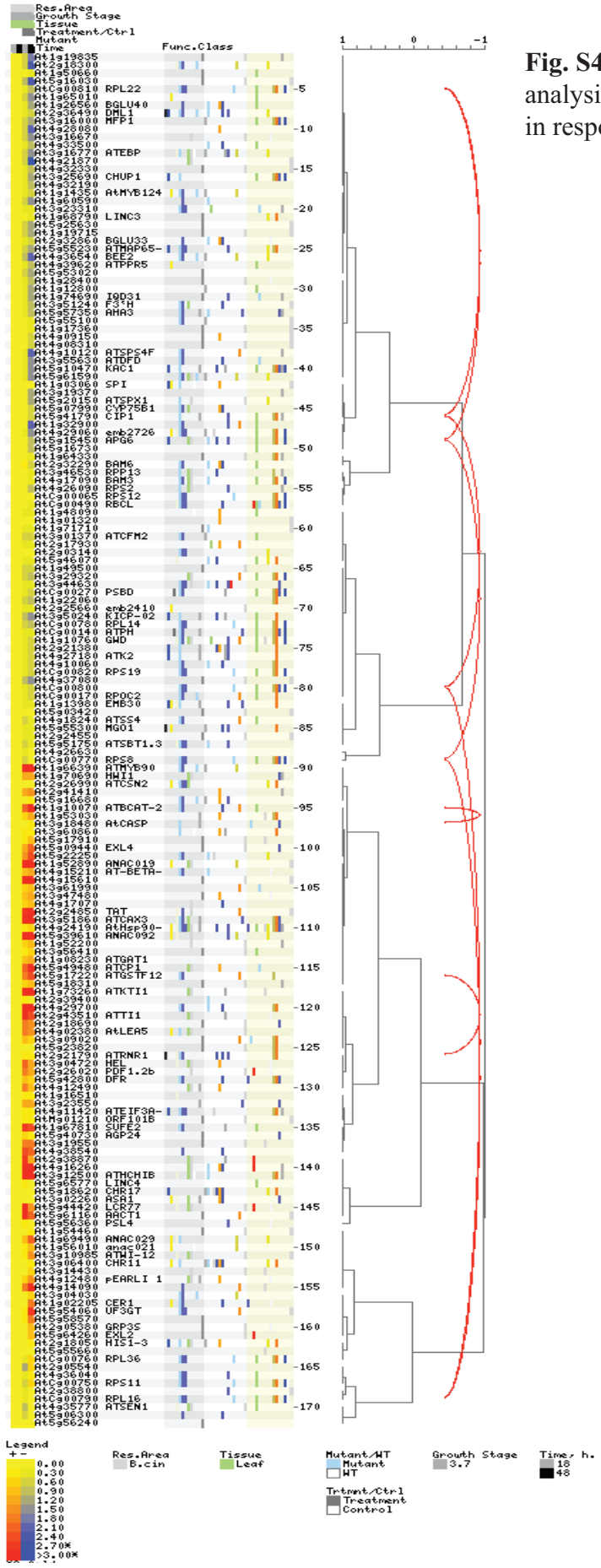

Fig. S4. Graphic display of hierarchical cluster analysis of *des1-1* up- or down-regulated genes in response to *Botrytis cinerea*. See legend S3.

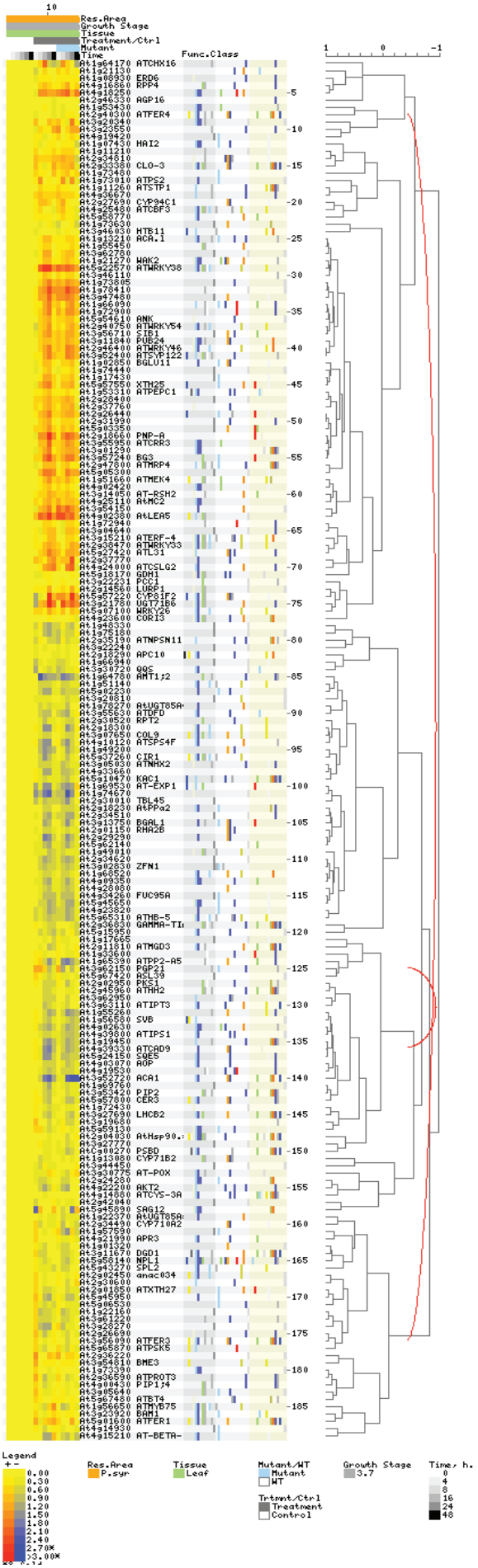

Fig. S5. Graphic display of hierarchical cluster analysis of *oas-a1.1* up- or down-regulated genes in response to *Pseudomonas* half leaf injection. See legend S3.

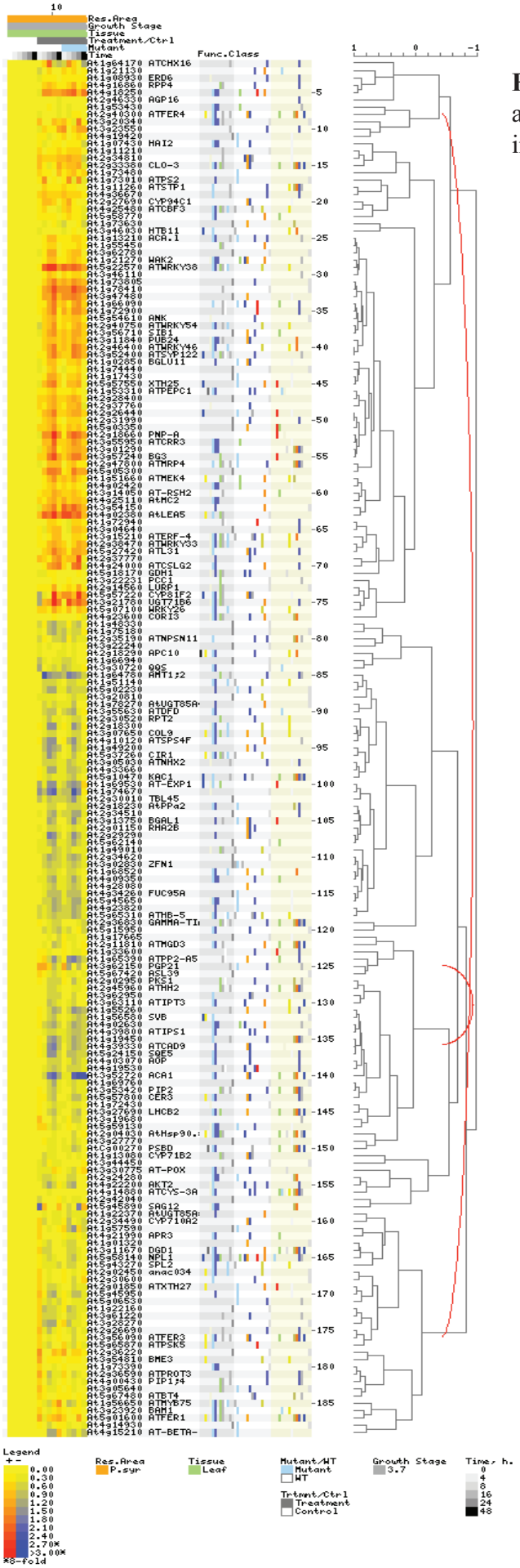

Fig. S6. Graphic display of hierarchical cluster analysis of *oas-a1.1* up- or down-regulated genes in response to *Botrytis cinerea*. See legend S3.

Table S1. Oligonucleotides used in this work. Efficiency of each pair of primers is also indicated.

Gene	**Primers efficiency (%)**	**Oligo name**	**Sequence (5´-3´)**
creA (*B. cinerea*)	92,7	creABOT -F	TCCTGCTCTTGCACCCATGGA
		creABOT -R	CCGGAGCTTGAGGAACTGGT
UBQ10 (At4g05320)	95,2	UBQ10-F	GGCCTTGTATAATCCCTGATGAATAAG
		UBQ10-R	AAAGAGATAACAGGAACGGAAACATAGT
DES1 (At5g28030)	97,9	DES1-F	TCGAGTCAAGTCAGATATGAAGCT
		DES1-R	TGTAACCTTGGTACCAACATCTCT
OASA1 (At4g14880)	100,2	OASA1-F	CACGAGCGATTTTCTCCATT
		OASA1-R	CAATTCTCGAGGCCATGATT
WRKY54 (At2g40750)	91,2	WRKY54-F	GGCAAGAGACGATGATGTTTG
		WRKY54-R	GCACTTGTTCTTTCATAATCAGC
RPM1 (At3g07040)	99,6	RPM1-F	CCTGCGATTAAGCATTATTTCA
		RPM1-R	TTATGTGAATAT CCATCCCTGA
PR1 (At2g14610)	102,0	PR1-F	TCAGTGAGACTCGGATGTG
		PR1-R	CCTGCATATGATGCTCCTT

Table S2. Oxidation degree of cysteine and glutahione in Col-0, No-0, *oas-a1.1*, *oas-a1.2*, *des1-1* and *des1-2* plants in control conditions.

	GSH	Cys
Col-0	5.9 (±0.3)	20.1 (±1.8)
des1.1	4.1 (±0.1)	12.6 (±0.4)
oas-a1.1	14.7 (±0.1)	27.8 (±0.8)
oas-a1.2	16.6 (±0.1)	30.3 (±1.4)
No-0	6.9 (±0.1)	13.4 (±0.3)
des1.2	4.2 (±0.1)	9.1 (±0.4)

Table S3. Conductivity measurements of Col-0, No-0, *oas-a1.1*, *oas-a1.2*, *des1-1* and *des1-2* 6 to 7 week-old plants under normal growth conditions. The data correspond to the mean ± standard deviation (SD) of at least three independent experiments.

	wt	*des1.1*	*oas-a1.1*	*oas-a1.2*	*cad2-1*
Conductivity ($\mu S\ cm^{-1}$)	64.3 ± 9.3	63.8 ± 4.5	75.3 ± 7.1	70.3 ± 5.4	65.3 ± 1.5

Capítulo IV

La isoforma mayoritaria citosólica O-acetilserina(tiol)liasa A1 (OAS-A1) de *A. thaliana* se inhibe por nitración

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Inhibition of *Arabidopsis O*-Acetylserine(thiol)lyase A1 by Tyrosine Nitration*

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The last step of sulfur assimilation is catalyzed by *O*-acetylserine(thiol)lyase (OASTL) enzymes. OASTLs are encoded by a multigene family in the model plant *Arabidopsis thaliana*. Cytosolic OASA1 enzyme is the main source of OASTL activity and thus crucial for cysteine homeostasis. We found that nitrating conditions after exposure to peroxynitrite strongly inhibited OASTL activity. Among OASTLs, OASA1 was markedly sensitive to nitration as demonstrated by the comparative analysis of OASTL activity in nitrated crude protein extracts from wild type and different *oastl* mutants. Furthermore, nitration assays on purified recombinant OASA1 protein led to 90% reduction of the activity due to inhibition of the enzyme, as no degradation of the protein occurred under these conditions. The reduced activity was due to nitration of the protein because selective scavenging of peroxynitrite with epicatechin impaired OASA1 nitration and the concomitant inhibition of OASTL activity. Inhibition of OASA1 activity upon nitration correlated with the identification of a modified OASA1 protein containing 3-nitroTyr^{302} residue. The essential role of the Tyr^{302} residue for the catalytic activity was further demonstrated by the loss of OASTL activity of a Y302A-mutated version of OASA1. Inhibition caused by Tyr^{302} nitration on OASA1 activity seems to be due to a drastically reduced *O*-acetylserine substrate binding to the nitrated protein, and also to reduced stabilization of the pyridoxal-5′-phosphate cofactor through hydrogen bonds. This is the first report identifying a Tyr nitration site of a plant protein with functional effect and the first post-translational modification identified in OASA1 enzyme.

Sulfur is an essential nutrient for all living organisms as it is a component of the amino acids cysteine and methionine required for protein synthesis. Moreover, a major determinant in plant cellular redox control such as glutathione (GSH) also contained sulfur. Most plant sulfur-containing compounds, including GSH, are derived from Cys, which is the final product of the primary sulfate assimilation pathway. The Cys biosynthetic pathway involves two sequential reactions catalyzed by Ser acetyltransferase (SAT),[4] which synthesizes the intermediary product, *O*-acetyl-Ser (OAS), from acetyl-CoA and Ser, and *O*-acetyl-Ser(thiol)lyase (OASTL), which incorporates sulfide, coming from the assimilatory reduction of sulfate, to OAS producing Cys. This reaction requires pyridoxal phosphate (PLP) as cofactor. There are nine genes coding for different isoforms of OASTL in the *Arabidopsis* genome (1). The most abundant OASTL transcripts correspond to the cytosolic OASA1, the plastidial OASB, and the mitochondrial OASC isoforms. Analysis of null alleles of different SAT and OASTL genes together with subcellular metabolite distributions in *A. thaliana* have recently shown that cysteine is predominantly formed in the cytosol, while OAS is produced in the mitochondria (2–6). The major cytosolic OASTL isoform and main responsible for cysteine biosynthesis, OASA1, is essential for heavy metal tolerance as its overexpression is sufficient to confer tolerance to elevated cadmium concentrations (7–9). By contrast, the mutant *oasa1.1* shows sensitivity to heavy metals but it is due to a constitutively reduced capacity to eliminate reactive oxygen species (ROS) under non-stressed conditions (9).

The uptake and assimilation of sulfate is strongly regulated by diverse regulatory mechanisms (for a recent review, see Ref. 10). Some components of the pathway are specifically regulated at the transcriptional level in plants, mainly the sulfate uptake and the reduction of 5′-adenylylsulfate (APS). It has been characterized that OASA1 is regulated at the transcriptional level in different abiotic stresses such as salinity and the presence of heavy metal (7, 11). Besides, SAT and OASTL form the hetero-oligomeric cysteine synthase complex in such a way that SAT requires binding to OASTL for full activity, while bound OASTL becomes inactivated (12). It has been proposed this complex acting as a sensor of the sulfur status of the plant. Moreover, the activity of the cysteine synthase complex is also regulated at the level of the rate-limiting step catalyzed by SAT through cysteine-mediated inhibition of this enzyme, although depending on subcellular local-

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[4] The abbreviations used are: SAT, serine acetyltransferase; APS, 5′-adenylsulfate; APR, adenosine 5′-phosphosulfate reductase; CE, crude extract; IP, immunoprecipitate; LC-MS/MS, liquid chromatography tandem mass spectrometry; MS, Murashige and Skoog medium; NO, nitric oxide; OAS, *O*-acetyl-Ser; OASTL, *O*-acetylserine(thiol)lyase; PLP, pyridoxal-5′-phosphate; PMSF, phenylmethanesulfonylfluoride; ROS, reactive oxygen species; SIN-1, 3-morpholinosydnonimine hydrochloride; Sup, supernatant; TCA, trichloroacetic acid.

ization and plant species (13). Because cysteine biosynthesis requires the reduced sulfur in form of sulfide, which is exclusively produced through sulfate assimilatory pathway in plastids (14), the mitochondria provide the bulk of OAS (2), and the main site for Cys production is cytosol in *Arabidopsis*, an exchange of sulfide and OAS between subcellular compartments must be important in controlling the function of the enzyme components of the cysteine synthase complex.

Post-translational modification represents an increasingly interesting level of regulation of protein function in all living organisms. Among more than hundred different post-translational modifications characterized, those mediated by the action of nitric oxide (NO)-derived modifiers have attracted lately the attention of plant biology researchers. The most important post-translational modifications related to NO action are *S*-nitrosylation of Cys (15) and nitration of Tyr residues (16). Nitration of Tyr residues under physiological conditions is mostly the result of protein interaction with the strong nitrating agent peroxynitrite, which is formed by the reaction of NO with superoxide anion (17). There is a second alternative mechanism of tyrosine nitration based on the generation of $NO_2^{\bullet}$ radicals by various hemoperoxydases in the presence of hydrogen peroxide and nitrite (18). Whatsoever, the nitration of tyrosine residues is a selective process with respect to the proteins that are nitrated and the affected tyrosine residues in a given protein (19, 20).

Despite the well documented regulation of OASTL and SAT function at the protein-protein interaction level, to our knowledge nothing has been reported about post-translational regulation of those proteins. Here we address whether post-translational modification of OASA1 may modulate its activity. Peroxynitrite-mediated nitration of crude extracts and purified recombinant protein as well as plant treatments with peroxynitrite inhibited OASTL activity through nitration of tyrosine residues. Mass spectrometry analysis of nitrated recombinant OASA1 protein allowed the identification of nitrated Tyr^{302}, a catalytically essential amino acid residue that is close to the previously reported key Asn^{77} in *O*-acetylserine binding site of OASA1 (21).

EXPERIMENTAL PROCEDURES

Plant Growth Conditions and Treatments—A. thaliana seeds of Col-0 wild type or different *oastl* mutants (9) were sown in moistened soil and grown under photoperiodic conditions (cycles of 16 h day and 8 h night for long days, at 22 °C and 20 °C). Plants were illuminated with 150 $\mu E\ m^{-2}\ s^{-1}$ cool-white fluorescent lamps and grown in 60% relative humidity. Alternatively, surface sterilized seeds were germinated and grown in agar-supplemented Murashige and Skoog (MS) medium (Duchefa, Haarlem, The Netherlands).

Expression, Extraction, and Purification of Recombinant His-tagged OASA1—The complete cDNA of OASA1 was cloned in pDEST17 vector (Invitrogen) to express a hexahistidine-tagged version of OASA1 by transformation of BL21-AI *Escherichia coli* competent cells (Invitrogen). Site-directed mutagenesis to generate Y203A and Y302A versions of OASA1 was performed as previously reported (22) with slight modifications. For protein induction, cell cultures with an OD of 0.4 were treated with 0.1% L-arabinose overnight at 22°. Recombinant protein production was checked by SDS-PAGE and Western blot analysis. Recombinant protein purification was carried out with the Ni-NTA purification system (Invitrogen) following manufacturers recommendations. His-tagged proteins were detected by Western blot with a polyclonal anti-His_6 antibody (Santa Cruz Biotechnology).

Protein Extraction, Immunoprecipitation, and Nitrating Treatments—For activity assays, around 100 mg of Col-0 and *oastl* mutant leaves were frozen and ground in liquid nitrogen and then extracted by adding extraction buffer (50 mM phosphate buffer, pH 7.5, 1 mM EDTA, 10 μM PLP, 0.5 mM PMSF, 1% (v/v) protease inhibitor mixture from Sigma) with or without 1 mM DTT, as described in each case, and briefly vortexing. Protein extracts were obtained by 13,000 $\times$ *g* centrifugation at 4 °C. For immunoprecipitation purposes, proteins were extracted in (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (v/v) protease inhibitor mixture from Sigma) buffer. Protein extracts (1 mg) were precleared with 50 μl of protein A-agarose (EZView Sigma) for 8 h at 4 °C. The unbound fractions were incubated overnight with 0.1 μg of monoclonal anti-3-NY antibody (Cayman) at 4 °C. To recover 3-NY-containing proteins, 60 μl of protein A-agarose were added and incubated for 8 h at 4 °C. After extensive washing with extraction buffer, proteins were eluted at 95 °C with elution buffer (1% SDS, 100 mM DTT, 50 mM Tris-HCl, pH 7.6) three times. Protein was quantified by Bradford's method (23).

Nitrating treatments on crude extracts or purified recombinant protein was performed by treatment with peroxynitrite generated from sodium nitrite plus hydrogen peroxide, or 3-morpholinosydnonimine hydrochloride (SIN-1; Invitrogen) at the indicated concentrations, as previously reported (24, 25). *In planta* peroxynitrite treatments were performed by infiltrating leaves with SIN-1. Selective scavenging of peroxynitrite with epicatechin (26) was used to assess the specificity of protein nitration and not oxidation and the subsequent inhibition of OASTL activity.

Measurement of OASTL Activity—OASTL activity was measured using the method described previously (27) in protein extracts obtained as described above. Cysteine was determined by measuring optical density at 560 nm after the formation of a complex with nynhydrin (28).

Western Blots—Protein extracts were separated by 10% SDS-PAGE, blotted onto nitrocellulose membrane, stained with Ponceau-S and probed with antibodies at the followed dilutions: monoclonal anti-3-NY (Cayman Chemicals) 1:1000, anti-His (Santa Cruz Biotechnology) 1:500, and custom-made polyclonal anti-recombinant OAS-A1 antibody (Biomedal S.L.) 1:10000. Secondary antibody was anti-mouse or anti-rabbit, for monoclonal or polyclonal primary antibodies, respectively, coupled to horseradish peroxidase (GE) at 1:10000 dilution, and ECL or ECL advance kit (GE) was used for immunoreactive protein detection.

MS Analysis—Samples were digested with sequencing grade trypsin, chymotrypsin, or the glutamic acid specific V8 protease (Promega) to improve peptide sequence coverage. Peptide separation by LC-MS/MS was performed using an Ultimate nano-LC system (LC Packings) and a QSTAR XL

Q-TOF hybrid mass spectrometer (MDS Sciex-Applied Biosystems). Samples (5 μl) were delivered to the system using a FAMOS autosampler (LC Packings) at 40 μl min^{-1}, and the peptides were trapped onto a PepMap C18 pre-column (5 mm, 300 m i.d; LC Packings). Peptides were then eluted onto the PepMap C18 analytical column (15 cm, 75 m i.d; LC Packings) at 200 nl min-1 and separated using a 55 min gradient of 15–50% ACN (120 min for the mixtures). The QSTAR XL was operated in information-dependent acquisition mode, in which a 1-s TOF MS scan from 400–2000 *m*/*z*, was performed, followed by 3-s product ion scans from 65–2000 *m*/*z* on the three most intense doubly or triply charged ions. Database search on Swiss-Prot and NCBInr databases was performed using MASCOT search engine (Matrix- Science). Searches were done with the different proteases specificity allowing one missed cleavage and a tolerance on the mass measurement of 100 ppm in MS mode and 0.8 Da for MS/MS ions. Carbamidomethylation of Cys was used as a fixed modification and oxidation of Met, deamidation of Asn and Gln, and nitration of Tyr as variable modifications.

Structural Analysis of OASA1—Three-dimensional structure of OASA1 (At4g14880) was obtained from the Protein Data Bank (PDB code access 1Z7W). The structure was visualized with Yasara or PyMol software. The distance between residues in Angstroms (Å) and the presence of hydrogen bonds were carried out with both programs with default settings.

RESULTS

Inhibition of OASTL Activity under Nitrating Conditions—Nitration of many different cellular acceptors including tyrosine residues of proteins is the result of either peroxynitrite action or $NO_2^{\bullet}$ radicals attack on the corresponding targets. Peroxynitrite can be produced *in situ* by different donors including 3-morpholinosydnonimine (SIN-1). Similarly, $NO_2^{\bullet}$ radicals can be produced *in vitro* by treatment with nitrite and hydrogen peroxide. We have tested whether nitrating treatments altered the OASTL activity of crude protein extracts from *Arabidopsis*. Fig. 1*A* shows that nitration with 1 mM nitrite/peroxide treatment led to a reduction of 81% in OASTL activity levels in wild-type extracts without DTT, and to around 37% in the presence of DTT, commonly used in the extraction buffer for OASTL activity (7–9). OASTL activity associated to OASA1 represented around 65–70% of total activity present in wild-type *Arabidopsis* crude extracts, as *oasa1.1* extracts had 30–35% of the activity present in wild-type plants (Fig. 1*A*) (9). Similar treatments on *oasa1.1* extracts led to a reduction of OASTL activity of 38 and 53% with and without DTT, respectively (Fig. 1*A*). The reduction of OASTL activity upon nitration must be due to inactivation of the enzyme as no changes in the OASA1 protein levels could be detected in any of the described treatments (Fig. 1*A*). Moreover, crude extracts from wild type and mutants of the most abundant OASTLs were also nitrated with 0.5 mM SIN-1 in the absence of DTT. Nitrated wild type, *oasb*, and *oasc* mutant extracts retained 22, 19, and 40%, respectively, of the OASTL activity present in non-nitrated controls (Fig. 1*B*). However, when nitrated the two allele mutants *oasa1.1* and

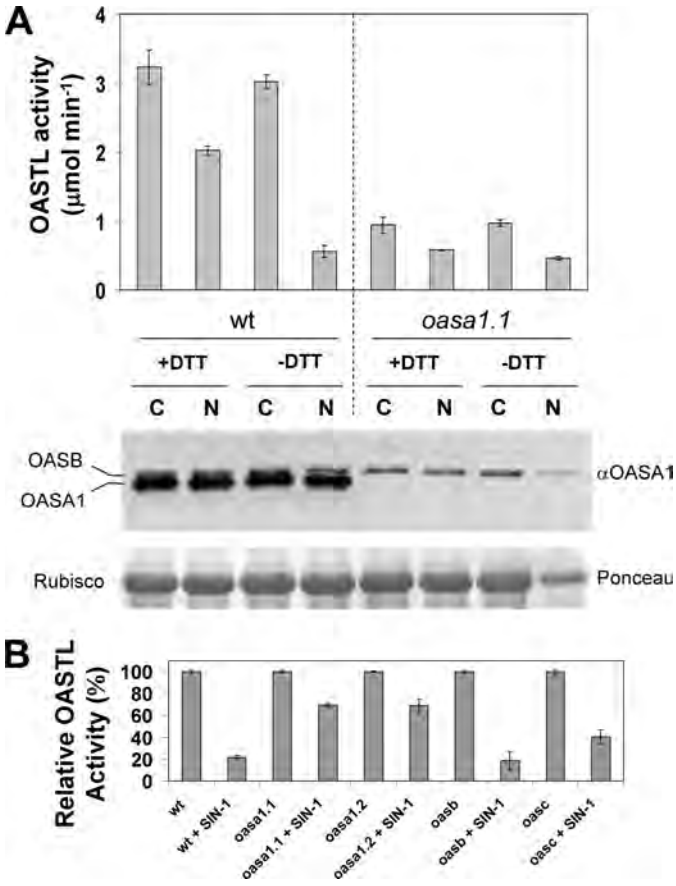

FIGURE 1. **Effect of nitration on OASTL activity and OASA1 protein levels of wild type and mutant extracts.** Total OASTL activity and OASA1 protein levels were analyzed in wild type and *oasa1.1* extracts obtained either in the presence (+*DTT*) or absence of (−*DTT*), and either non-nitrated as control (*C*) or nitrated (*N*) by treatment with 1 mM nitrite/hydrogen peroxide mixture for 1 h. Anti-OASA1 antibody crossreacted preferentially with OASA1 but showed also a weaker crossreaction with OASB isoform. Ponceau S staining of Rubisco is shown as loading control. OASTL activities are the mean values of three replicates ± S.D. (*A*). OASTL activity in 0.5 mM SIN-1 treated extracts from wild type, *oasa1.1*, *oas1.2*, *oasb*, and *oasc* mutants was measured after 1 h. After SIN-1 removal, activity was measured along with non-treated samples as controls. Values are the mean ± S.D. of three replicates and are expressed relative to the corresponding non-nitrated control for every genotype (*B*).

oasa1.2, extracts retained 69% of the activity of its corresponding control (Fig. 1*B*). As shown with nitrite/peroxide-treated samples, SIN-1 treatment did not produce OASA1 protein degradation as tested by Western blot (data not shown). These data suggest that OASA1 is more sensitive to nitration-mediated inhibition than other OASTLs. Based on wild type and *oasa1* allele activities upon nitration, SIN-1, and nitrite/peroxide treatments inhibited 37 and 47% of the OASTL activity catalyzed by non OASA1 proteins (Table 1). In turn, OASA1 activity was 88 and 95% inhibited by both nitrating treatments (Table 1). Differential effect of nitration in inhibiting activity of OASA1 and other OASTLs moved us to analyze the effects of nitrating treatment on purified recombinant OASA1.

Samples of purified recombinant OASA1 obtained from *E. coli* expressing an N-terminal hexahistidine-tagged version were tested for OASTL activity after treatment with increasing concentrations of SIN-1. At 250 μM SIN-1 significant in-

TABLE 1

Sensitivity of OASA1 and other OASTL enzymes to nitration-mediated inhibition

OASTL activity associated to OASA1 was estimated by the difference between the total activity measured in wild-type seedlings and that measured in *oasa1.1* mutant. Nitration (*N*) was performed by treatments for 1 h with nitrite/peroxide or SIN-1, and no DTT as indicated. Untreated samples were used as controls (C). Values (nmol min^{-1}) are the mean of three replicates ± standard deviation. Relative percentages of inhibition were calculated for OASA1 and the rest of OASTLs after nitration by both treatments.

	wt C	*oasa1.1* C	OASA1 C	Other OASTLs C	wt N	*oasa1.1* N	OASA1 N	Other OASTLs N	Inhibition OASA1	Inhibition other OASTLs
									%	%
Nitrite/H_2O_2	3020 ± 99	970 ± 49	2050	970	559 ± 85	458 ± 22	101	458	95	47
SIN-1	603 ± 3	231 ± 3	372	231	189 ± 25	145 ± 30	44	145	88	37

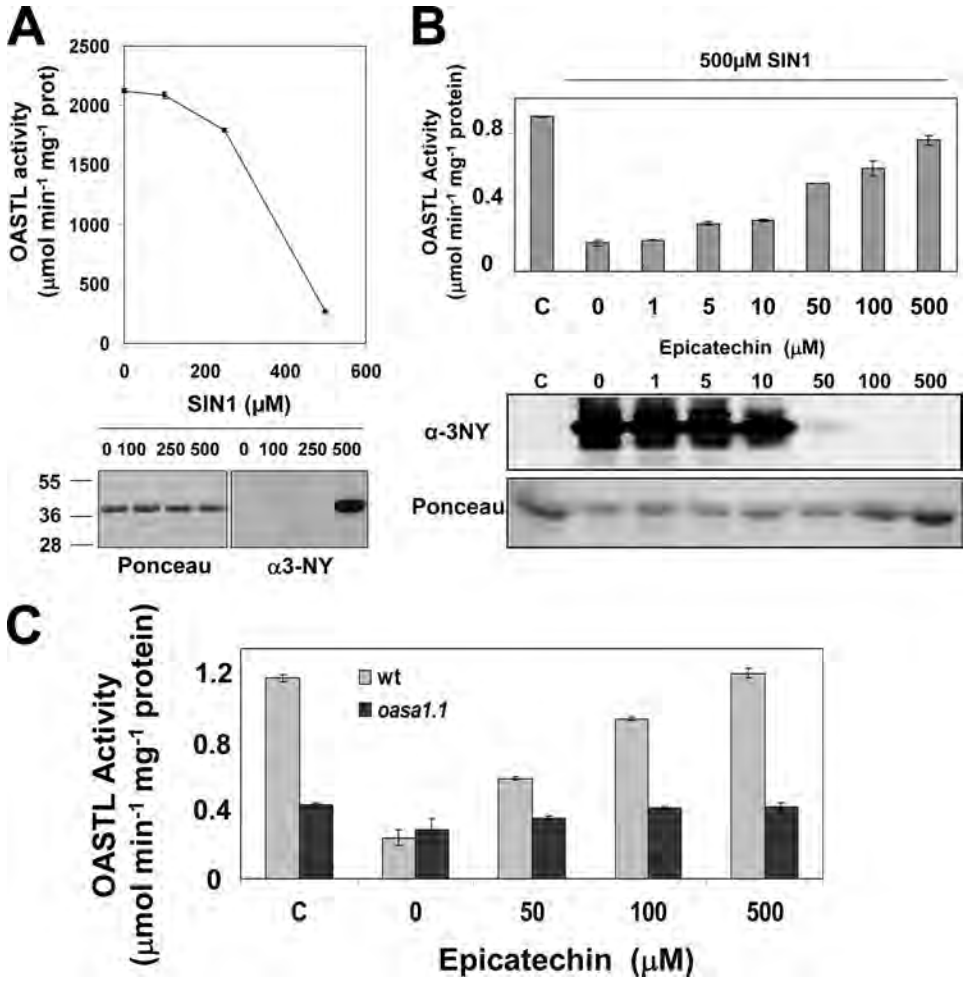

FIGURE 2. **OASTL activity and nitrated OASA1 protein levels in purified OASA1 protein treated with increasing concentrations of the nitrating reagent SIN-1.** Samples of 12 μg of His-tagged purified recombinant OASA1 protein were treated with the indicated concentration of SIN-1 for 1 h. After SIN-1 removal, OASTL activity, and total and nitrated protein levels were quantified (*A*). Activity and protein levels in samples treated with 0.5 mM SIN-1 or untreated as a control (*c*) and epicatechin at the indicated concentrations (*B*). Western blot with anti-3nitroY antibody (α-3NY) and Ponceau staining as loading control are shown in *panels A* and *B*. Scavenging effect of epicatechin on the OASTL activity of SIN-1 (0.5 mM)-nitrated crude extracts from wild type (*clear gray bars*) and *oasa1.1* (*dark gray bars*) leaves (*C*). OASTL activities are the mean values of three replicates ± S.D.

hibition was already detected and 87% inhibition occurred at 500 μM (Fig. 2*A*). This percentage of inhibition is fully consistent with the estimation of inhibition of the OASTL activity corresponding to OASA1 detected in nitrated crude extracts (Table 1). Reduction of OASTL activity under these nitrating conditions was not due to degradation of protein as demonstrated by unaltered levels of proteins present in samples treated with increasing SIN-1 concentrations (Fig. 2*B*). Correlating with the strong inhibition of OASTL activity of OASA1 the corresponding Tyr-nitrated form of OASA1 was detected by Western blot using an anti-3-nitroY antibody (Fig. 2*A*). We checked that inhibition of OASA1 activity was specifically associated to nitration and not to oxidation by using the peroxynitrite scavenger epicatechin (26). Increasing concentrations of epicatechin blocked SIN-1 mediated OASA1 nitration and the concomitant inhibition of OASTL activity (Fig. 2*B*). Moreover, the protective effect of epicatechin specifically on OASA1 was supported by the negligible effect caused by epicatechin on crude extracts from the *oasa1.1* mutant (Fig. 2*C*).

Identification of Tyr-nitration Site in Nitrated OASA1—Nitrated and non-nitrated samples of purified OASA1 used for inhibition assays described above were further analyzed by

A

Peptide	Score	Expect	[MH^{2+}]	[MH]	Shift	Treatment
YLSTVLFDATR	82	7.1 10^{-6}	643.33	1286.66		Control
^{NO2}YLSTVLFDATR	76	1.5 10^{-6}	665.82	1331.64	+44.98	Nitrated

B

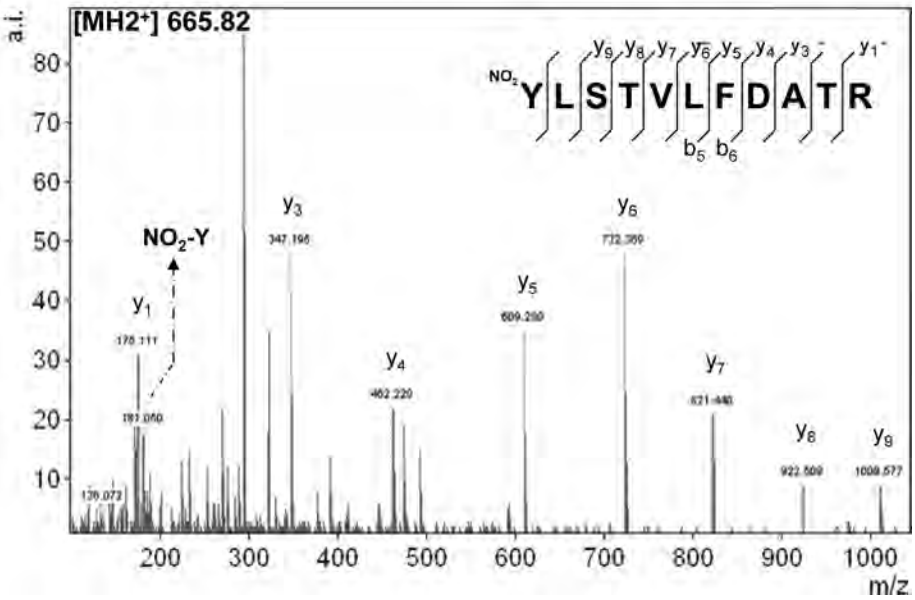

FIGURE 3. **Identification of a Tyr-nitration site in OASA1.** LC-MS/MS analysis of nitrated OASA1 and non-nitrated control allowed the identification of Y*LSTVLFDATR peptide with nitrated or non-nitrated Tyr^{302}, respectively (*A*). The 22.49 Da shift of the double charged peptide indicates nitration of Tyr^{302}. Ion score (MASCOT) of the nitrated and non-nitrated peptides are shown. The MS spectra corresponding to Y(NO_2)LSTVLFDATR ($Z = 2$; $m/z = 665.82$) is shown (*B*). NO_2-Y indicates the presence of the nitrated tyrosine immonium ion mass in the LC-MS/MS spectra. Identified y and b ion series are *pointed out*.

mass spectrometry. Samples were digested with chymotrypsin or trypsin and analyzed by LC-MS/MS. Nitrated and non-nitrated samples were confirmed to be OASA1 with MASCOT scores (sequence coverages) of 725 (79%) and 1207 (88%) for trypsin-digested samples and 248 (24%) and 163 (40%) for chymotrypsin-digested samples, respectively. By LC-MS/MS, the nitrated peptide Y(NO_2)LSTVLFDATR ($Z = 2$; $m/z = 665.828096$; MASCOT Ion Score: 76; Expect: 1.5e-006; Monoisotopic mass of neutral peptide: 1329.6565) was identified with an increase mass of 44.99 Da compatible with acquisition of a nitro group in Tyr^{302} (Fig. 3). OASA1 contains 7 Tyr residues distributed all along the amino acid sequence but most of them are located far away from the active site in the three-dimensional structure, except Tyr^{302}, which is directed to the active site (Fig. 4*A*). Tyr^{302} is located at 4.7 Å from the Asn^{77} (Fig. 4*B*), and forming a hydrogen bond with this amino acid (Fig. 4*C*), which has been previously reported to be essential for the activity (21). Asn^{77} interacts with the *O*-acetylserine and SAT binding site and through hydrogen bond stabilizes pyridoxal phosphate (PLP) cofactor anchored by Lys^{46} (21). Changes in spatial conformation of PLP and substrate microenvironment sites may also alter the efficiency of substrate binding to the active site. Upon binding of *O*-acetylserine to the active site of the enzyme, the substrate reacts with PLP yielding the α-aminoacrylate intermediate with an absorbance maximum at 470 nm (21, 29). We measured the increase in absorbance at 470 nm of purified OASA1 in the presence of increasing concentrations of *O*-acetylserine and compared with the values obtained using the same amount of nitrated protein. The non-nitrated OASA1 increases its absorbance at 470 nm with increasing OAS concentration as expected. By contrast, no increase in absorbance was detected in nitrated OASA1 (Fig. 4*D*), suggesting that either binding of *O*-acetylserine is severely hindered by nitration of Tyr^{302} or binding occurs but far enough from the PLP site to avoid productive interaction between substrate and cofactor.

We have further confirmed the essential role of Tyr^{302} for OASTL activity of OASA1 by constructing a site-directed Y302A mutant version that retained less than 3% of the activity of the unmodified recombinant OASA1 protein (Fig. 5). This effect was specific for Tyr^{302} as another mutated version Y203A, with a mutation in a Tyr residue that was not identified above as a functional target of nitration, retained 88% of the activity and responded to SIN-1-nitrating treatment with nitration of the mutated protein and consequent inhibition of the activity (Fig. 5) similarly to that observed for unmodified OASA1 (Fig. 2*A*).

In Vivo Detection of Nitrated OASA1—To test whether the nitration of Tyr detected in OASA1 *in vitro* could be physiologically relevant, we performed an immunoprecipitation assay of wild-type protein extracts with anti-3-nitroY antibody. The immunoprecipitated proteins were analyzed by Western blot with an antibody against OASA1. Fig. 6*A* shows that a small fraction of the OASA1 protein present in the crude extract was detected in the immunoprecipitated fraction, sug-

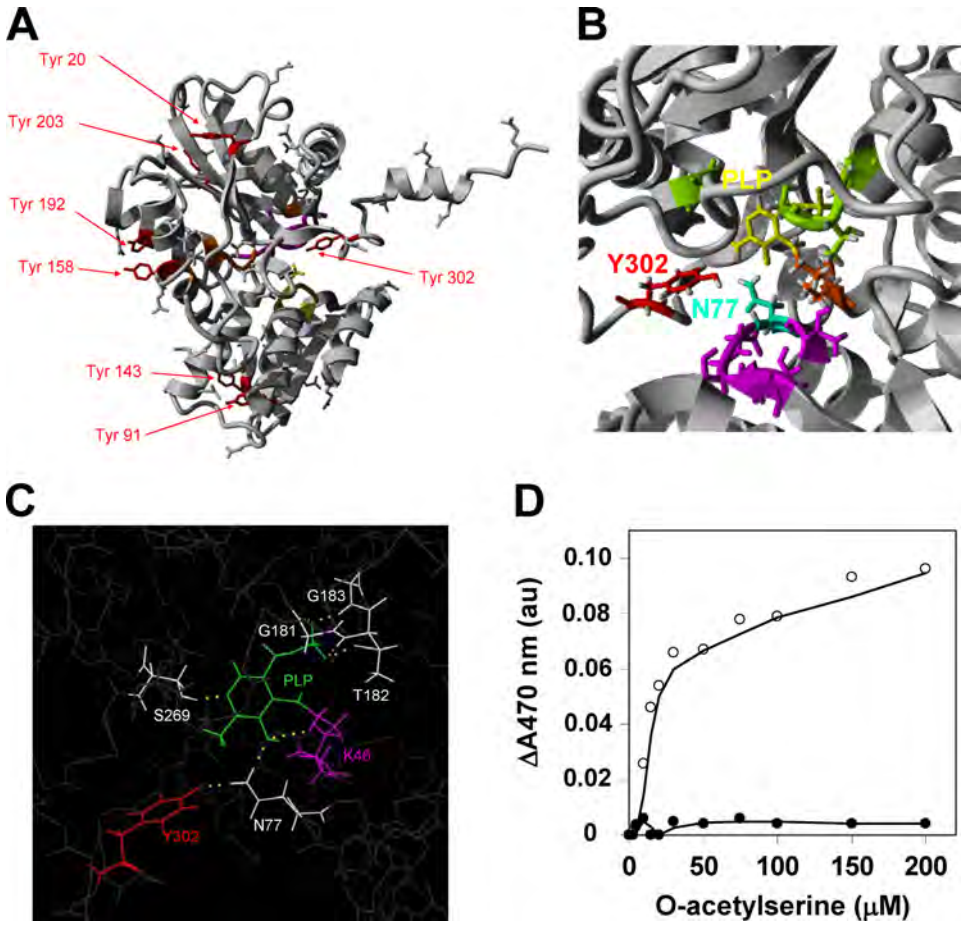

FIGURE 4. **Three-dimensional model of OASA1 showing the position and potential interactions of Tyr residues.** The conformation of OASA1 molecule showing the position of the seven Tyr residues (in *red*), the PLP binding site (in *orange*), *O*-acetylserine substrate binding site (in *yellow*) and SAT protein interaction site (in *purple*) are shown (*A*). Detail of the three-dimensional structure showing Tyr^{302} and the Asn^{77} residues at a 4.7 Å distance, the amino acid residues interacting with the Lys^{46} (in *orange*)-linked PLP (in *yellow*) through hydrogen bonds (in *green*), and the OAS and SAT binding site (in *purple*) (*B*). *In silico* analysis, using PyMol software, of the potential hydrogen bonds (yellow *dashed line*) between amino acid residues surrounding PLP (*C*). Spectrophotometric detection of the reaction of OASA1-bound *O*-acetylserine with PLP to form an α-aminoacrylate intermediate. Samples of 200 μg of non-nitrated (*open circles*) or nitrated (*close circles*) purified OASA1 were incubated with increasing concentrations of *O*-acetylserine as indicated, and the increase in the absorbance at 470 nm registered (*D*).

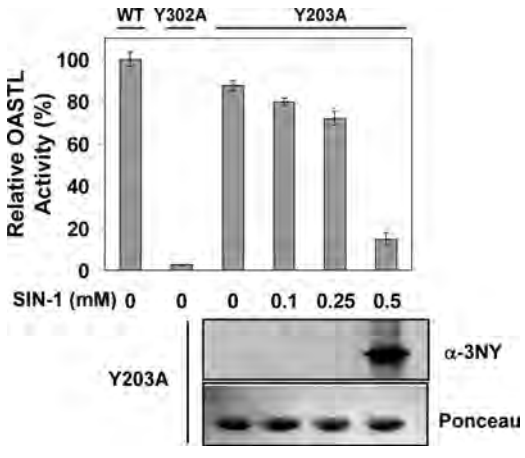

FIGURE 5. **OASTL activity of mutated recombinant versions of OASA1.** Purified samples of WT and mutated (Y302A and Y203A) versions of OASA1 were assayed for OASTL activity in the absence or presence of the indicated concentrations of SIN-1. OASTL activities are the mean values of three replicates ± standard deviation. Western blot with anti-3nitroY antibody (α-3NY) and Ponceau staining as loading control (Ponceau) are shown for nitrated Y203A samples.

gesting that OASA1 may be Tyr-nitrated under physiological conditions in the absence of an exogenous nitrating treatment. Despite efforts to identify *in vivo* Tyr-nitrated peptides we did not success, likely because of the low amounts of nitrated protein that are expected under physiological conditions. We then proceed to perform *in planta* nitration experiments by infiltrating leaves with SIN-1 and further protein extraction and OASTL activity analysis. Fig. 6*B* shows that by 90 min after treatment with 2 mM SIN-1, OASTL activity was 34% inhibited. Inhibition of activity correlated with detection of a significant fraction of nitrated OASA1 in SIN-1-treated leaf samples (Fig. 6*B*).

DISCUSSION

Cysteine biosynthesis is a crucial process because this amino acid is a constituent of proteins. Moreover, Cys is a precursor for a huge number of essential bio-molecules, such as many plant defense compounds formed in response to different environmental adverse conditions. Among them, glutathione is the most determinant molecule in controlling the redox state of the cells. The main site of cysteine biosynthesis

is the cytosol in *A. thaliana*, and therefore the major contributor to this synthesis is OASA1, but in its unbound form, when it is fully active. Association and dissociation of the cysteine synthase complex appears to be a level of regulation of cysteine biosynthesis in response to sulfur nutrition perturbations (12). The gene coding for OASA1 is also transcriptionally regulated by stress conditions (7, 11). Sulfate assimilation has been proposed to be extensively regulated by abiotic stress at the post-transcriptional level acting on plant adenosine 5′-phosphosulfate reductase (APR) through a complex network of multiple signals (30). However, besides transcriptional control of *OASTL* genes and interaction with SAT, no other level of regulation has been reported for OASTL function. A rapid way to modulate the function of OASTL may be through post-translational modification of the protein. However, to date no such modification has been identified in plants. Many stress activated responses have in common the participation of redox processes. Under oxidative, nitrosative, or nitrative conditions plant proteins can undergo several modifications including oxidation of Met, Cys, or Trp residues, nitration of Tyr, and nitrosylation of Cys (31). The main physiological nitrating molecules are the radicals $NO_2^{\bullet}$ and $ONOO^-$, which are in turn produced from nitric oxide by further oxidation (32). Although $NO_2^{\bullet}$ and $ONOO^-$ are extremely reactive, their interactions with proteins do not lead to stochastic nitration of Tyr residues of the target protein. Factors determining the selectivity of Tyr nitration in proteins include the exposure of the aromatic ring to the surface of the protein, the location of the Tyr on a loop structure, its association with a neighboring negative charge, the proximity of the proteins to the site of generation of nitrating agents, their abundance and the local environments of the Tyr residues (19, 33). Moreover, the physiological significance of a regulatory mechanism based on the differential effect of a post-translationally modified protein relies on the conservation of that residue in others proteins with the same activity. This work reports the negative effect of Tyr^{302} nitration on the OASTL activity of OASA1. This protein, which represents the main isoform in *Arabidopsis*, contains 7 Tyr residues spanning the whole primary sequence of the protein. Three-dimensional structure of OASA1 allowed the spatial location of different Tyr residues respect to the surface molecule and proximity to the catalytic site (Fig. 4). Table 2 summarizes the parameters defining the position and the microenvironment of every Tyr residue in OASA1 protein. Among seven Tyr residues of OASA1 only two, Tyr^{91} and Tyr^{302}, are located in loops, are conserved in the family of OASTL *Arabidopsis* proteins (3) and have both negatively and positively charged amino acids close enough (Table 2) to fulfill theoretical requirements for being targets of nitration (19, 33). In our approach using purified recombinant OASA1 protein nitrated *in vitro*, only Tyr^{302} was identified as 3-nitroTyr (Fig. 3). The identification of Tyr^{302} in nitrated OASA1 that lost most of its OASTL activity is consistent with the location of this residue very close to the active site of the enzyme (Fig. 4). In fact, Tyr^{302} is only 4.7 Å from the Asn^{77}, which is a key amino acid for the efficient binding of the substrate *O*-acetylserine to OASA1 (21). Moreover, Asn^{77} and other closely located amino acids including Ser^{269}, Gly^{181}, Gly^{183}, Thr^{183}, and

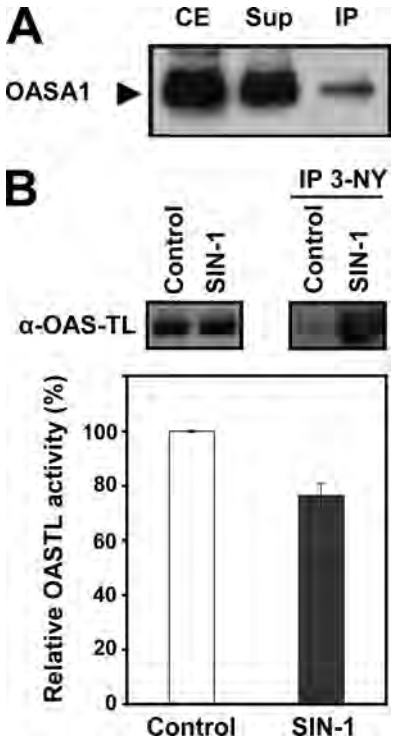

FIGURE 6. **Analysis of *in vivo* Tyr-nitration of OASA1.** Crude extracts from wild-type *A. thaliana* seedlings were immunoprecipitated with anti-3-nitroY antibody, and the presence of OASA1 in crude extract (*CE*) input and the resulting supernatant (*Sup*) and immunoprecipitate (*IP*) was analyzed by Western blot with anti-OASA1 antibody (*A*). Leaves were infiltrated with 2 mM SIN-1 for 90 min and then crude protein extracts were prepared for OASTL activity measurement (*bottom panel*) and for immunoprecipitation with 3-nitroTyr (IP 3-NY) antibodies. Western blot with anti-OASA1 (α-OASA1) was performed with protein extracts (*left up panel*) or IP (*right up panel*). OASTL activity values are the mean values of three replicates ± standard deviation (*B*).

TABLE 2

Theoretical requirements of Tyr residues of OASA1 to be nitrated and *in silico* prediction of phosphorylation potential

Distances between residues were calculated from the three-dimensional structure model shown in Fig. 4 with the analysis performed with Yasara software as indicated under "Experimental Procedures." Prediction of phosphorylation potential was analyzed with the NetPhos 2.0 software from CBS.

Tyr	Conserved	Distance to Asp/Glu	Proximal basic amino acids in primary sequence	Location in loop	Tyr phosphorylation score
20	No	-	No	No	0.156
91	Yes	3.9 Å to Glu^{58}	Lys^{92}	Yes	0.043
143	Yes	8.1 Å to Asp^{56}	No	No	0.050
158	No	4.3 Å to Glu^{195}	His^{157}	No	0.912[a]
192	Yes	7.6 Å to Glu^{195}	Lys^{191}	No	0.138
203	No	5.7 Å to Glu^{242}	Lys^{201}	No	0.032
302	Yes	11.2 Å to Glu^{300}	Arg^{301}	Yes	0.718[a]

[a] Significant score for Tyr phosphorylation site prediction.

Thr^{185} are all involved in stabilizing PLP through hydrogen bonds with the phosphate group of the cofactor (21). It is tempting to propose that 3-nitroY^{302} might introduce a perturbation in the microenvironment of PLP cofactor leading to a more restricted accessibility of the substrates to the catalytic site or to unproductive substrate binding. Productive *O*-acetylserine binding to OASA1 can be easily tested because the conversion to cysteine is mediated by the formation of an α-aminoacrylate intermediate with PLP that show a maximum of absorbance at 470 nm. An increase in A_{470} should be detected when appropriate *O*-acetylserine binding occurs and that is what we observed with purified OASA1 (Fig. 4*C*). However, a similar assay with the nitrated Tyr^{302}-containing protein led to no increase in A_{470} (Fig. 4*C*), suggesting that modification of this amino acid either drastically hinder binding of the substrate to the enzyme, or the conformation of the active site has been altered enough to allow binding of the substrate but not close enough to PLP to form the α-aminoacrylate intermediate required to complete the transformation of *O*-acetylserine to cysteine. By contrast, Tyr^{91} is located in a surface loop far from the catalytic site of the enzyme (Fig. 3*A*) and thus, even if nitrated under certain conditions, less effect on catalytic activity should be expected. Because phosphorylation of Tyr is a post-translational modification with a big impact on general plant signaling (34), and particularly in hormone-derived signaling (35–37), nitration of Tyr may have not only an effect itself but may interfere also with phosphorylation, thus being doubly important in regulating function of the modified protein. Interestingly, prediction of Tyr phosphorylation sites in OASA1 gave a high score only to Tyr^{158} and Tyr^{302} (Table 2). The potential modification of Tyr^{302} by either phosphorylation or nitration may represent also a new still unexplored regulatory mechanism in controlling OASA1 function. Moreover, it has been demonstrated that *O*-acetylserine and the C terminus of SAT compete for the same binding site (38), and the modification of Tyr^{302} (Fig. 4*B*) may have influence on the formation or disruption of the Cys synthase complex. It is clear whatsoever that Tyr^{302} is an essential residue for OASTL activity of OASA1 as demonstrated by the drastically reduced activity detected in a Y302A-mutated version of OASA1 (Fig. 5).

The fact that even under an extensive *in vitro* nitration assay on OASA1 only one of the Tyr residues of the protein has been identified as nitrated tells about the selectivity of the Tyr nitration process. Alternatively, the ratio of nitrated molecules to the non-nitrated protein molecules present in the sample may be very low thus making the MS-based identification difficult. This may explain why we could not identify the 3-nitro Tyr^{302} modification *in vivo*. Nevertheless, crude extracts that were not exogenously nitrated should contain nitrated forms of OASA1, as immunoprecipitation with anti-3-nitroTyr antibody and further detection by Western blot with a specific anti-OASA1 antibody allowed the recovery and detection of OASA1 protein in the immunoprecipitated fraction (Fig. 6*A*). Because immunoprecipitation did not certainly led to an OASA1 enrichment (Fig. 6*A*) compared with levels detected in the crude extracts, it is likely that the amount of nitrated forms *in vivo* are far below the levels of non-nitrated form of the protein. We have anyway confirmed the *in vivo* effect of nitration on OASA1 by infiltration of leaves with SIN-1 and further protein and activity analysis.

Post-translational modification of OASA1 by Y-nitration may represent a rapid and efficient regulatory mechanism to control the biosynthesis of cysteine and glutathione in response to stress factors. Both abiotic and biotic stress factors lead often to the production of reactive oxygen and nitrogen species in the stressed plants. Simultaneous production of NO (39) and superoxide anion in cells of stressed plants correlates with the production of strong nitrating peroxynitrite, which can nitrate Tyr residues of proteins. Cytosolic OASA1 is the main OASTL enzyme so an efficient control of its activity is crucial for controlling cysteine homeostasis under stress conditions. Recently, new insights on the function of this enzyme as a determinant of the antioxidative capacity of the cytosol have been proposed (9). Heavy metals have been characterized as inducers of cysteine biosynthesis (7, 8), and also as activators of NO production in eukaryotic algae and plants (40, 41). NO-derived and peroxynitrite-mediated Tyr-nitration of OASA1 may represent a rapid mechanism to control the amplitude and duration of the responses triggered by heavy metals or any other stress factors in plants. Alternatively, this sort of rapid inactivation mechanism could be operative only in tissues undergoing the direct effect of the stress factor, thus allowing the execution of the stress-activated responses in the rest of the plant. This local inactivation mechanism of OASA1 could be useful to avoid an extra production of cysteine and/or glutathione, which may act as a strong scavenger of the reactive oxygen and nitrogen species in the stressed area, thus limiting the activation of downstream signaling processes required for an efficient stress-related response in the whole plant. Rapid and local inactivation of enzymes could represent not only an efficient mechanism to modulate responses to stress, but also as a way to restrict enzyme activities in certain plant organs or under specific developmental stages, what makes this post-translational modification a relevant level for the regulatory effects exerted by sulfur-containing compounds on development and responses to stress. The identification of the Tyr nitration site in OASA1 protein and the subsequent inhibition of enzyme activity represent, to our knowledge, the first report identifying a post-translational modification of plant OASTLs and the first identification and further characterization of a nitration site in a plant protein with strong impact on its function.

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REFERENCES

1. Wirtz, M., Droux, M., and Hell, R. (2004) *J. Exp. Bot.* **55,** 1785–1798
2. Haas, F. H., Heeg, C., Queiroz, R., Bauer, A., Wirtz, M., and Hell, R. (2008) *Plant Physiol.* **148,** 1055–1067
3. Heeg, C., Kruse, C., Jost, R., Gutensohn, M., Ruppert, T., Wirtz, M., and Hell, R. (2008) *Plant Cell* **20,** 168–185
4. Krueger, S., Niehl, A., Lopez Martin, M. C., Steinhauser, D., Donath, A.,

Hildebrandt, T., Romero, L. C., Hoefgen, R., Gotor, C., and Hesse, H. (2009) *Plant Cell Environ.* **32,** 349–367
5. Watanabe, M., Kusano, M., Oikawa, A., Fukushima, A., Noji, M., and Saito, K. (2008) *Plant Physiol.* **146,** 310–320
6. Watanabe, M., Mochida, K., Kato, T., Tabata, S., Yoshimoto, N., Noji, M., and Saito, K. (2008) *Plant Cell* **20,** 2484–2496
7. Dominguez-Solís, J. R., Gutierrez-Alcalá, G., Vega, J. M., Romero, L. C., and Gotor, C. (2001) *J. Biol. Chem.* **276,** 9297–9302
8. Dominguez-Solís, J. R., López-Martin, M. C., Ager, F. J., Ynsa, M. D., Romero, L. C., and Gotor, C. (2004) *Plant Biotechnol. J.* **2,** 469–476
9. López-Martín, M. C., Becana, M., Romero, L. C., and Gotor, C. (2008) *Plant Physiol.* **147,** 562–572
10. Yi, H., Galant, A., Ravilious, G. E., Preuss, M. L., and Jez, J. M. (2010) *Mol. Plant* **3,** 269–279
11. Barroso, C., Romero, L. C., Cejudo, F. J., Vega, J. M., and Gotor, C. (1999) *Plant Mol. Biol.* **40,** 729–736
12. Wirtz, M., and Hell, R. (2006) *J. Plant Physiol.* **163,** 273–286
13. Droux, M. (2004) *Photosynth. Res.* **79,** 331–348
14. Leustek, T., Martin, M. N., Bick, J. A., and Davies, J. P. (2000) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **51,** 141–165
15. Lindermayr, C., and Durner, J. (2009) *J. Proteomics* **73,** 1–9
16. Corpas, F. J., Chaki, M., Leterrier, M., and Barroso, J. B. (2009) *Plant Signal. Behav.* **4,** 920–923
17. Szabó, C., Ischiropoulos, H., and Radi, R. (2007) *Nat. Rev. Drug Discov.* **6,** 662–680
18. Thomas, D. D., Espey, M. G., Vitek, M. P., Miranda, K. M., and Wink, D. A. (2002) *Proc. Natl. Acad. Sci. U.S.A.* **99,** 12691–12696
19. Ischiropoulos, H. (2003) *Biochem. Biophys. Res. Commun.* **305,** 776–783
20. Abello, N., Kerstjens, H. A., Postma, D. S., and Bischoff, R. (2009) *J. Proteome Res.* **8,** 3222–3238
21. Bonner, E. R., Cahoon, R. E., Knapke, S. M., and Jez, J. M. (2005) *J. Biol. Chem.* **280,** 38803–38813
22. Atanassov, I. I., Atanassov, I. I., Etchells, J. P., and Turner, S. R. (2009) *Plant Methods* **5,** 14
23. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
24. Radi, R. (2004) *Proc. Natl. Acad. Sci. U.S.A.* **101,** 4003–4008
25. Zhang, Y., Lu, N., and Gao, Z. (2009) *Int. J. Biochem. Cell Biol.* **41,** 907–915
26. Schroeder, P., Klotz, L. O. Buchczyk, D. P., Sadik, C. D. Schewe, T., and Sies, H. (2001) *Biochem. Biophys. Res. Commun.* **285,** 782–787
27. Barroso, C., Vega, J. M., and Gotor, C. (1995) *FEBS Lett.* **363,** 1–5
28. Gaitonde, M. K. (1967) *Biochem. J.* **104,** 627–633
29. Tai, C. H., and Cook, P. F. (2001) *Acc. Chem. Res.* **34,** 49–59
30. Koprivova, A., North, K. A., and Kopriva, S. (2008) *Plant Physiol.* **146,** 1408–1420
31. Rinalducci, S., Murgiano, L., and Zolla, L. (2008) *J. Exp. Bot.* **59,** 3781–3801
32. Durzan, D. J., and Pedroso, M. C. (2002) *Biotechnol. Genet. Eng. Rev.* **19,** 293–337
33. Souza, J. M., Daikhin, E., Yudkoff, M., Raman, C. S., and Ischiropoulos, H. (1999) *Arch. Biochem. Biophys.* **371,** 169–178
34. de la Fuente van Bentem, S., and Hirt, H. (2009) *Trends Plant Sci.* **14,** 71–76
35. Ghelis, T., Bolbach, G., Clodic, G., Habricot, Y., Miginiac, E., Sotta, B., and Jeannette, E. (2008) *Plant Physiol.* **148,** 1668–1680
36. Oh, M. H., Wang, X., Kota, U., Goshe, M. B., Clouse, S. D., and Huber, S. C. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106,** 658–663
37. Kim, T. W., Guan, S., Sun, Y., Deng, Z., Tang, W., Shang, J. X., Sun, Y., Burlingame, A. L., and Wang, Z. Y. (2009) *Nat. Cell Biol.* **11,** 1254–1260
38. Francois, J. A., Kumaran, S., and Jez, J. M. (2006) *Plant Cell* **18,** 3647–3655
39. Lozano-Juste, J., and León, J. (2010) *Plant Physiol.* **152,** 891–903
40. Zhang, L. P., Mehta, S. K., Liu, Z. P., and Yang, Z. M. (2008) *Plant Cell Physiol.* **49,** 411–419
41. De Michele, R., Vurro, E., Rigo, C., Costa, A., Elviri, L., Di Valentin, M., Careri, M., Zottini, M., Sanità di Toppi, L., and Lo Schiavo, F. (2009) *Plant Physiol.* **150,** 217–228

Conclusiones

1. DES1 es una L-Cisteína desulfhidrasa localizada en el citosol que cataliza la desulfuración de L-cisteína a sulfuro, amonio y piruvato, y es la única L-Cys desulfhidrasa citosólica descrita hasta la fecha en *Arabidopsis thaliana*. DES1 está involucrada en el mantenimiento de la homeostasis de cisteína en el citosol, principalmente durante los estadíos más tardíos del desarrollo o en respuesta a perturbaciones ambientales.

2. La deficiencia en la proteína DES1 provoca una senescencia prematura, la activación de la autofagia y una alteración significativa del perfil transcripcional en estadíos tardíos de crecimiento en *Arabidopsis thaliana*.

3. El sulfuro revierte la senescencia prematura, la inducción de la autofagia en los mutantes *des1,* así como en plantas silvestres y mutantes donde se ha inducido la autofagia por limitación de carbono, y también revierte la alteración transcripcional observada. Por tanto, proponemos que el H_2S actúa como una molécula señalizadora que regula negativamente la autofagia y modula el perfil transcripcional en *Arabidopsis thaliana*.

4. La cisteína citosólica juega un papel importante (directa o indirectamente) en la respuesta inmune de la planta. Una regulación precisa de la homeostasis de cisteína citosólica es crítica para que se dé correctamente la respuesta de la planta a patógenos. En concreto, posee un papel protector durante la resistencia basal o inducida por PAMPs, y es esencial para iniciar la respuesta hipersensible durante la resistencia inducida por efector.

5. La enzima más importante implicada en la biosíntesis de cisteína en el citosol de *Arabidopsis thaliana*, OAS-A1, sufre un proceso de inactivación producido por la modificación post-traduccional específica del residuo de tirosina 302 mediante nitración. Es la primera vez que se identifica y caracteriza un sitio de nitración en una proteína de plantas, afectando específicamente a su función.

Bibliografía

Aarts N., Metz M., Holub E., Staskawicz B. J., Daniels M. J. y Parker J. E. (1998)."Different requirements for EDS1 and NDR1 by disease resistance genes define at least two R gene-mediated signaling pathways in Arabidopsis". Proc Natl Acad Sci U S A. 95 (17): 10306-10311.

Alamillo J. M. y Garcia-Olmedo F. (2001)."Effects of urate, a natural inhibitor of peroxynitrite-mediated toxicity, in the response of Arabidopsis thaliana to the bacterial pathogen Pseudomonas syringae". Plant J. 25 (5): 529-540.

Alvarez C., Bermudez M. A., Romero L. C., Gotor C. y Garcia I. (2011a)."Cysteine homeostasis plays an essential role in plant immunity". New Phytol. doi: 10.1111/j.1469-8137.2011.03889.x.

Alvarez C., Calo L., Romero L. C., Garcia I. y Gotor C. (2010)."An O-acetylserine(thiol)lyase homolog with L-cysteine desulfhydrase activity regulates cysteine homeostasis in Arabidopsis". Plant Physiol. 152 (2): 656-669.

Alvarez C., Lozano-Juste J., Romero L. C., Garcia I., Gotor C. y Leon J. (2011b)."Inhibition of Arabidopsis O-acetylserine(thiol)lyase A1 by tyrosine nitration". J Biol Chem. 286 (1): 578-586.

Asai S. y Yoshioka H. (2008)."The role of radical burst via MAPK signaling in plant immunity". Plant Signal Behav. 3 (11): 920-922.

Asai T., Tena G., Plotnikova J., Willmann M. R., Chiu W. L., Gomez-Gomez L., Boller T., Ausubel F. M. y Sheen J. (2002)."MAP kinase signalling cascade in Arabidopsis innate immunity". Nature. 415 (6875): 977-983.

Ausubel F. M. (2005)."Are innate immune signaling pathways in plants and animals conserved?". Nat Immunol. 6 (10): 973-979.

Bari R. y Jones J. D. (2009)."Role of plant hormones in plant defence responses". Plant Mol Biol. 69 (4): 473-488.

Barroso C., Romero L. C., Cejudo F. J., Vega J. M. y Gotor C. (1999)."Salt-specific regulation of the cytosolic O-acetylserine(thiol)lyase gene from Arabidopsis thaliana is dependent on abscisic acid". Plant Mol Biol. 40 (4): 729-736.

Barroso C., Romero L. C., Vega J. M. y Gotor C. (1997)."Molecular characterization of the sulfur metabolism in plants ". Current Topics Phytochem. 1: 19-29.

Barroso C., Vega J. M. y Gotor C. (1995)."A new member of the cytosolic O-acetylserine(thiol)lyase gene family in Arabidopsis thaliana". FEBS Lett. 363 (1-2): 1-5.

Bassham D. C. (2007)."Plant autophagy--more than a starvation response". Curr Opin Plant Biol. 10 (6): 587-593.

Bassham D. C., Laporte M., Marty F., Moriyasu Y., Ohsumi Y., Olsen L. J. y Yoshimoto K. (2006)."Autophagy in development and stress responses of plants". Autophagy. 2 (1): 2-11.

Baxter I., Muthukumar B., Park H. C., Buchner P., Lahner B., Danku J., Zhao K., Lee J., Hawkesford M. J., Guerinot M. L. y Salt D. E. (2008)."Variation in molybdenum content across broadly distributed populations of Arabidopsis thaliana is controlled by a mitochondrial molybdenum transporter (MOT1)". PLoS Genet. 4 (2): e1000004.

Bednarek P., Pislewska-Bednarek M., Svatos A., Schneider B., Doubsky J., Mansurova M., Humphry M., Consonni C., Panstruga R., Sanchez-Vallet A., Molina A. y Schulze-Lefert P. (2009)."A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense". Science. 323 (5910): 101-106.

Belkhadir Y., Subramaniam R. y Dangl J. L. (2004)."Plant disease resistance protein signaling: NBS-LRR proteins and their partners". Curr Opin Plant Biol. 7 (4): 391-399.

Bermudez M. A., Paez-Ochoa M. A., Gotor C. y Romero L. C. (2010)."Arabidopsis S-sulfocysteine synthase activity is essential for chloroplast function and long-day light-dependent redox control". Plant Cell. 22 (2): 403-416.

Berrocal-Lobo M., Molina A. y Solano R. (2002)."Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in Arabidopsis confers resistance to several necrotrophic fungi". Plant J. 29 (1): 23-32.

Bick J. A., Dennis J. J., Zylstra G. J., Nowack J. y Leustek T. (2000)."Identification of a new class of 5'-adenylylsulfate (APS) reductases from sulfate-assimilating bacteria". J Bacteriol. 182 (1): 135-142.

Bick J. A., Setterdahl A. T., Knaff D. B., Chen Y., Pitcher L. H., Zilinskas B. A. y Leustek T. (2001)."Regulation of the plant-type 5'-adenylyl sulfate reductase by oxidative stress". Biochemistry. 40 (30): 9040-9048.

Bloem E., Haneklaus S., Salac I., Wickenhauser P. y Schnug E. (2007)."Facts and fiction about sulfur metabolism in relation to plant-pathogen interactions". Plant Biol (Stuttg). 9 (5): 596-607.

Bogdanova N. y Hell R. (1997)."Cysteine synthesis in plants: protein-protein interactions of serine acetyltransferase from Arabidopsis thaliana". Plant J. 11 (2): 251-262.

Bolchi A., Petrucco S., Tenca P. L., Foroni C. y Ottonello S. (1999)."Coordinate modulation of maize sulfate permease and ATP sulfurylase mRNAs in response to variations in sulfur nutritional status: stereospecific down-regulation by L-cysteine". Plant Mol Biol. 39 (3): 527-537.

Boller T. y Felix G. (2009)."A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors". Annu Rev Plant Biol. 60: 379-406.

Bonner E. R., Cahoon R. E., Knapke S. M. y Jez J. M. (2005)."Molecular basis of cysteine biosynthesis in plants: structural and functional analysis of O-acetylserine sulfhydrylase from Arabidopsis thaliana". J Biol Chem. 280 (46): 38803-38813.

Buchner P., Takahashi H. y Hawkesford M. J. (2004)."Plant sulphate transporters: co-ordination of uptake, intracellular and long-distance transport". J Exp Bot. 55 (404): 1765-1773.

Clay N. K., Adio A. M., Denoux C., Jander G. y Ausubel F. M. (2009)."Glucosinolate metabolites required for an Arabidopsis innate immune response". Science. 323 (5910): 95-101.

Cobbett C. y Goldsbrough P. (2002)."Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis". Annu Rev Plant Biol. 53: 159-182.

Codogno P. (2004)."[ATG genes and macroautophagy]". Med Sci (Paris). 20 (8-9): 734-736.

Chen S. X. y Schopfer P. (1999)."Hydroxyl-radical production in physiological reactions. A novel function of peroxidase". Eur J Biochem. 260 (3): 726-735.

Chinchilla D., Bauer Z., Regenass M., Boller T. y Felix G. (2006)."The Arabidopsis receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception". Plant Cell. 18 (2): 465-476.

Chinchilla D., Zipfel C., Robatzek S., Kemmerling B., Nurnberger T., Jones J. D., Felix G. y Boller T. (2007)."A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence". Nature. 448 (7152): 497-500.

Chini A., Fonseca S., Fernandez G., Adie B., Chico J. M., Lorenzo O., Garcia-Casado G., Lopez-Vidriero I., Lozano F. M., Ponce M. R., Micol J. L. y Solano R. (2007)."The JAZ family of repressors is the missing link in jasmonate signalling". Nature. 448 (7154): 666-671.

Chisholm S. T., Coaker G., Day B. y Staskawicz B. J. (2006)."Host-microbe interactions: shaping the evolution of the plant immune response". Cell. 124 (4): 803-814.

Dangl J. L. y Jones J. D. (2001)."Plant pathogens and integrated defence responses to infection". Nature. 411 (6839): 826-833.

Darsow T., Rieder S. E. y Emr S. D. (1997)."A multispecificity syntaxin homologue, Vam3p, essential for autophagic and biosynthetic protein transport to the vacuole". J Cell Biol. 138 (3): 517-529.

Davidian J. C. y Kopriva S. (2010)."Regulation of sulfate uptake and assimilation--the same or not the same?". Mol Plant. 3 (2): 314-325.

Delledonne M., Zeier J., Marocco A. y Lamb C. (2001)."Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response". Proc Natl Acad Sci U S A. 98 (23): 13454-13459.

Diaz-Troya S., Perez-Perez M. E., Florencio F. J. y Crespo J. L. (2008)."The role of TOR in autophagy regulation from yeast to plants and mammals". Autophagy. 4 (7): 851-865.

Dickinson M. (2003). "Molecular Plant Pathology". London and New York, Taylor & Francis Group.

Dixon D. P., Lapthorn A. y Edwards R. (2002)."Plant glutathione transferases". Genome Biol. 3 (3): REVIEWS3004.

Doelling J. H., Walker J. M., Friedman E. M., Thompson A. R. y Vierstra R. D. (2002)."The APG8/12-activating enzyme APG7 is required for proper nutrient recycling and senescence in Arabidopsis thaliana". J Biol Chem. 277 (36): 33105-33114.

Dominguez-Solis J. R., Gutierrez-Alcala G., Vega J. M., Romero L. C. y Gotor C. (2001)."The cytosolic O-acetylserine(thiol)lyase gene is regulated by heavy metals and can function in cadmium tolerance". J Biol Chem. 276 (12): 9297-9302.

Dominguez-Solis J. R., Lopez-Martin M. C., Ager F. J., Ynsa M. D., Romero L. C. y Gotor C. (2004)."Increased cysteine availability is essential for cadmium tolerance and accumulation in Arabidopsis thaliana". Plant Biotechnol J. 2 (6): 469-476.

Droux M. (2004)."Sulfur assimilation and the role of sulfur in plant metabolism: a survey". Photosynth Res. 79 (3): 331-348.

Droux M., Ruffet M. L., Douce R. y Job D. (1998)."Interactions between serine acetyltransferase and O-acetylserine (thiol) lyase in higher plants--structural and kinetic properties of the free and bound enzymes". Eur J Biochem. 255 (1): 235-245.

Ducruix C., Junot C., Fievet J. B., Villiers F., Ezan E. y Bourguignon J. (2006)."New insights into the regulation of phytochelatin biosynthesis in A. thaliana cells from metabolite profiling analyses". Biochimie. 88 (11): 1733-1742.

Durrant W. E. y Dong X. (2004)."Systemic acquired resistance". Annu Rev Phytopathol. 42: 185-209.

Fan W. y Dong X. (2002)."In vivo interaction between NPR1 and transcription factor TGA2 leads to salicylic acid-mediated gene activation in Arabidopsis". Plant Cell. 14 (6): 1377-1389.

Feys B. J., Moisan L. J., Newman M. A. y Parker J. E. (2001)."Direct interaction between the Arabidopsis disease resistance signaling proteins, EDS1 and PAD4". EMBO J. 20 (19): 5400-5411.

Fluhr R. (2001)."Sentinels of disease. Plant resistance genes". Plant Physiol. 127 (4): 1367-1374.

Forouhar F., Yang Y., Kumar D., Chen Y., Fridman E., Park S. W., Chiang Y., Acton T. B., Montelione G. T., Pichersky E., Klessig D. F. y Tong L. (2005)."Structural and biochemical studies identify tobacco SABP2 as a methyl salicylate esterase and implicate it in plant innate immunity". Proc Natl Acad Sci U S A. 102 (5): 1773-1778.

Foyer C. H. y Noctor G. (2011)."Ascorbate and glutathione: the heart of the redox hub". Plant Physiol. 155 (1): 2-18.

Foyer C. H., Theodoulou F. L. y Delrot S. (2001)."The functions of inter- and intracellular glutathione transport systems in plants". Trends Plant Sci. 6 (10): 486-492.

Francois J. A., Kumaran S. y Jez J. M. (2006)."Structural basis for interaction of O-acetylserine sulfhydrylase and serine acetyltransferase in the Arabidopsis cysteine synthase complex". Plant Cell. 18 (12): 3647-3655.

Fujioka Y., Noda N. N., Fujii K., Yoshimoto K., Ohsumi Y. y Inagaki F. (2008)."In vitro reconstitution of plant Atg8 and Atg12 conjugation systems essential for autophagy". J Biol Chem. 283 (4): 1921-1928.

Garcia I., Castellano J. M., Vioque B., Solano R., Gotor C. y Romero L. C. (2010)."Mitochondrial beta-cyanoalanine synthase is essential for root hair formation in Arabidopsis thaliana". Plant Cell. 22 (10): 3268-3279.

Giles G. I. y Jacob C. (2002)."Reactive sulfur species: an emerging concept in oxidative stress". Biol Chem. 383 (3-4): 375-388.

Glazebrook J. (2001)."Genes controlling expression of defense responses in Arabidopsis--2001 status". Curr Opin Plant Biol. 4 (4): 301-308.

Goldsbrough P. (2000). "Metal tolerance in plants: the role of phytochelatins and metallothioneins.". CRC Press LLC. Boca Ratón.

Gomez-Gomez L. y Boller T. (2000)."FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis". Mol Cell. 5 (6): 1003-1011.

Gotor C., Cejudo F. J., Barroso C. y Vega J. M. (1997)."Tissue-specific expression of ATCYS-3A, a gene encoding the cytosolic isoform of O-acetylserine(thiol)lyase in Arabidopsis". Plant J. 11 (2): 347-352.

Gozuacik D. y Kimchi A. (2004)."Autophagy as a cell death and tumor suppressor mechanism". Oncogene. 23 (16): 2891-2906.

Gromes R., Hothorn M., Lenherr E. D., Rybin V., Scheffzek K. y Rausch T. (2008)."The redox switch of gamma-glutamylcysteine ligase via a reversible monomer-dimer transition is a mechanism unique to plants". Plant J. 54 (6): 1063-1075.

Gross A., Brückner G., Heldt H. W. y Flügge U. I. (1990)."Comparison of the kinetic properties, inhibition and labelling of the phosphate translocators from maize and spinach mesophyll chloroplasts". Planta. 180 (2): 262-271.

Grzam A., Martin M. N., Hell R. y Meyer A. J. (2007)."gamma-Glutamyl transpeptidase GGT4 initiates vacuolar degradation of glutathione S-conjugates in Arabidopsis". FEBS Lett. 581 (17): 3131-3138.

Gullner G., Tobias I., Fodor J. y Komives T. (1999)."Elevation of glutathione level and activation of glutathione-related enzymes affect virus infection in tobacco". Free Radic Res. 31 Suppl: S155-161.

Gutierrez-Alcala G., Gotor C., Meyer A. J., Fricker M., Vega J. M. y Romero L. C. (2000)."Glutathione biosynthesis in Arabidopsis trichome cells". Proc Natl Acad Sci U S A. 97 (20): 11108-11113.

Gutierrez-Marcos J. F., Roberts M. A., Campbell E. I. y Wray J. L. (1996)."Three members of a novel small gene-family from Arabidopsis thaliana able to complement functionally an Escherichia coli mutant defective in PAPS reductase activity encode proteins with a thioredoxin-like domain and "APS reductase" activity". Proc Natl Acad Sci U S A. 93 (23): 13377-13382.

Haas F. H., Heeg C., Queiroz R., Bauer A., Wirtz M. y Hell R. (2008)."Mitochondrial serine acetyltransferase functions as a pacemaker of cysteine synthesis in plant cells". Plant Physiol. 148 (2): 1055-1067.

Hammerschmidt R. (1999)."PHYTOALEXINS: What Have We Learned After 60 Years?". Annu Rev Phytopathol. 37: 285-306.

Hammond-Kosack K. E. y Jones J. D. (1997)."Plant Disease Resistance Genes". Annu Rev Plant Physiol Plant Mol Biol. 48: 575-607.

Hanaoka H., Noda T., Shirano Y., Kato T., Hayashi H., Shibata D., Tabata S. y Ohsumi Y. (2002)."Leaf senescence and starvation-induced chlorosis are accelerated by the disruption of an Arabidopsis autophagy gene". Plant Physiol. 129 (3): 1181-1193.

Harrington H. M. y Smith I. K. (1980)."Cysteine metabolism in cultured tobacco cells". Plant Physiol. 65 (1): 151-155.

Hatzfeld Y., Lee S., Lee M., Leustek T. y Saito K. (2000a)."Functional characterization of a gene encoding a fourth ATP sulfurylase isoform from Arabidopsis thaliana". Gene. 248 (1-2): 51-58.

Hatzfeld Y., Maruyama A., Schmidt A., Noji M., Ishizawa K. y Saito K. (2000b)."beta-Cyanoalanine synthase is a mitochondrial cysteine synthase-like protein in spinach and Arabidopsis". Plant Physiol. 123 (3): 1163-1171.

Hawkesford M. J. y De Kok L. J. (2006)."Managing sulphur metabolism in plants". Plant Cell Environ. 29 (3): 382-395.

Heath M. C. (2000)."Hypersensitive response-related death". Plant Mol Biol. 44 (3): 321-334.

Heeg C., Kruse C., Jost R., Gutensohn M., Ruppert T., Wirtz M. y Hell R. (2008)."Analysis of the Arabidopsis O-acetylserine(thiol)lyase gene family demonstrates compartment-specific differences in the regulation of cysteine synthesis". Plant Cell. 20 (1): 168-185.

Heidenreich T., Wollers S., Mendel R. R. y Bittner F. (2005)."Characterization of the NifS-like domain of ABA3 from Arabidopsis thaliana provides insight into the mechanism of molybdenum cofactor sulfuration". J Biol Chem. 280 (6): 4213-4218.

Heldt W. H., Werdan K., Milovancev M. y Geller G. (1973)."Alkalization of the chloroplast stroma caused by light-dependent proton flux into the thylakoid space". Biochim Biophys Acta. 314 (2): 224-241.

Hell R. (1997)."Molecular physiology of plant sulfur metabolism". Planta. 202 (2): 138-148.

Hell R., Jost R., Berkowitz O. y Wirtz M. (2002)."Molecular and biochemical analysis of the enzymes of cysteine biosynthesis in the plant Arabidopsis thaliana". Amino Acids. 22 (3): 245-257.

Hicks L. M., Cahoon R. E., Bonner E. R., Rivard R. S., Sheffield J. y Jez J. M. (2007)."Thiol-based regulation of redox-active glutamate-cysteine ligase from Arabidopsis thaliana". Plant Cell. 19 (8): 2653-2661.

Hilpert B., Bohlmann H., op den Camp R. O., Przybyla D., Miersch O., Buchala A. y Apel K. (2001)."Isolation and characterization of signal transduction mutants of Arabidopsis thaliana that constitutively activate the octadecanoid pathway and form necrotic microlesions". Plant J. 26 (4): 435-446.

Hindson V. J. y Shaw W. V. (2003)."Random-order ternary complex reaction mechanism of serine acetyltransferase from Escherichia coli". Biochemistry. 42 (10): 3113-3119.

Hirai M. Y., Fujiwara T., Awazuhara M., Kimura T., Noji M. y Saito K. (2003)."Global expression profiling of sulfur-starved Arabidopsis by DNA macroarray reveals the role of O-acetyl-l-serine as a general regulator of gene expression in response to sulfur nutrition". Plant J. 33 (4): 651-663.

Hirai M. Y. y Saito K. (2008)."Analysis of systemic sulfur metabolism in plants using integrated '-omics' strategies". Mol Biosyst. 4 (10): 967-973.

Hofius D., Schultz-Larsen T., Joensen J., Tsitsigiannis D. I., Petersen N. H., Mattsson O., Jorgensen L. B., Jones J. D., Mundy J. y Petersen M. (2009)."Autophagic components contribute to hypersensitive cell death in Arabidopsis". Cell. 137 (4): 773-783.

Holler K., Kiraly L., Kunstler A., Muller M., Gullner G., Fattinger M. y Zechmann B. (2010)."Enhanced glutathione metabolism is correlated with sulfur-induced resistance in Tobacco mosaic virus-infected genetically susceptible Nicotiana tabacum plants". Mol Plant Microbe Interact. 23 (11): 1448-1459.

Hothorn M., Wachter A., Gromes R., Stuwe T., Rausch T. y Scheffzek K. (2006)."Structural basis for the redox control of plant glutamate cysteine ligase". J Biol Chem. 281 (37): 27557-27565.

Howarth J. R., Dominguez-Solis J. R., Gutierrez-Alcala G., Wray J. L., Romero L. C. y Gotor C. (2003a)."The serine acetyltransferase gene family in Arabidopsis thaliana and the regulation of its expression by cadmium". Plant Mol Biol. 51 (4): 589-598.

Howarth J. R., Fourcroy P., Davidian J. C., Smith F. W. y Hawkesford M. J. (2003b)."Cloning of two contrasting high-affinity sulfate transporters from tomato induced by low sulfate and infection by the vascular pathogen Verticillium dahliae". Planta. 218 (1): 58-64.

Howe G. y Merchant S. (1992)."Heavy Metal-Activated Synthesis of Peptides in Chlamydomonas reinhardtii". Plant Physiol. 98 (1): 127-136.

Huckelhoven R. (2007)."Cell wall-associated mechanisms of disease resistance and susceptibility". Annu Rev Phytopathol. 45: 101-127.

Hwang C. S., Shemorry A. y Varshavsky A. (2010)."N-terminal acetylation of cellular proteins creates specific degradation signals". Science. 327 (5968): 973-977.

Ishida H., Yoshimoto K., Izumi M., Reisen D., Yano Y., Makino A., Ohsumi Y., Hanson M. R. y Mae T. (2008)."Mobilization of rubisco and stroma-localized fluorescent proteins of chloroplasts to the vacuole by an ATG gene-dependent autophagic process". Plant Physiol. 148 (1): 142-155.

Jacob C., Giles G. I., Giles N. M. y Sies H. (2003)."Sulfur and selenium: the role of oxidation state in protein structure and function". Angew Chem Int Ed Engl. 42 (39): 4742-4758.

Jez J. M., Cahoon R. E. y Chen S. (2004)."Arabidopsis thaliana glutamate-cysteine ligase: functional properties, kinetic mechanism, and regulation of activity". J Biol Chem. 279 (32): 33463-33470.

Johnson C., Boden E. y Arias J. (2003)."Salicylic acid and NPR1 induce the recruitment of trans-activating TGA factors to a defense gene promoter in Arabidopsis". Plant Cell. 15 (8): 1846-1858.

Jones A. M., Thomas V., Bennett M. H., Mansfield J. y Grant M. (2006)."Modifications to the Arabidopsis defense proteome occur prior to

significant transcriptional change in response to inoculation with Pseudomonas syringae". Plant Physiol. 142 (4): 1603-1620.

Jones J. D. y Dangl J. L. (2006)."The plant immune system". Nature. 444 (7117): 323-329.

Kajava A. V. (1998)."Structural diversity of leucine-rich repeat proteins". J Mol Biol. 277 (3): 519-527.

Kaku H., Nishizawa Y., Ishii-Minami N., Akimoto-Tomiyama C., Dohmae N., Takio K., Minami E. y Shibuya N. (2006)."Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor". Proc Natl Acad Sci U S A. 103 (29): 11086-11091.

Kamada Y., Funakoshi T., Shintani T., Nagano K., Ohsumi M. y Ohsumi Y. (2000)."Tor-mediated induction of autophagy via an Apg1 protein kinase complex". J Cell Biol. 150 (6): 1507-1513.

Kataoka T., Watanabe-Takahashi A., Hayashi N., Ohnishi M., Mimura T., Buchner P., Hawkesford M. J., Yamaya T. y Takahashi H. (2004)."Vacuolar sulfate transporters are essential determinants controlling internal distribution of sulfate in Arabidopsis". Plant Cell. 16 (10): 2693-2704.

Katsir L., Chung H. S., Koo A. J. y Howe G. A. (2008)."Jasmonate signaling: a conserved mechanism of hormone sensing". Curr Opin Plant Biol. 11 (4): 428-435.

Keller T., Damude H. G., Werner D., Doerner P., Dixon R. A. y Lamb C. (1998)."A plant homolog of the neutrophil NADPH oxidase gp91phox subunit gene encodes a plasma membrane protein with Ca2+ binding motifs". Plant Cell. 10 (2): 255-266.

Kendrick M. D. y Chang C. (2008)."Ethylene signaling: new levels of complexity and regulation". Curr Opin Plant Biol. 11 (5): 479-485.

Kesarwani M., Yoo J. y Dong X. (2007)."Genetic interactions of TGA transcription factors in the regulation of pathogenesis-related genes and disease resistance in Arabidopsis". Plant Physiol. 144 (1): 336-346.

Ketelaar T., Voss C., Dimmock S. A., Thumm M. y Hussey P. J. (2004)."Arabidopsis homologues of the autophagy protein Atg8 are a novel family of microtubule binding proteins". FEBS Lett. 567 (2-3): 302-306.

Khan M. S., Haas F. H., Samami A. A., Gholami A. M., Bauer A., Fellenberg K., Reichelt M., Hansch R., Mendel R. R., Meyer A. J., Wirtz M. y Hell R. (2010)."Sulfite reductase defines a newly discovered bottleneck for assimilatory sulfate reduction and is essential for growth and development in Arabidopsis thaliana". Plant Cell. 22 (4): 1216-1231.

Kihara A., Noda T., Ishihara N. y Ohsumi Y. (2001)."Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and

carboxypeptidase Y sorting in Saccharomyces cerevisiae". J Cell Biol. 152 (3): 519-530.

Kim I., Rodriguez-Enriquez S. y Lemasters J. J. (2007)."Selective degradation of mitochondria by mitophagy". Arch Biochem Biophys. 462 (2): 245-253.

Kliebenstein D. (2004)."Secondary metabolites and plant/environment interactions: a view through Arabidopsis thaliana tinged glasses.". Plant Cell Environ. 27: 675–684.

Kopriva S. y Koprivova A. (2004)."Plant adenosine 5'-phosphosulphate reductase: the past, the present, and the future". J Exp Bot. 55 (404): 1775-1783.

Kopriva S. y Rennenberg H. (2004)."Control of sulphate assimilation and glutathione synthesis: interaction with N and C metabolism". J Exp Bot. 55 (404): 1831-1842.

Koprivova A., North K. A. y Kopriva S. (2008)."Complex signaling network in regulation of adenosine 5'-phosphosulfate reductase by salt stress in Arabidopsis roots". Plant Physiol. 146 (3): 1408-1420.

Krueger S., Niehl A., Lopez Martin M. C., Steinhauser D., Donath A., Hildebrandt T., Romero L. C., Hoefgen R., Gotor C. y Hesse H. (2009)."Analysis of cytosolic and plastidic serine acetyltransferase mutants and subcellular metabolite distributions suggests interplay of the cellular compartments for cysteine biosynthesis in Arabidopsis". Plant Cell Environ. 32 (4): 349-367.

Kumaran S., Yi H., Krishnan H. B. y Jez J. M. (2009)."Assembly of the cysteine synthase complex and the regulatory role of protein-protein interactions". J Biol Chem. 284 (15): 10268-10275.

Kunze G., Zipfel C., Robatzek S., Niehaus K., Boller T. y Felix G. (2004)."The N terminus of bacterial elongation factor Tu elicits innate immunity in Arabidopsis plants". Plant Cell. 16 (12): 3496-3507.

Kushnir S., Babiychuk E., Storozhenko S., Davey M. W., Papenbrock J., De Rycke R., Engler G., Stephan U. W., Lange H., Kispal G., Lill R. y Van Montagu M. (2001)."A mutation of the mitochondrial ABC transporter Sta1 leads to dwarfism and chlorosis in the Arabidopsis mutant starik". Plant Cell. 13 (1): 89-100.

Lamb C. y Dixon R. A. (1997)."The Oxidative Burst in Plant Disease Resistance". Annu Rev Plant Physiol Plant Mol Biol. 48: 251-275.

Lambrix V., Reichelt M., Mitchell-Olds T., Kliebenstein D. J. y Gershenzon J. (2001)."The Arabidopsis epithiospecifier protein promotes the hydrolysis of glucosinolates to nitriles and influences Trichoplusia ni herbivory". Plant Cell. 13 (12): 2793-2807.

Lappartient A. G., Vidmar J. J., Leustek T., Glass A. D. y Touraine B. (1999)."Inter-organ signaling in plants: regulation of ATP sulfurylase and

sulfate transporter genes expression in roots mediated by phloem-translocated compound". Plant J. 18 (1): 89-95.

Laurie-Berry N., Joardar V., Street I. H. y Kunkel B. N. (2006)."The Arabidopsis thaliana JASMONATE INSENSITIVE 1 gene is required for suppression of salicylic acid-dependent defenses during infection by Pseudomonas syringae". Mol Plant Microbe Interact. 19 (7): 789-800.

Lee M. y Leustek T. (1999)."Identification of the gene encoding homoserine kinase from Arabidopsis thaliana and characterization of the recombinant enzyme derived from the gene". Arch Biochem Biophys. 372 (1): 135-142.

Lenz H. D., Haller E., Melzer E., Kober K., Wurster K., Stahl M., Bassham D. C., Vierstra R. D., Parker J. E., Bautor J., Molina A., Escudero V., Shindo T., van der Hoorn R. A., Gust A. A. y Nurnberger T. (2011)."Autophagy differentially controls plant basal immunity to biotrophic and necrotrophic pathogens". Plant J.

Leon J., Romero L. C., Galvan F. y Vega J. M. (1987)."Purification and physicochemical characterization of O-acetyl-L-serine sulfhydrylase from Chlamydomonas reinhardtii". Plant Sci. 53: 93-99.

Leon J., Yalpani N., Raskin I. y Lawton M. A. (1993)."Induction of Benzoic Acid 2-Hydroxylase in Virus-Inoculated Tobacco". Plant Physiol. 103 (2): 323-328.

Leon S., Touraine B., Briat J. F. y Lobreaux S. (2002)."The AtNFS2 gene from Arabidopsis thaliana encodes a NifS-like plastidial cysteine desulphurase". Biochem J. 366 (Pt 2): 557-564.

Leustek T., Martin M. N., Bick J. A. y Davies J. P. (2000)."Pathways and Regulation of Sulfur Metabolism Revealed through Molecular and Genetic Studies". Annu Rev Plant Physiol Plant Mol Biol. 51: 141-165.

Li J., Brader G., Kariola T. y Palva E. T. (2006)."WRKY70 modulates the selection of signaling pathways in plant defense". Plant J. 46 (3): 477-491.

Lin J. F. y Wu S. H. (2004)."Molecular events in senescing Arabidopsis leaves". Plant J. 39 (4): 612-628.

Liu Y. y Bassham D. C. (2010)."TOR is a negative regulator of autophagy in Arabidopsis thaliana". PLoS One. 5 (7): e11883.

Liu Y., Schiff M., Czymmek K., Talloczy Z., Levine B. y Dinesh-Kumar S. P. (2005)."Autophagy regulates programmed cell death during the plant innate immune response". Cell. 121 (4): 567-577.

Lopez-Martin M. C., Becana M., Romero L. C. y Gotor C. (2008a)."Knocking out cytosolic cysteine synthesis compromises the antioxidant capacity of the cytosol to maintain discrete concentrations of hydrogen peroxide in Arabidopsis". Plant Physiol. 147 (2): 562-572.

Lopez-Martin M. C., Romero L. C. y Gotor C. (2008b)."Cytosolic cysteine in redox signaling". Plant Signal Behav. 3 (10): 880-881.

Lopez-Martín M. C., Romero L. C. y Gotor C. (2005)."Differential involvement of cysteine biosynthesis genes in cadmium tolerance in Arabidopsis thaliana. In "Sulfur Transport and Assimilation in Plants in the Post Genomic Era", K. Saito, L.J. De Kok, I. Stulen, M.J. Hawkesford, E. Schnug, A. Sirko, H. Rennenberg, eds., ". Backhuys Publishers, Leiden. 225-227.

Lorenzo O., Chico J. M., Sanchez-Serrano J. J. y Solano R. (2004)."JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis". Plant Cell. 16 (7): 1938-1950.

Lorenzo O. y Solano R. (2005)."Molecular players regulating the jasmonate signalling network". Curr Opin Plant Biol. 8 (5): 532-540.

Lozano-Juste J., Colom-Moreno R. y Leon J. (2011)."In vivo protein tyrosine nitration in Arabidopsis thaliana". J Exp Bot. 62 (10): 3501-3517.

Lunn J. E., Droux M., Martin J. y Douce R. (1990)."Localization of ATP Sulfurylase and O-Acetylserine(thiol)lyase in Spinach Leaves". Plant Physiol. 94 (3): 1345-1352.

Martin G. B., Brommonschenkel S. H., Chunwongse J., Frary A., Ganal M. W., Spivey R., Wu T., Earle E. D. y Tanksley S. D. (1993)."Map-based cloning of a protein kinase gene conferring disease resistance in tomato". Science. 262 (5138): 1432-1436.

Martinez D. E., Costa M. L., Gomez F. M., Otegui M. S. y Guiamet J. J. (2008)."'Senescence-associated vacuoles' are involved in the degradation of chloroplast proteins in tobacco leaves". Plant J. 56 (2): 196-206.

Maruyama-Nakashita A., Nakamura Y., Watanabe-Takahashi A., Inoue E., Yamaya T. y Takahashi H. (2005)."Identification of a novel cis-acting element conferring sulfur deficiency response in Arabidopsis roots". Plant J. 42 (3): 305-314.

Maruyama-Nakashita A., Nakamura Y., Yamaya T. y Takahashi H. (2004)."Regulation of high-affinity sulphate transporters in plants: towards systematic analysis of sulphur signalling and regulation". J Exp Bot. 55 (404): 1843-1849.

Meyer A. J. y Hell R. (2005)."Glutathione homeostasis and redox-regulation by sulfhydryl groups". Photosynth Res. 86 (3): 435-457.

Meyer A. J., May M. J. y Fricker M. (2001)."Quantitative in vivo measurement of glutathione in Arabidopsis cells". Plant J. 27 (1): 67-78.

Meyer T., Burow M., Bauer M. y Papenbrock J. (2003)."Arabidopsis sulfurtransferases: investigation of their function during senescence and in cyanide detoxification". Planta. 217 (1): 1-10.

Meyers B. C., Dickerman A. W., Michelmore R. W., Sivaramakrishnan S., Sobral B. W. y Young N. D. (1999)."Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding superfamily". Plant J. 20 (3): 317-332.

Meyers B. C., Kozik A., Griego A., Kuang H. y Michelmore R. W. (2003)."Genome-wide analysis of NBS-LRR-encoding genes in Arabidopsis". Plant Cell. 15 (4): 809-834.

Mishina T. E. y Zeier J. (2006)."The Arabidopsis flavin-dependent monooxygenase FMO1 is an essential component of biologically induced systemic acquired resistance". Plant Physiol. 141 (4): 1666-1675.

Mitou G., Budak H. y Gozuacik D. (2009)."Techniques to study autophagy in plants". Int J Plant Genomics. 2009: 451357.

Miya A., Albert P., Shinya T., Desaki Y., Ichimura K., Shirasu K., Narusaka Y., Kawakami N., Kaku H. y Shibuya N. (2007)."CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis". Proc Natl Acad Sci U S A. 104 (49): 19613-19618.

Mizushima N., Noda T. y Ohsumi Y. (1999)."Apg16p is required for the function of the Apg12p-Apg5p conjugate in the yeast autophagy pathway". EMBO J. 18 (14): 3888-3896.

Mizushima N., Yamamoto A., Hatano M., Kobayashi Y., Kabeya Y., Suzuki K., Tokuhisa T., Ohsumi Y. y Yoshimori T. (2001)."Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells". J Cell Biol. 152 (4): 657-668.

Morris K., MacKerness S. A., Page T., John C. F., Murphy A. M., Carr J. P. y Buchanan-Wollaston V. (2000)."Salicylic acid has a role in regulating gene expression during leaf senescence". Plant J. 23 (5): 677-685.

Mou Z., Fan W. y Dong X. (2003)."Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes". Cell. 113 (7): 935-944.

Mugford S. G., Lee B. R., Koprivova A., Matthewman C. y Kopriva S. (2011)."Control of sulfur partitioning between primary and secondary metabolism". Plant J. 65 (1): 96-105.

Mugford S. G., Yoshimoto N., Reichelt M., Wirtz M., Hill L., Mugford S. T., Nakazato Y., Noji M., Takahashi H., Kramell R., Gigolashvili T., Flugge U. I., Wasternack C., Gershenzon J., Hell R., Saito K. y Kopriva S. (2009)."Disruption of adenosine-5'-phosphosulfate kinase in Arabidopsis reduces levels of sulfated secondary metabolites". Plant Cell. 21 (3): 910-927.

Mullineaux P. M. y Rausch T. (2005)."Glutathione, photosynthesis and the redox regulation of stress-responsive gene expression". Photosynth Res. 86 (3): 459-474.

Mur L. A., Brown I. R., Darby R. M., Bestwick C. S., Bi Y. M., Mansfield J. W. y Draper J. (2000)."A loss of resistance to avirulent bacterial pathogens in tobacco is associated with the attenuation of a salicylic acid-potentiated oxidative burst". Plant J. 23 (5): 609-621.

Natarajan K., Meyer M. R., Jackson B. M., Slade D., Roberts C., Hinnebusch A. G. y Marton M. J. (2001)."Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast". Mol Cell Biol. 21 (13): 4347-4368.

Nibbe M., Hilpert B., Wasternack C., Miersch O. y Apel K. (2002)."Cell death and salicylate- and jasmonate-dependent stress responses in Arabidopsis are controlled by single cet genes". Planta. 216 (1): 120-128.

Noctor G., Gomez L., Vanacker H. y Foyer C. H. (2002)."Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signalling". J Exp Bot. 53 (372): 1283-1304.

Noda T. y Ohsumi Y. (1998)."Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast". J Biol Chem. 273 (7): 3963-3966.

Noji M., Goulart Kawashima C., Obayashi T. y Saito K. (2006)."In silico assessment of gene function involved in cysteine biosynthesis in Arabidopsis: expression analysis of multiple isoforms of serine acetyltransferase". Amino Acids. 30 (2): 163-171.

Noji M., Inoue K., Kimura N., Gouda A. y Saito K. (1998)."Isoform-dependent differences in feedback regulation and subcellular localization of serine acetyltransferase involved in cysteine biosynthesis from Arabidopsis thaliana". J Biol Chem. 273 (49): 32739-32745.

Nomura K., Debroy S., Lee Y. H., Pumplin N., Jones J. y He S. Y. (2006)."A bacterial virulence protein suppresses host innate immunity to cause plant disease". Science. 313 (5784): 220-223.

Nurnberger T., Brunner F., Kemmerling B. y Piater L. (2004)."Innate immunity in plants and animals: striking similarities and obvious differences". Immunol Rev. 198: 249-266.

Otegui M. S., Noh Y. S., Martinez D. E., Vila Petroff M. G., Staehelin L. A., Amasino R. M. y Guiamet J. J. (2005)."Senescence-associated vacuoles with intense proteolytic activity develop in leaves of Arabidopsis and soybean". Plant J. 41 (6): 831-844.

Pan Q., Wendel J. y Fluhr R. (2000)."Divergent evolution of plant NBS-LRR resistance gene homologues in dicot and cereal genomes". J Mol Evol. 50 (3): 203-213.

Papenbrock J., Riemenschneider A., Kamp A., Schulz-Vogt H. N. y Schmidt A. (2007)."Characterization of cysteine-degrading and H2S-releasing enzymes of higher plants - from the field to the test tube and back". Plant Biol (Stuttg). 9 (5): 582-588.

Park S. y Imlay J. A. (2003)."High levels of intracellular cysteine promote oxidative DNA damage by driving the fenton reaction". J Bacteriol. 185 (6): 1942-1950.

Park S. W., Kaimoyo E., Kumar D., Mosher S. y Klessig D. F. (2007)."Methyl salicylate is a critical mobile signal for plant systemic acquired resistance". Science. 318 (5847): 113-116.

Petiot A., Ogier-Denis E., Blommaart E. F., Meijer A. J. y Codogno P. (2000)."Distinct classes of phosphatidylinositol 3'-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells". J Biol Chem. 275 (2): 992-998.

Phillips A. R., Suttangkakul A. y Vierstra R. D. (2008)."The ATG12-conjugating enzyme ATG10 Is essential for autophagic vesicle formation in Arabidopsis thaliana". Genetics. 178 (3): 1339-1353.

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

Pilon-Smits E. A., Garifullina G. F., Abdel-Ghany S., Kato S., Mihara H., Hale K. L., Burkhead J. L., Esaki N., Kurihara T. y Pilon M. (2002)."Characterization of a NifS-like chloroplast protein from Arabidopsis. Implications for its role in sulfur and selenium metabolism". Plant Physiol. 130 (3): 1309-1318.

Prosser I. M., Purves J. V., Saker L. R. y Clarkson D. T. (2001)."Rapid disruption of nitrogen metabolism and nitrate transport in spinach plants deprived of sulphate". J Exp Bot. 52 (354): 113-121.

Rausch T. y Wachter A. (2005)."Sulfur metabolism: a versatile platform for launching defence operations". Trends Plant Sci. 10 (10): 503-509.

Rea P. A., Li Z. S., Lu Y. P., Drozdowicz Y. M. y Martinoia E. (1998)."From Vacuolar Gs-X Pumps to Multispecific Abc Transporters". Annu Rev Plant Physiol Plant Mol Biol. 49: 727-760.

Rennenberg H., Arabatzis N. y Grundel I. (1987)."Cysteine desulphydrase activity in higher plants: evidence for the action of L- and D-cysteine specific enzymes". phytochemistry. 26 (6): 1583-1589.

Rennenberg H. y Filner P. (1983)."Developmental changes in the potential for h(2)s emission in cucurbit plants". Plant Physiol. 71 (2): 269-275.

Riemenschneider A., Riedel K., Hoefgen R., Papenbrock J. y Hesse H. (2005a)."Impact of reduced O-acetylserine(thiol)lyase isoform contents on potato plant metabolism". Plant Physiol. 137 (3): 892-900.

Riemenschneider A., Wegele R., Schmidt A. y Papenbrock J. (2005b)."Isolation and characterization of a D-cysteine desulfhydrase protein from Arabidopsis thaliana". FEBS J. 272 (5): 1291-1304.

Romero L. C., Domínguez-Solís J. R., Gutiérrez-Alcalá G. y Gotor C. (2001)."Salt regulation of O-acetylserine(thiol)lyase in Arabidopsis thaliana and increased tolerance in yeast ". Plant Physiol. Biochem. . 39: 643-647

Ronald P. C., Salmeron J. M., Carland F. M. y Staskawicz B. J. (1992)."The cloned avirulence gene avrPto induces disease resistance in tomato cultivars containing the Pto resistance gene". J Bacteriol. 174 (5): 1604-1611.

Rotte C. y Leustek T. (2000)."Differential subcellular localization and expression of ATP sulfurylase and 5'-adenylylsulfate reductase during ontogenesis of Arabidopsis leaves indicates that cytosolic and plastid forms of ATP sulfurylase may have specialized functions". Plant Physiol. 124 (2): 715-724.

Rouached H., Berthomieu P., El Kassis E., Cathala N., Catherinot V., Labesse G., Davidian J. C. y Fourcroy P. (2005)."Structural and functional analysis of the C-terminal STAS (sulfate transporter and anti-sigma antagonist) domain of the Arabidopsis thaliana sulfate transporter SULTR1.2". J Biol Chem. 280 (16): 15976-15983.

Saito K. (2004)."Sulfur assimilatory metabolism. The long and smelling road". Plant Physiol. 136 (1): 2443-2450.

Saito K., Yokoyama H., Noji M. y Murakoshi I. (1995)."Molecular cloning and characterization of a plant serine acetyltransferase playing a regulatory role in cysteine biosynthesis from watermelon". J Biol Chem. 270 (27): 16321-16326.

Schmidt A. (1982)."Z. Pjfanrenphysiol. 107 (301).

Schmidt A. (2005)."Metabolic background of H2S release from plants.". In LJ De Kok, E Schnug, eds, Proceedings of the 1st Sino-German Workshop on Aspects of Sulfur Nutrition of Plants. Landbauforschung Vo¨ lkenrode, Braunschweig, Germany,. 121-129.

Seay M., Patel S. y Dinesh-Kumar S. P. (2006)."Autophagy and plant innate immunity". Cell Microbiol. 8 (6): 899-906.

Seskar M., Shulaev V. y Raskin I. (1998)."Endogenous methyl salicylate in pathogen-inoculated tobacco plants". Plant Physiol. 116: 387-392.

Setya A., Murillo M. y Leustek T. (1996)."Sulfate reduction in higher plants: molecular evidence for a novel 5'-adenylylsulfate reductase". Proc Natl Acad Sci U S A. 93 (23): 13383-13388.

Shibagaki N. y Grossman A. R. (2004)."Probing the function of STAS domains of the Arabidopsis sulfate transporters". J Biol Chem. 279 (29): 30791-30799.

Shintani T., Mizushima N., Ogawa Y., Matsuura A., Noda T. y Ohsumi Y. (1999)."Apg10p, a novel protein-conjugating enzyme essential for autophagy in yeast". EMBO J. 18 (19): 5234-5241.

Shulaev V., Leon J. y Raskin I. (1995)."Is Salicylic Acid a Translocated Signal of Systemic Acquired Resistance in Tobacco?". Plant Cell. 7 (10): 1691-1701.

Simon-Plas F., Elmayan T. y Blein J. P. (2002)."The plasma membrane oxidase NtrbohD is responsible for AOS production in elicited tobacco cells". Plant J. 31 (2): 137-147.

Sirko A., Gotor, C. (2007). " Molecular links between toxic metals in the environment and plant sulfur metabolism. Sulfur in Plants - an Ecophysiological Perspective.". M.J. Hawkesford & L.J. De Kok.

Smith F. W., Hawkesford M. J., Ealing P. M., Clarkson D. T., Vanden Berg P. J., Belcher A. R. y Warrilow A. G. (1997)."Regulation of expression of a cDNA from barley roots encoding a high affinity sulphate transporter". Plant J. 12 (4): 875-884.

Staskawicz B. J., Ausubel F. M., Baker B. J., Ellis J. G. y Jones J. D. (1995)."Molecular genetics of plant disease resistance". Science. 268 (5211): 661-667.

Takahashi H. (2010)."Regulation of sulfate transport and assimilation in plants". Int Rev Cell Mol Biol. 281: 129-159.

Takahashi H., Kopriva S., Giordano M., Saito H. y Hell R. (2011)."Sulfur assimilation in photosynthetic organisms: molecular functions and regulations of transporters and assimilatory enzymes". Annu. Rev. Plant Biol. 62: 13.11-13.28.

Takahashi H., Watanabe-Takahashi A., Smith F. W., Blake-Kalff M., Hawkesford M. J. y Saito K. (2000)."The roles of three functional sulphate transporters involved in uptake and translocation of sulphate in Arabidopsis thaliana". Plant J. 23 (2): 171-182.

Takken F. L. y Tameling W. I. (2009)."To nibble at plant resistance proteins". Science. 324 (5928): 744-746.

Tanida I., Mizushima N., Kiyooka M., Ohsumi M., Ueno T., Ohsumi Y. y Kominami E. (1999)."Apg7p/Cvt2p: A novel protein-activating enzyme essential for autophagy". Mol Biol Cell. 10 (5): 1367-1379.

Tao Y., Xie Z., Chen W., Glazebrook J., Chang H. S., Han B., Zhu T., Zou G. y Katagiri F. (2003)."Quantitative nature of Arabidopsis responses during

compatible and incompatible interactions with the bacterial pathogen Pseudomonas syringae". Plant Cell. 15 (2): 317-330.

Thomma B. P., Cammue B. P. y Thevissen K. (2002)."Plant defensins". Planta. 216 (2): 193-202.

Thompson A. R. y Vierstra R. D. (2005)."Autophagic recycling: lessons from yeast help define the process in plants". Curr Opin Plant Biol. 8 (2): 165-173.

Tomatsu H., Takano J., Takahashi H., Watanabe-Takahashi A., Shibagaki N. y Fujiwara T. (2007)."An Arabidopsis thaliana high-affinity molybdate transporter required for efficient uptake of molybdate from soil". Proc Natl Acad Sci U S A. 104 (47): 18807-18812.

Ton J., Van Pelt J. A., Van Loon L. C. y Pieterse C. M. (2002)."Differential effectiveness of salicylate-dependent and jasmonate/ethylene-dependent induced resistance in Arabidopsis". Mol Plant Microbe Interact. 15 (1): 27-34.

Torres M. A. (2010)."ROS in biotic interactions". Physiol Plant. 138 (4): 414-429.

Torres M. A. y Dangl J. L. (2005)."Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development". Curr Opin Plant Biol. 8 (4): 397-403.

Torres M. A., Jones J. D. y Dangl J. L. (2006)."Reactive oxygen species signaling in response to pathogens". Plant Physiol. 141 (2): 373-378.

Tsuda K. y Katagiri F. (2010)."Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity". Curr Opin Plant Biol. 13 (4): 459-465.

Tsuda K., Sato M., Glazebrook J., Cohen J. D. y Katagiri F. (2008)."Interplay between MAMP-triggered and SA-mediated defense responses". Plant J. 53 (5): 763-775.

Ungermann C. y Langosch D. (2005)."Functions of SNAREs in intracellular membrane fusion and lipid bilayer mixing". J Cell Sci. 118 (Pt 17): 3819-3828.

Vaara M. (1992)."Eight bacterial proteins, including UDP-N-acetylglucosamine acyltransferase (LpxA) and three other transferases of Escherichia coli, consist of a six-residue periodicity theme". FEMS Microbiol Lett. 76 (3): 249-254.

Van der Zaal B. J., Neuteboom L. W., Pinas J. E., Chardonnens A. N., Schat H., Verkleij J. A. y Hooykaas P. J. (1999)."Overexpression of a novel Arabidopsis gene related to putative zinc-transporter genes from animals can lead to enhanced zinc resistance and accumulation". Plant Physiol. 119 (3): 1047-1055.

Van Hoewyk D., Pilon M. y Pilon-Smits E. A. H. (2008)."The functions of NifSlike proteins in plant sulfur and selenium metabolism". Plant Sci. 174: 117-123.

Vanlerberghe G. C., Robson C. A. y Yip J. Y. (2002)."Induction of mitochondrial alternative oxidase in response to a cell signal pathway down-regulating the

cytochrome pathway prevents programmed cell death". Plant Physiol. 129 (4): 1829-1842.

Vauclare P., Kopriva S., Fell D., Suter M., Sticher L., von Ballmoos P., Krahenbuhl U., den Camp R. O. y Brunold C. (2002)."Flux control of sulphate assimilation in Arabidopsis thaliana: adenosine 5'-phosphosulphate reductase is more susceptible than ATP sulphurylase to negative control by thiols". Plant J. 31 (6): 729-740.

Vidmar J. J., Tagmount A., Cathala N., Touraine B. y Davidian J. E. (2000)."Cloning and characterization of a root specific high-affinity sulfate transporter from Arabidopsis thaliana". FEBS Lett. 475 (1): 65-69.

Vlot A. C., Klessig D. F. y Park S. W. (2008)."Systemic acquired resistance: the elusive signal(s)". Curr Opin Plant Biol. 11 (4): 436-442.

Wachter A., Wolf S., Steininger H., Bogs J. y Rausch T. (2005)."Differential targeting of GSH1 and GSH2 is achieved by multiple transcription initiation: implications for the compartmentation of glutathione biosynthesis in the Brassicaceae". Plant J. 41 (1): 15-30.

Wang C. L. y Oliver D. J. (1997)."Identification of a putative flexible loop in Arabidopsis glutathione synthetase". Biochem J. 322 (Pt 1): 241-244.

Wang D., Amornsiripanitch N. y Dong X. (2006)."A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants". PLoS Pathog. 2 (11): e123.

Watanabe M., Kusano M., Oikawa A., Fukushima A., Noji M. y Saito K. (2008a)."Physiological roles of the beta-substituted alanine synthase gene family in Arabidopsis". Plant Physiol. 146 (1): 310-320.

Watanabe M., Mochida K., Kato T., Tabata S., Yoshimoto N., Noji M. y Saito K. (2008b)."Comparative genomics and reverse genetics analysis reveal indispensable functions of the serine acetyltransferase gene family in Arabidopsis". Plant Cell. 20 (9): 2484-2496.

Weber M., Suter M., Brunold C. y Kopriva S. (2000)."Sulfate assimilation in higher plants characterization of a stable intermediate in the adenosine 5'-phosphosulfate reductase reaction". Eur J Biochem. 267 (12): 3647-3653.

Wiermer M., Feys B. J. y Parker J. E. (2005)."Plant immunity: the EDS1 regulatory node". Curr Opin Plant Biol. 8 (4): 383-389.

Williams J. S. y Cooper R. M. (2004)."The oldest fungicide and newest phytoalexin – a reappraisal of the fungitoxicity of elemental sulphur". Plant Pathol. 53: 263–279.

Williams J. S., Hall S. A., Hawkesford M. J., Beale M. H. y Cooper R. M. (2002)."Elemental sulfur and thiol accumulation in tomato and defense against a fungal vascular pathogen". Plant Physiol. 128 (1): 150-159.

Wirthmueller L., Zhang Y., Jones J. D. y Parker J. E. (2007)."Nuclear accumulation of the Arabidopsis immune receptor RPS4 is necessary for triggering EDS1-dependent defense". Curr Biol. 17 (23): 2023-2029.

Wirtz M., Berkowitz O., Droux M. y Hell R. (2001)."The cysteine synthase complex from plants. Mitochondrial serine acetyltransferase from Arabidopsis thaliana carries a bifunctional domain for catalysis and protein-protein interaction". Eur J Biochem. 268 (3): 686-693.

Wirtz M. y Droux M. (2005)."Synthesis of the sulfur amino acids: cysteine and methionine". Photosynth Res. 86 (3): 345-362.

Wirtz M., Droux M. y Hell R. (2004)."O-acetylserine (thiol) lyase: an enigmatic enzyme of plant cysteine biosynthesis revisited in Arabidopsis thaliana". J Exp Bot. 55 (404): 1785-1798.

Wirtz M., Heeg C., Samami A. A., Ruppert T. y Hell R. (2010)."Enzymes of cysteine synthesis show extensive and conserved modifications patterns that include N(alpha)-terminal acetylation". Amino Acids. 39 (4): 1077-1086.

Wirtz M. y Hell R. (2006)."Functional analysis of the cysteine synthase protein complex from plants: structural, biochemical and regulatory properties". J Plant Physiol. 163 (3): 273-286.

Wirtz M. y Hell R. (2007)."Dominant-negative modification reveals the regulatory function of the multimeric cysteine synthase protein complex in transgenic tobacco". Plant Cell. 19 (2): 625-639.

Wittstock U. y Halkier B. A. (2002)."Glucosinolate research in the Arabidopsis era". Trends Plant Sci. 7 (6): 263-270.

Wu W. y Berkowitz G. A. (1992)."Stromal pH and Photosynthesis Are Affected by Electroneutral K and H Exchange through Chloroplast Envelope Ion Channels". Plant Physiol. 98 (2): 666-672.

Xiang C. y Oliver D. J. (1998)."Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in Arabidopsis". Plant Cell. 10 (9): 1539-1550.

Xie Z. y Klionsky D. J. (2007)."Autophagosome formation: core machinery and adaptations". Nat Cell Biol. 9 (10): 1102-1109.

Xiong Y., Contento A. L. y Bassham D. C. (2005)."AtATG18a is required for the formation of autophagosomes during nutrient stress and senescence in Arabidopsis thaliana". Plant J. 42 (4): 535-546.

Xiong Y., Contento A. L., Nguyen P. Q. y Bassham D. C. (2007)."Degradation of oxidized proteins by autophagy during oxidative stress in Arabidopsis". Plant Physiol. 143 (1): 291-299.

Yamaguchi Y., Nakamura T., Kusano T. y Sano H. (2000)."Three Arabidopsis genes encoding proteins with differential activities for cysteine synthase and beta-cyanoalanine synthase". Plant Cell Physiol. 41 (4): 465-476.

Yi H., Galant A., Ravilious G. E., Preuss M. L. y Jez J. M. (2010)."Sensing sulfur conditions: simple to complex protein regulatory mechanisms in plant thiol metabolism". Mol Plant. 3 (2): 269-279.

Yonekura-Sakakibara K., Onda Y., Ashikari T., Tanaka Y., Kusumi T. y Hase T. (2000)."Analysis of reductant supply systems for ferredoxin-dependent sulfite reductase in photosynthetic and nonphotosynthetic organs of maize". Plant Physiol. 122 (3): 887-894.

Yoo S. D. y Sheen J. (2008)."MAPK signaling in plant hormone ethylene signal transduction". Plant Signal Behav. 3 (10): 848-849.

Yoshimoto K., Jikumaru Y., Kamiya Y., Kusano M., Consonni C., Panstruga R., Ohsumi Y. y Shirasu K. (2009)."Autophagy negatively regulates cell death by controlling NPR1-dependent salicylic acid signaling during senescence and the innate immune response in Arabidopsis". Plant Cell. 21 (9): 2914-2927.

Yoshimoto K., Takano Y. y Sakai Y. (2010)."Autophagy in plants and phytopathogens". FEBS Lett. 584 (7): 1350-1358.

Yoshimoto N., Inoue E., Saito K., Yamaya T. y Takahashi H. (2003)."Phloem-localizing sulfate transporter, Sultr1;3, mediates re-distribution of sulfur from source to sink organs in Arabidopsis". Plant Physiol. 131 (4): 1511-1517.

Yoshimoto N., Takahashi H., Smith F. W., Yamaya T. y Saito K. (2002)."Two distinct high-affinity sulfate transporters with different inducibilities mediate uptake of sulfate in Arabidopsis roots". Plant J. 29 (4): 465-473.

Yoshioka H., Asai S., Yoshioka M. y Kobayashi M. (2009)."Molecular mechanisms of generation for nitric oxide and reactive oxygen species, and role of the radical burst in plant immunity". Mol Cells. 28 (4): 321-329.

Yoshioka H., Numata N., Nakajima K., Katou S., Kawakita K., Rowland O., Jones J. D. y Doke N. (2003)."Nicotiana benthamiana gp91phox homologs NbrbohA and NbrbohB participate in H2O2 accumulation and resistance to Phytophthora infestans". Plant Cell. 15 (3): 706-718.

Yoshioka H., Sugie K., Park H. J., Maeda H., Tsuda N., Kawakita K. y Doke N. (2001)."Induction of plant gp91 phox homolog by fungal cell wall, arachidonic acid, and salicylic acid in potato". Mol Plant Microbe Interact. 14 (6): 725-736.

Zechmann B., Zellnig G., Urbanek-Krajnc A. y Muller M. (2007)."Artificial elevation of glutathione affects symptom development in ZYMV-infected Cucurbita pepo L. plants". Arch Virol. 152 (4): 747-762.

Zheng L., White R. H., Cash V. L., Jack R. F. y Dean D. R. (1993)."Cysteine desulfurase activity indicates a role for NIFS in metallocluster biosynthesis". Proc Natl Acad Sci U S A. 90 (7): 2754-2758.

Zipfel C. (2008)."Pattern-recognition receptors in plant innate immunity". Curr Opin Immunol. 20 (1): 10-16.

Zipfel C. y Felix G. (2005)."Plants and animals: a different taste for microbes?". Curr Opin Plant Biol. 8 (4): 353-360.

Zipfel C., Kunze G., Chinchilla D., Caniard A., Jones J. D., Boller T. y Felix G. (2006)."Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation". Cell. 125 (4): 749-760.

Zuber H., Davidian J. C., Aubert G., Aime D., Belghazi M., Lugan R., Heintz D., Wirtz M., Hell R., Thompson R. y Gallardo K. (2010)."The seed composition of Arabidopsis mutants for the group 3 sulfate transporters indicates a role in sulfate translocation within developing seeds". Plant Physiol. 154 (2): 913-926.