Journal of Experimental Botany, Vol. 63, No. 8, pp. 3219–3227, 2012
doi:10.1093/jxb/ers043 Advance Access publication 27 February, 2012
This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

S-Nitrosoglutathione is a component of wound- and salicylic acid-induced systemic responses in *Arabidopsis thaliana*

M. Carme Espunya[1], Roberto De Michele[1,*], Aurelio Gómez-Cadenas[2] and M. Carmen Martínez[1,†]

[1] Departament de Bioquímica i Biologia Molecular, Facultat de Biociències, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain

[2] Departamento de Ciencias Agrarias y del Medio Natural, Universitat Jaume I, 12071 Castellón, Spain

* Present address: Istituto di Genetica Vegetale, Consiglio Nazionale delle Ricerche, Corso Calatafimi 414, 90129 Palermo, Italy
† To whom correspondence should be addressed. E-mail: carmen.martinez@uab.es

Received 27 October 2011; Revised 25 January 2012; Accepted 30 January 2012

Abstract

S-Nitrosoglutathione (GSNO) is a bioactive, stable, and mobile reservoir of nitric oxide (NO), and an important player in defence responses to herbivory and pathogen attack in plants. It has been demonstrated previously that GSNO reductase (GSNOR) is the main enzyme responsible for the *in vivo* control of intracellular levels of GSNO. In this study, the role of *S*-nitrosothiols, in particular of GSNO, in systemic defence responses in *Arabidopsis thaliana* was investigated further. It was shown that GSNO levels increased rapidly and uniformly in injured *Arabidopsis* leaves, whereas in systemic leaves GSNO was first detected in vascular tissues and later spread over the parenchyma, suggesting that GSNO is involved in the transmission of the wound mobile signal through the vascular tissue. Moreover, GSNO accumulation was required to activate the jasmonic acid (JA)-dependent wound responses, whereas the alternative JA-independent wound-signalling pathway did not involve GSNO. Furthermore, extending previous work on the role of GSNOR in pathogenesis, it was shown that GSNO acts synergistically with salicylic acid in systemic acquired resistance activation. In conclusion, GSNOR appears to be a key regulator of systemic defence responses, in both wounding and pathogenesis.

Key words: *Arabidopsis thaliana*, jasmonic acid, *S*-nitrosoglutathione reductase (GSNOR), *S*-nitrosothiols (SNOs), systemic acquired resistance (SAR), wounding.

Introduction

Plants respond rapidly to pathogens and herbivores by triggering several defence mechanisms. Three plant hormones, salicylic acid (SA), jasmonic acid (JA), and ethylene (E), play important signalling functions in plant immunity, inducing the expression of specific genes (López *et al.*, 2008; Bari and Jones, 2009). A general feature of defence processes is the activation of a rapid local response, followed by a systemic protection to future attacks. The identification of signalling molecules conferring these long-distance responses has been the object of considerable efforts (Grant and Lamb, 2006; Vlot *et al.*, 2008; Shah, 2009).

A wealth of evidence demonstrates that the jasmonate pathway plays a central role in regulation of wound- and herbivory-induced defence responses in species throughout the plant kingdom (reviewed by Koo and Howe, 2009; Sun *et al.*, 2011). In this context, JA biosynthesis and signalling have been studied in the model systems of *Arabidopsis* and tomato, showing that the two models differ significantly. In tomato, insect attack or wounding triggers accumulation of systemin by cleavage from its precursor, prosystemin. Systemin interaction with its receptor in the plasma membrane activates synthesis of JA, which induces the expression of defensive proteins, such as proteinase inhibitor II (Pin2) (Rojo *et al.*, 2003). Recently, mutant analyses and grafting experiments indicate that JA, but not systemin, is the long-hunted mobile signal for systemic

Abbreviations: DTT, dithiothreitol; E, ethylene; GSNO, S-nitrosoglutathione; GSNOR, GSNO reductase; JA, jasmonic acid; NO, nitric oxide; RT-qPCR, reverse transcriptase quantitative PCR; SA, salicylic acid; SAR, systemic acquired resistance; SD, standard deviation; SNP, sodium nitroprusside; WT, wild type.

wound response in tomato (Sun *et al.*, 2011). In *Arabidopsis*, no homologous sequence of prosystemin could be identified in its genome, but a 23 aa peptide (called AtPep1) that activates transcription of the antimicrobial defensin *PDF1* gene has been characterized (Huffaker *et al.*, 2006). The peptide is derived from a 92 aa precursor encoded by a small gene that is inducible by wounding, JA, and E. Therefore, AtPep1 may be the functional homologue of systemin in *Arabidopsis*. Wound-induced systemic responses in *Arabidopsis* are mediated by two distinct pathways involving JA, the cell-non-autonomous pathway (in which JA is produced in the damaged leaf and transported to distal sites) and the cell-autonomous pathway (a mobile signal other than JA is produced in the damaged leaf and transported to the distal tissue, where it triggers JA synthesis). The two pathways may work synergistically to elicit the distal responses. In addition, a poorly understood but well-established JA-independent wound-signalling pathway is also operative in *Arabidopsis*. This pathway is activated by oligosaccharides released at the wounded tissues and induces the expression of a set of JA-independent genes (Rojo *et al.*, 1999).

Whereas JA is generally involved in the response to herbivory, SA plays a central role in the defence processes activated by pathogens. However, the separation between the signalling networks activated by herbivory/wounding and pathogens is not very neat, with some responses sharing elements of both mechanisms (Clarke *et al.*, 2000; Shah, 2003). Pathogen challenging stimulates SA synthesis, and SA accumulation at distal tissues is essential for the establishment of systemic acquired resistance (SAR) (reviewed by Vlot *et al.*, 2009). The nature of the mobile signal for SAR is still unknown. Once SA was discarded as the phloem-mobile signal, other molecules have been proposed, such as small peptides and lipid derivatives, methyl-SA, the apoplastic lipid transfer protein (DIR1), and *S*-nitrosoglutathione (GSNO) (Durner and Klessig, 1999; Maldonado *et al.*, 2002; Xia *et al.*, 2004; Park *et al.*, 2007; Rustérucci *et al.*, 2007). GSNO derives from the spontaneous reaction of glutathione (the major intracellular low-molecular-mass antioxidant) with nitric oxide (NO). The reaction is reversible, and GSNO might act both as NO reservoir and NO donor (Stamler *et al.*, 1992; Lindermayr *et al.*, 2005). NO, which is a gaseous reactive radical, regulates immunity in animals and plants, and is a common component of wound and pathogen responses (Durner and Klessig, 1999; Huang *et al.*, 2004; Wendehenne *et al.*, 2004; Feechan *et al.* 2005, Rustérucci *et al.* 2007; Chaki *et al.*, 2011). NO reacts with protein and non-protein thiols to form nitrosothiols (SNOs) (Stamler, 1994), and protein nitrosylation results in many cases in the regulation of protein activity (Lindermayr *et al.*, 2005; Romero-Puertas *et al.*, 2008; Tada *et al.*, 2008; Holzmeister *et al.*, 2011). Levels of SNOs *in vivo* are controlled by NO synthesis (which in plants is achieved by different routes) and by GSNO turnover, which is performed mainly by the enzyme GSNO reductase (GSNOR) (Liu *et al.*, 2001). GSNOR is an evolutionarily conserved enzyme that controls intracellular levels of both GSNO and *S*-nitrosylated proteins in eukaryotes (Liu *et al.*, 2001; Feechan *et al.*, 2005; Rustérucci *et al.*, 2007; Chaki *et al.*, 2011).

Arabidopsis GSNOR, previously known as glutathione-dependent formaldehyde dehydrogenase, is encoded by a single-copy gene (*ADH2*; GenBank accession no. X82647; Martínez *et al.*, 1996). We have described elsewhere the generation of *Arabidopsis* transgenic plants with higher or lower levels of GSNOR by overexpression of the *ADH2* gene or its antisense sequence, respectively (Achkor *et al.*, 2003; Rustérucci *et al.*, 2007). These transgenic plants showed modified levels of SNOs, which influence plant basal resistance and gene-mediated resistance. Moreover, SAR is compromised in plants overexpressing GSNOR and enhanced in antisense plants, suggesting an important role of GSNOR in SAR establishment (Rustérucci *et al.*, 2007). The current study further investigated the role of GSNOR in modulating GSNO levels *in vivo*, and the implications for wound and SAR responses. It was shown that GSNO accumulation is required to activate the JA-dependent wound responses, at both local and systemic sites. Moreover, GSNO acted synergistically with SA to mount the SAR response. It was concluded that GSNOR is a key regulator of systemic defence responses, in both wounding and pathogenesis.

Materials and methods

Plant growth

Wild-type (WT) *Arabidopsis thaliana* (Col-0) plants and the transgenic lines were germinated and grown in soil at 22°C under a 16 h light photoperiod. Four-week-old plants were used for all experiments. Wounding was performed in alternate rosette leaves using forceps. SA and sodium nitroprusside (SNP) were dissolved in water and infiltrated in alternate leaves at concentrations of 1 and 0.5 mM, respectively. Infiltrations with 0.5 mM potassium ferrocyanide were also performed as controls for SNP. All plant tissues were frozen in liquid N_2 after harvesting and kept at –80°C until use. In all treatments, leaves from several plants were mixed to avoid individual variations.

Transcriptional analysis

Total RNA was extracted with Ultraspec RNA (Biotech Laboratories, Texas, USA), and first-strand cDNA was synthesized with Moloney murine leukemia virus High Performance Reverse Transcriptase (RT; Epicentre Biotechnologies, Wisconsin, USA), following the manufacturer's instructions. Real-time quantitative PCR (qPCR) was performed using a Bio-Rad MyiQ single colour Real-Time PCR Detection System and SYBR Green Master Mix (BioRad Laboratories, CA, USA). The specificity of the PCRs was confirmed by melting-curve analysis at 55–95°C. The $-\Delta\Delta C_T$ values were calculated relative to those of the reference gene *EF1-α* (At5g60390) (Livak and Schmittgen, 2001). The gene-specific primer pairs are listed in Table 1.

Determination of SNO content

The concentration of total SNOs in WT *Arabidopsis* and in the transgenic lines was determined following the method of Saville (1958), as described by Rustérucci *et al.* (2007).

Determination of JA and SA concentration

Hormone extraction and analysis were carried out essentially as described by Durgbanshi *et al.* (2005), with slight modifications. Plant tissue was homogenized in distilled water using an

Ultra-Turrax tissue homogenizer (Ika-Werke, Staufen, Germany). Before homogenization, samples were spiked with a deuterated standard of SA (D_4-SA) and dihydrojasmonic acid as internal standards. After homogenization and centrifugation, the pH of the supernatant was adjusted to 3.0 and partitioned twice against diethyl ether. The organic layers were combined and evaporated in a centrifuge vacuum evaporator. The dry residue was thereafter resuspended in water:methanol (9:1) solution, filltered, and injected into an HPLC system (Alliance 2695; Waters Corporation, Massachusetts, USA). Hormones were separated in a reversed-phase C18 column using methanol and 0.01% acetic acid as solvents. The mass spectrometer, a triple quadrupole (Quattro LC; Micromass Ltd, Manchester, UK), was operated in negative ionization electrospray mode and the different plant hormones were detected according to their specific transitions using a multi-residue mass spectrometric method.

Immunolocalization of GSNO

Leaves were fixed with 4% (w/v) paraformaldehyde in 1× PBS buffer (3.2 mM Na_2HPO_4, 0,5 mM KH_2PO_4, 135 mM NaCl, 1.3 mM KCl, pH 7.3) for 3 d at 4°C. After fixation, transverse sections of 0.5–1 mm thickness were obtained with a blade and placed onto polylysine-coated slides. The sections were washed with PBSX solution [1× PBS, 0.05% (v/v) Triton-X-100] and treated with 2% Driselase in PBSX solution for 30 min at 37°C to digest the cell walls, followed by three washes with PBSX solution and two washes with 1× PBS. All further antibody incubations and washes were performed with blocking solution (1× PBS with 3% BSA and 0.1% Tween 20). Sections were blocked overnight at 4°C. Incubation with primary anti-GSNO antibody (diluted 1:500; Agrisera, Vännäs, Sweden) was performed for 2 h at 37°C. After several washes, the sections were further incubated with a biotinylated anti-rat IgG (diluted 1:200, Invitrogen, Paisley, UK, or diluted 1:5000, Sigma-Aldrich Co, St Louis, Missouri, USA) for 1.5 h at room temperature, washed, and finally incubated with Alexa Fluor 488–streptavidin conjugate (diluted 1:500; Invitrogen) for 1.5 h at room temperature. After extensive washes with blocking solution and 1× PBS, 0.1% Tween 20, slides were mounted on Fluoprep (bioMérieux, Marcy l'Etoile, France). Sections were observed with a Leica TCS-SP2 AOBS confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany), using standard filters (excitation 488 nm; emission 498–515 nm). Controls for background staining were performed by omitting the primary antiserum. Additional controls to test that the antibody reacted specifically with GSNO were performed by pre-infiltration of leaves with reducing agents. Four-week-old leaves from GSNOR antisense plants were infiltrated with 10 mM dithiothreitol (DTT) or 10 mM ascorbate, or both. After 30 min, GSNO inmunolocalization was performed as described above.

Table 1. Primers pairs used for RT-qPCR All amplifications were performed at an annealing temperature of 60°C.

Gene name	Accession number	Forward/ reverse	Primer sequence (5′→3′)
WR3	At5g50200	Forward	CTTCTCATATGCTCACTGATCCA
		Reverse	CGAGCTTAGCGTCCATGTAA
PDF1.2	At5g44420	Forward	TTTGCTGCTTTCGACGCAC
		Reverse	CGCAAACCCCTGACCATG
PR1	At2g14610	Forward	GCTACGCAGAACAACTAAGAGG
		Reverse	GCCTTCTCGCTAACCCACAT
EF1-α	At5g60390	Forward	TGAGCACGCTCTTCTTGCTTTCA
		Reverse	GGTGGTGGCATCCATCTTGTTACA

Results

GSNOR regulates SNO levels in wounded and systemic leaves

It has recently been described that wounding of sunflower hypocotyls leads to an increase in SNO content and a decrease in GSNOR protein (Chaki *et al.*, 2011). Wounding is also known to downregulate GSNOR expression and enzymatic activity in *Arabidopsis* (Díaz *et al.*, 2003). To see whether these observations were linked, we measured SNO levels following wounding in WT *Arabidopsis* plants and in the transgenic lines with modified GSNOR levels, described previously (Achkor *et al.*, 2003; Rustérucci *et al.*, 2007). Basal SNO levels in GSNOR-overexpressing plants and in antisense plants were 83 and 131%, respectively, of those in control plants (Fig. 1). These values are similar to those reported previously by our group (Rustérucci *et al.*, 2007). Upon wounding, SNO levels increased significantly in both WT and antisense plants but not in the overexpressing line. Moreover, antisense plants showed significantly higher SNO increments (120% in wounded leaves and 129% in systemic leaves) than those in WT plants (no significant increase in wounded leaves and 117% in systemic leaves) (Fig. 1). These data showed that wounding induced SNO accumulation and that GSNOR modulated SNO levels.

Immunolocalization of GSNO in WT and transgenic plants

The total SNOs measured comprised GSNO, which is the actual target of degradation by GSNOR. In order to

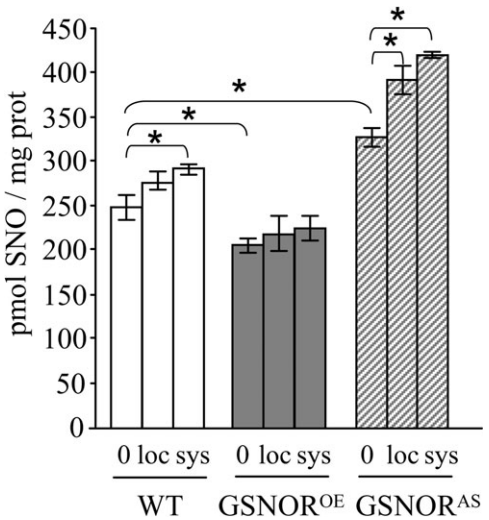

Fig. 1. SNO levels upon plant wounding. Four-week old *Arabidopsis* leaves were wounded and the tissues were harvested after 1 h (local injured leaves) or 4 h (systemic leaves). The results shown are the mean of three independent experiments ±standard deviation (SD). WT, wild-type *Arabidopsis* plants; $GSNOR^{OE}$, GSNOR-overexpressing line; $GSNOR^{AS}$, GSNOR antisense line; 0, unwounded plants; loc, local injured leaves; sys, systemic leaves of wounded plants. Pairs for Student's *t*-test are indicated with brackets: *, $P < 0.05$.

analyse GSNO levels in basal conditions and after wounding, immunolocalization experiments were performed on leaf sections using a GSNO antibody. GSNO was visualized as green immunofluorescence by confocal microscopy. Under these experimental conditions, GSNO localized uniformly throughout the parenchyma and the vascular tissue in WT plants (Fig. 2A, B). Immunofluorescence was considerably reduced in plants overexpressing GSNOR (Fig. 2C, D) and enhanced in antisense plants (Fig. 2E, F). These results were in agreement with the total levels of SNOs measured in leaf extracts of the different lines (see Fig. 1). Controls for background labelling, which was always negligible, were performed by omitting the incubation with primary anti-GSNO antibody (Fig. 2G, H). Upon wounding, GSNO labelling clearly increased in the injured leaves, with higher accumulation in the proximity of the damaged regions (Fig. 3E–H). Moreover, GSNO labelling also increased in systemic leaves, first in the vascular tissue (30 min after wounding; Fig. 3I, J) and later (2 h after wounding) spreading all over the parenchyma cells (Fig. 3K, L). Background staining in controls deprived of primary antibody was always negligible (Fig. 3C, D). Additional controls to test the specificity of the anti-GSNO antibody were performed by pre-infiltration of leaves from GSNOR antisense plants (the line with the stronger GSNO signal) with reducing agents, such as DTT and/or ascorbate. GSNO labelling clearly decreased after treatments with the reducing agents, confirming that the antibody reacts specifically with GSNO (Supplementary Fig. S1 in *JXB* online). Taken together, these results, combined with the increased SNO levels determined in systemic leaves in response to wounding, strongly suggested that GSNO is involved in transmission of the wound signal from injured to systemic tissues through the vascular tissue.

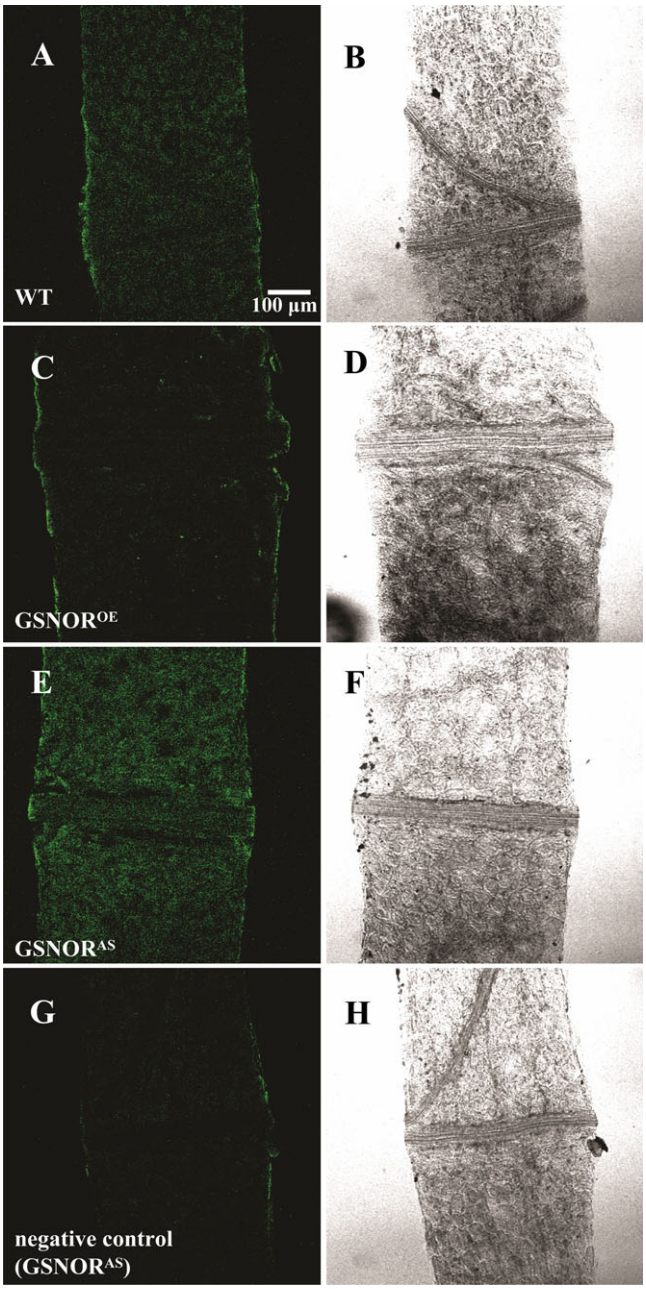

Fig. 2. GSNO immunolocalization in leaf sections. Pictures show confocal laser-scanning microscopy images of GSNO immunolabelling with a specific anti-GSNO antibody (A, C, E, and G), and the corresponding transmission light microscopy images (B, D, F, and H). A negative control for background staining (G, H) was also included. See Fig. 1 legend for abbreviations.

GSNOR activity modulates the JA-dependent wound responses

To further study the role of GSNOR in the wound response, we measured the expression levels of wound-responsive gene 3, *WR3* (At5g50200), which is activated by the JA-independent pathway, and of defensin *PDF1.2* (At5g44420), a molecular marker of the E- and JA-dependent pathways.

All three *Arabidopsis* lines analysed showed similar patterns of *WR3* induction, at both local and distal sites (Fig. 4A), suggesting that GSNOR activity, and by inference GSNO, is not a component of the JA-independent signalling pathway. In contrast, the pattern of wound-induced *PDF1.2* expression was very different in WT and transgenic lines. WT plants showed low basal levels of *PDF1.2*, which were significantly induced upon wounding (up to 9- and 5-fold in wounded and systemic leaves, respectively) (Fig. 4B). GSNOR-overexpressing plants showed lower *PDF1.2* basal levels and impaired *PDF1.2* induction upon wounding, in both local and systemic leaves. Finally, antisense GSNOR plants showed constitutive high expression of *PDF1.2* in unwounded leaves (up to 164-fold compared with WT plants), which further increased upon wounding in systemic tissues (4-fold). Taken together, these data suggested that GSNO is involved in activation of the E- and JA-dependent pathways, and that increased levels of GSNO are able to constitutively keep the response activated. The more pronounced induction of *PDF1.2* expression in systemic tissue is in agreement with the higher SNOs levels under the same conditions, and confirmed the idea that SNOs play an important role in wound-induced systemic responses.

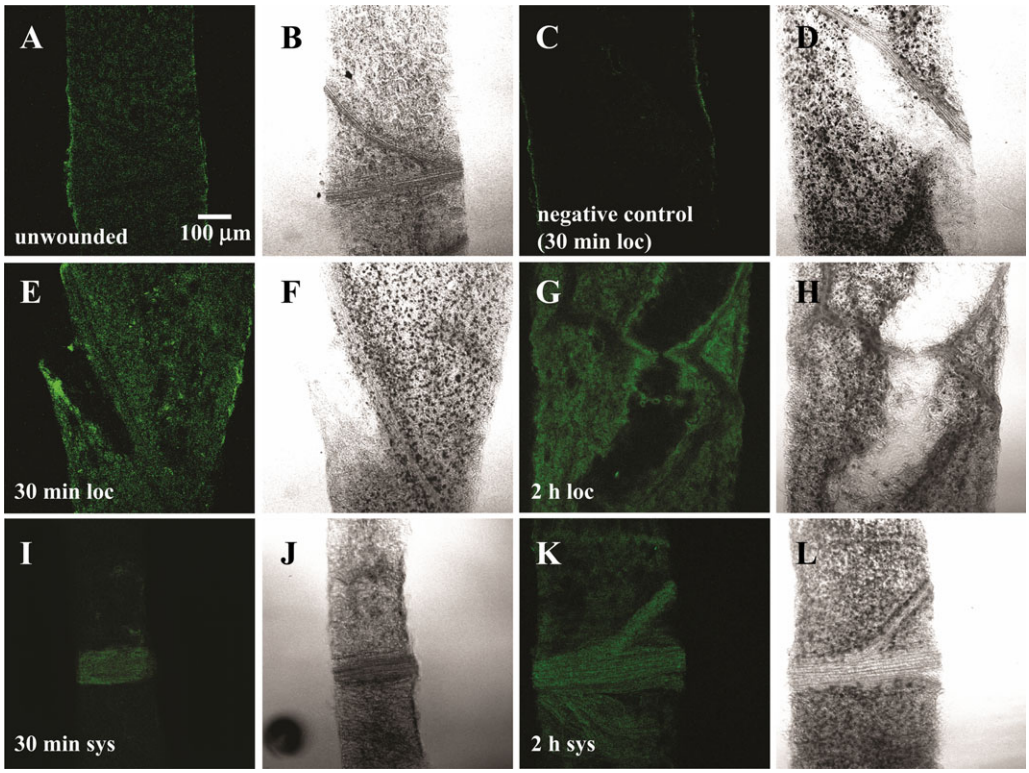

Fig. 3. GSNO immunolocalization after wounding. Pictures show confocal laser-scanning microscopy images of GSNO immunolabelling in unwounded (A) and wounded (E, G, I, K) leaves in both local and systemic tissues, as indicated. The corresponding transmission light microscopy images are also shown (B, F, H, J and L). A negative control for background staining (C, D) was also included. See Fig. 1 legend for abbreviations.

GSNOR activity modulates SAR

According to the results above, GSNO appeared to be involved in both local and systemic responses to wounding. We wondered whether the pathways triggered in defence against pathogens were similarly affected. To this purpose, a pathogen attack was mimicked by treatments with SA, as it is known that SA induces local and systemic defence responses in the absence of pathogen infection (Ward *et al.*, 1991). The expression of the SA-signalling marker gene *PR1* (At2g14610) was measured in SA-infiltrated leaves (local response) and in untouched leaves from the same plants (systemic response). As a control, plants were mock infiltrated with water and showed no changes in *PR1* levels under these conditions (data not shown). Fig. 5A shows that all the lines were able to induce a similar strong response in SA-infiltrated leaves, although, strikingly, *PR1* induction was more rapid in both transgenic lines than in WT plants. To assess the effects of GSNOR on SAR establishment, we measured *PR1* expression in systemic leaves (Fig. 5B). *PR1* was significantly induced in WT plants, with a peak at 48 h after infiltration. However, plants overexpressing GSNOR failed to induce PR1 systemically, suggesting that SAR was impaired in these plants, which is in agreement with our previous data shown in Rustérucci *et al.* (2007). Moreover, antisense plants showed a rapid and stronger systemic induction of *PR1*, attaining up to 9-fold the transcript levels of WT plants at 24 h. On the whole, these results corroborated the idea that GSNOR plays an important role in SAR modulation.

Determination of JA and SA content in WT and transgenic lines

It has been reported that both wounding and SA are able to induce NO synthesis (Wendehenne, *et al.*, 2004; Zottini *et al.*, 2007), suggesting that NO acts downstream of JA and SA in plant-induced defences. However, it has also been proposed that GSNOR may control SA synthesis (Feechan *et al.*, 2005), as loss-of-function GSNOR mutants (*atgsnor1-3* plants) showed reduced basal SA content, whereas gain-of-function GSNOR mutants (*atgsnor1-1*) showed enhanced SA content. In order to ascertain whether our transgenic lines contained altered hormone levels, SA and JA were measured in all the lines. The results showed that SA levels were nearly equivalent in the WT and the transgenic lines (Fig. 6A). In contrast, JA levels were increased in both the overexpressing (122%) and the antisense line (117%) compared with the levels found in WT plants grown under the same conditions (Fig. 6B).

Discussion

This work studied the roles of GSNOR in modulating *in vivo* GSNO levels, and its implications for wound-induced

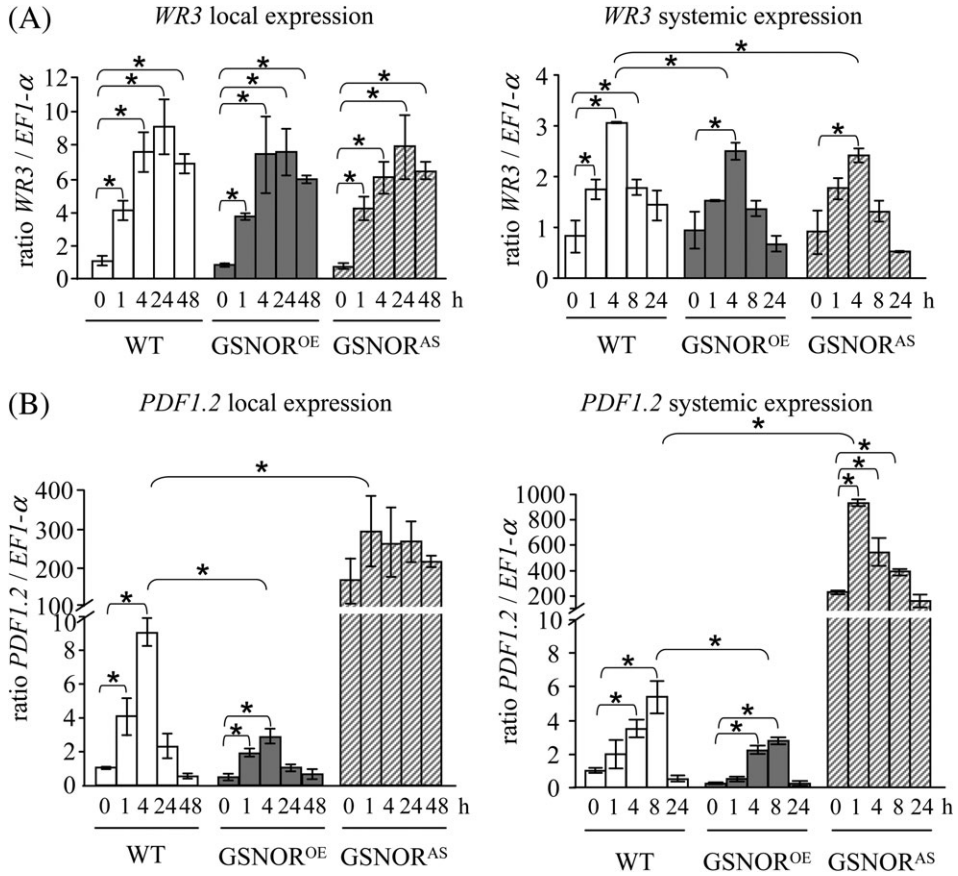

Fig. 4. Transcriptional analysis of the wound response. (A) Transcriptional activation of the JA-independent wound-responsive *WR3* gene, in local (left) and systemic (right) tissues. (B) Transcriptional activation of the JA-dependent wound-responsive *PDF1.2* gene, measured under the same conditions as in (A). Scales on the *y*-axes in (B) have been interrupted for a better representation. Measures were performed by RT-qPCR and the corresponding values calculated relative to those of the constitutively expressed *EF1-α* gene. The results shown are the mean of three independent experiments ±SD. Pairs for Student's *t*-test are indicated with brackets: *, $P < 0.05$. See Fig. 1 legend for abbreviations.

responses and SAR. *Arabidopsis* transgenic plants were used with modified levels of GSNOR activity, obtained by overexpression of sense or antisense constructs of the *ADH2* gene encoding GSNOR (Achkor *et al.*, 2003). We have reported previously that these transgenic plants exhibit changes in both basal SNO concentrations and their capability to induce SNO accumulation after pathogen challenge (Rustérucci *et al.*, 2007). In this work, it was shown that wounding induced SNO accumulation in WT and antisense GSNOR plants, in both local and systemic tissues, whereas overexpressing GSNOR plants were impaired in this response. Moreover, SNO levels in antisense plants were always higher than in WT plants, in both wounded and systemic leaves. Taken together, these results strongly suggested that continuous and unregulated overexpression of GSNOR hindered the normal increments of SNO production upon wounding and pathogen attack, whereas downregulation of GSNOR using antisense constructs facilitated its accumulation under the same conditions.

These results also showed that GSNOR activity modulated the E/JA-dependent wound responses, as the transcriptional levels of the JA-inducible *PDF1.2* gene showed a strict correlation with SNO levels (higher induction in the antisense line and lower induction in the overexpressing line compared with WT plants). Surprisingly, the JA basal concentration was slightly enhanced in both transgenic lines, suggesting that increased JA levels were not sufficient for *PDF1.2* induction. Thus, GSNO might act synergistically with JA to modulate *PDF1.2* expression in response to wounding. In support of this, it was shown that *PDF1.2* transcript levels increased after SNP treatment (a NO donor), in both SNP-infiltrated leaves and systemic leaves (Supplementary Fig. S2A, in *JXB* online).

In contrast, expression of the JA-insensitive *WR3* gene was unaffected by changes in intracellular GSNOR activity, indicating that NO/GSNO is not a signalling component of this alternative wound-signalling pathway.

Immunolocalization of GSNO in leaf sections revealed interesting results. In agreement with the SNO levels measured in the different lines, enhanced GSNO staining was detected in the antisense GSNOR plants and very faint labelling in the overexpressing lines compared with GSNO levels in WT plants. In addition, GSNO labelling increased rapidly and uniformly in wounded leaves, and also increased in systemic leaves, but in the latter case GSNO accumulation

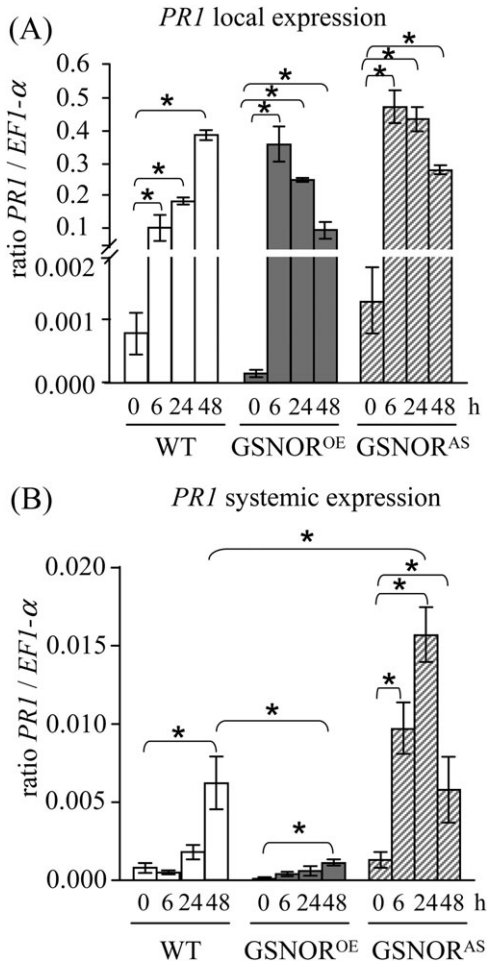

Fig. 5. SA-induced *PR1* gene expression. *PR1* transcript levels were measured by RT-qPCR in SA-infiltrated leaves (A) and in systemic leaves (B). The values are represented relative to those of the constitutively expressed *EF1-α* gene. The scale on the *y*-axis in (A) has been interrupted for a better representation. The results shown are the mean of three independent experiments ±SD. Pairs for Student's *t*-test are indicated with brackets: *, $P < 0.05$. See Fig. 1 legend for abbreviations.

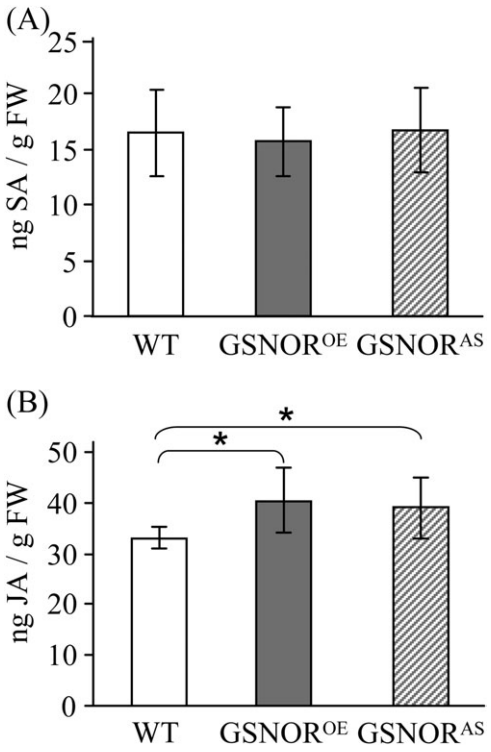

Fig. 6. SA and JA content. SA and JA were measured in WT *Arabidopsis* plants and in the GSNOR transgenic lines ($GSNOR^{OE}$, overexpressing line; $GSNOR^{AS}$, antisense line). The results are the mean of three independent experiments ±SD. Pairs for Student's *t*-test are indicated with brackets: *, $P < 0.05$. FW, fresh weight.

was first detected only in vascular tissues and later spread all over the parenchyma. These results are in agreement with results in sunflower hypocotyls, showing that wounding triggers accumulation of GSNO and reduction of GSNOR content (Chaki *et al.*, 2011), and with previous results from our group showing the downregulation of *Arabidopsis* GSNOR expression by wounding and JA (Díaz *et al.*, 2003). Moreover, the pattern of GSNO systemic accumulation favours the idea of GSNO as the mobile signal being transported through the phloem, a hypothesis that has been formulated previously and that is supported by the reported localization of GSNOR protein in the phloem (Rustérucci *et al.*, 2007) and of its substrate, GSNO, in the collenchyma cells located adjacent to the vascular cambium (Barroso *et al.*, 2006). However, it remains to be elucidated whether GSNO itself, or a secondary signal generated at the wounded leaves, is the long-distance signal responsible of GSNO increments at distal sites.

We also showed that SA-mediated SAR activation required GSNO accumulation at the distal sites: SA infiltration failed to induce *PR1* systemically in GSNOR-overexpressing lines, whereas it induced *PR1* systemically in the antisense line. Moreover, SNP treatments in WT plants induced *PR1* expression, in both local and systemic leaves (Supplementary Fig. S2B). These results corroborated our previous data showing the impairment of antisense GSNOR plants in SAR establishment (Rustérucci *et al.*, 2007).

An important aspect of this study was that, in contrast to the *atgsnor1-1* and *atgsnor1-3* plants reported by Feechan *et al.* (2005), both our transgenic lines contained SA levels that were similar to those in WT plants (Fig. 6A). This implies that responses in our mutants derived from the modified GSNOR activity and GSNO content, and were not an indirect effect of SA accumulation. Moreover, both our lines were able to induce PR1 expression by exogenous SA, indicating that the SA-signalling pathway was not impaired.

It is commonly accepted that SA signalling is regulated through the activity and subcellular localization of NPR1 (Tada *et al.*, 2008; Vlot *et al.*, 2009). NPR1 resides in the cytoplasm in an oxidized oligomeric form (Mou *et al.*, 2003; Tada *et al.*, 2008), and SA accumulation after pathogen attacks triggers its reduction and subsequent monomerization. NPR1 monomer is translocated to the nucleus, where it interacts with the transcription factor TGA1, activating *PR1* expression. The NPR1/TGA1-mediated signalling pathway is also regulated by NO/GSNO via *S*-nitrosylation and possibly

glutathionylation of both proteins (Tada *et al.*, 2008; Lindermayr *et al.*, 2010). Some authors have shown that *S*-nitrosylation of NPR1 promotes its oligomerization and thus cytoplasmic localization (Tada *et al.*, 2008). However, other authors have shown that NO promotes NPR1 translocation to the nucleus, where it interacts with *S*-nitrosylated TGA1, enhancing TGA1 DNA-binding activity (Lindermayr *et al.*, 2010). To conciliate these disparate results, it has been proposed that the *S*-nitrosylation-mediated oligomerization might not have an inhibitory effect on NPR1 but may constitute a step prior to monomer accumulation, favouring the idea of a positive effect of NO/GSNO on plant defence. Additionally, Lindermayr *et al.* (2010) propose that a secondary, activating *S*-nitrosylation of NPR1 might occur once this protein is already in the nucleus. These complex and apparently contradictory effects of NO/GSNO on NPR1 regulation might explain why the results obtained with mutants completely devoid of GSNOR activity (Feechan *et al.*, 2005) were the opposite of those obtained in mutants with 50% of GSNOR activity (Rustérucci *et al.*, 2007). A GSNOR knockout might entirely hinder activation of the NPR1/TGA1-signalling pathway, whereas diminished levels of GSNOR activity might favour the existence of the appropriate ratio of *S*-nitrosylated/glutathionylated NPR1/TGA1 forms, with a positive effect on plant defence. Moreover, defence responses of GSNOR knockout mutants obtained in different *Arabidopsis* ecotypes are strikingly contradictory (Feechan *et al.*, 2005; Holzmeister *et al.*, 2011). Our data support the idea that low, but not null, sustained levels of GSNOR activity have a positive effect on plant defence, and particularly on SAR establishment.

In summary, the results presented in this work highlight the importance of GSNOR activity in modulating systemic responses to wounding and pathogens. The results show that GSNO acts synergistically with classical hormones involved in plant defence (particularly SA and JA) to activate gene responses at local and systemic sites.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Specificity of the anti-GSNO antibody. Leaves from GSNOR antisense plants were untreated (A, B), or infiltrated with 10 mM DTT (C, D), 10 mM ascorbate (E, F), or both (G, H), prior to GSNO immunolocalization. Confocal microscopy was used to show the disappearance of GSNO labelling in the presence of the reducing agents compared with the untreated control. A negative control without primary GSNO antibody (I, J) was also included.

Supplementary Fig. S2. Induction of *PDF1.2* and *PR1* expression by SNP. Alternate leaves were infiltrated with 0.5 M SNP (an NO donor), and local and systemic leaves were harvested at the times indicated. Transcript levels were measured by RT-qPCR and the values were calculated relative to those of the constitutive *EF1-α* gene. The results shown are the mean of three independent experiments ±SD. Pairs for Student's *t*-test are indicated with brackets: *, $P < 0.05$. To test that NO, and not cyanide, had mediated these responses, *PR1* and *PDF1.2* expression was also measured at different times after leaf infiltration with potassium ferrocyanide. No significant expression changes after ferrocyanide infiltration were found (data not shown).

Acknowledgements

This work was supported by grants BFU2007-60569, BFU2010-15090, and Consolider Ingenio 2010 CSD2007-00036 (Ministerio de Educación y Ciencia, Spain) and by grant 2009SGR 0795 (Generalitat de Catalunya, Catalunya, Spain). We thank the Servei de Microscopia and the Laboratori d'anàlisi i fotodocumentació (Universitat Autònoma de Barcelona, Spain) for technical support.

References

Achkor H, Díaz M, Fernandez MR, Biosca JA, Pares X, Martínez MC. 2003. Enhanced formaldehyde detoxification by overexpression of glutathione-dependent formaldehyde dehydrogenase from Arabidopsis. *Plant Physiology* **132,** 2248–2255.

Bari R, Jones JDG. 2009. Role of plant hormones in plant defence responses. *Plant Molecular Biology* **69,** 473–488.

Barroso JB, Corpas FJ, Carreras A, *et al*. 2006. Localization of *S*-nitrosoglutahione and expression of *S*-nitrosoglutathione reductase in pea plants under cadmium stress. *Journal of Experimental Botany* **57,** 1785–1793.

Chaki M, Valderrama R, Fernández-Ocaña AM, *et al*. 2011. Mechanical wounding induces a nitrosative stress by down-regulation of GSNO reductase and an increase in *S*-nitrosothiols in sunflower (*Helianthus annuus*) seedlings. *Journal of Experimental Botany* **62,** 1803–1813.

Clarke JD, Volko SM, Ledford H, Ausubel FM, Dong X. 2000. Roles of salicylic acid, jasmonic acid, and ethylene in *cpr*-induced resistance in Arabidopsis. *The Plant Cell* **12,** 2175–2190.

Díaz M, Achkor H, Titarenko E, Martínez MC. 2003. The gene encoding glutathione-dependent formaldehyde dehydrogenase/GSNO reductase is responsive to wounding, jasmonic acid and salicylic acid. *FEBS Letters* **543,** 136–139.

Durgbanshi A, Arbona V, Pozo O, Miersch O, Sancho JV, Gomez-Cadenas A. 2005. Simultaneous determination of multiple phytohormones in plant extracts by liquid chromatography-electrospray tandem mass spectrometry. *Journal of Agricultural and Food Chemistry* **53,** 8437–8442.

Durner J, Klessig DF. 1999. Nitric oxide as a signal in plants. *Current Opinion in Plant Biology* **2,** 369–374.

Feechan A, Kwon E, Yun BW, Wang Y, Pallas JA, Loake GJ. 2005. A central role for *S*-nitrosothiols in plant disease resistance. *Proceedings of the National Academy of Sciences, USA* **102,** 8054–8059.

Grant M, Lamb C. 2006. Systemic immunity. *Current Opinion in Plant Biology* **9,** 414–420.

Holzmeister C, Fröhlich A, Sariogu H, Bauer N, Durner J, Lindermayr C. 2011. Proteomic analysis of defence response of wild type *Arabidopsis thaliana* and plants with impaired NO^- homeostasis. *Proteomics* **11,** 1664–1683.

Huang X, Stettmaier K, Michel C, Hutzler P, Mueller MJ, Durner J. 2004. Nitric oxide is induced by wounding and influences jasmonic acid signaling in *Arabidopsis thaliana*. *Planta* **218,** 938–946.

Huffaker A, Pearce G, Ryan CA. 2006. An endogenous peptide signal in *Arabidopsis* activates components of the innate immune response. *Proceedings of the National Academy of Sciences, USA* **103,** 10098–10103.

Koo AJ, Howe GA. 2009. The wound hormone jasmonate. *Phytochemistry* **70,** 1571–1580.

Lindermayr C, Saalbach G, Durner J. 2005. Proteomic identification of *S*-nitrosylated proteins in Arabidopsis. *Plant Physiology* **137,** 921–930.

Lindermayr C, Sell S, Müller B, Leister D, Durner J. 2010. Redox regulation of the NPR1–TGA1 system of *Arabidopsis thaliana* by nitric oxide. *The Plant Cell* **22,** 2894–2907.

Liu L, Hausladen A, Zeng M, Que L, Heitman J, Stamler JS. 2001. A metabolic enzyme for *S*-nitrosothiol conserved from bacteria to humans. *Nature* **410,** 490–494.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta\ C}{}_{T}$ method. *Methods* **25,** 402–408.

López MA, Bannenberg G, Castresana C. 2008. Controlling hormone signaling is a plant and pathogen challenge for growth and survival. *Current Opinion in Plant Biology* **11,** 420–427.

Maldonado AM, Doerner P, Dixon RA, Lamb CJ, Cameron RK. 2002. A putative lipid transfer protein involved in systemic resistance signalling in *Arabidopsis*. *Nature* **419,** 399–403.

Martínez MC, Achkor H, Persson B, Fernández MR, Shafqat J, Farrés J, Jörnvall H, Parés X. 1996. *Arabidopsis* formaldehyde dehydrogenase: molecular properties of plant class III alcohol dehydrogenase provide further insights into the origins, structure and function of plant class P and liver class I alcohol dehydrogenases. *European Journal of Biochemistry* **241,** 849–857.

Mou Z, Fan W, Dong X. 2003. Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* **113,** 935–944.

Park SW, Kaimoyo E, Kumar D, Mosher S, Klessig DF. 2007. Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. *Science* **318,** 113–116.

Rojo E, León J, Sánchez-Serrano JJ. 1999. Cross-talk between wound signalling pathways determines local versus systemic gene expression in *Arabidopsis thaliana*. *The Plant Journal* **20,** 135–142.

Rojo E, Solano R, Sánchez-Serrano JJ. 2003. Interactions between signaling compounds involved in plant defense. *Journal of Plant Growth Regulation* **22,** 82–98.

Romero-Puertas MC, Campostrini N, Mattè A, Righetti PG, Perazolli M, Zolla L, Roepstorff P, Delledonne M. 2008. Proteomic analysis of *S*-nitrosylated proteins in *Arabidopsis thaliana* undergoing hypersensitive response. *Proteomics* **8,** 1459–1469.

Rustérucci C, Espunya MC, Díaz M, Chabannes M, Martínez MC. 2007. *S*-Nitrosoglutathione reductase affords protection against pathogens in Arabidopsis, both locally and systemically. *Plant Physiology* **143,** 1282–1292.

Saville B. 1958. A scheme for the colorimetric determination of microgram amounts of thiols. *Analyst* **83,** 670–672.

Shah J. 2003. The salicylic acid loop in plant defense. *Current Opinion in Plant Biology* **6,** 365–371.

Shah J. 2009. Plants under attack: systemic signals in defence. *Current Opinion in Plant Biology* **12,** 459–464.

Stamler JS. 1994. Redox signalling: nitrosylation and related target interactions of nitric oxide. *Cell* **78,** 931–936.

Stamler JS, Jaraki O, Osborne J, Simon DI, Keaney K, Vita J, Singel D, Valeri CR, Loscalzo J. 1992. Nitric oxide circulates in mammalian plasma primarily as an *S*-nitroso adduct of serum albumin. *Proceedings of the National Academy of Sciences, USA* **89,** 7674–7677.

Sun JQ, Jiang HL, Li CY. 2011. Systemin/jasmonate-mediated systemic defense signaling in tomato. *Molecular Plant* **4,** 607–615.

Tada Y, Spoel SH, Pajerowska-Mukhtar K, Mou Z, Song J, Wang C, Zuo J, Dong X. 2008. Plant immunity requires conformational charges of NPR1 via *S*-nitrosylation and thioredoxins. *Science* **321,** 952–956.

Vlot AC, Dempsey A, Klessig DF. 2009. Salicylic acid, a multifaceted hormone to combat disease. *Annual Review of Phytopathology* **47,** 177–206.

Vlot AC, Klessig DF, Park SW. 2008. Systemic acquired resistance, the elusive signal(s). *Current Opinion in Plant Biology* **11,** 436–442.

Ward ER, Uknes SJ, Williams SC, Dincher SS, Wiederhold DL, Alexander DC, Ahl-Goy P, Metraux JP, Ryals JA. 1991. Coordinate gene activity in response to agents that induce systemic acquired resistance. *The Plant Cell* **3,** 1085–1094.

Wendehenne D, Durner J, Klessig DF. 2004. Nitric oxide, a new player in plant signalling and defence responses. *Current Opinion in Plant Biology* **7,** 449–455.

Xia Y, Suzuki H, Borevitz J, Blount J, Guo Z, Patel K, Dixon RA, Lamb C. 2004. An extracellular aspartic protease functions in *Arabidopsis* disease resistance signalling. *EMBO Journal* **23,** 980–988.

Zottini M, Costa A, De Michele R, Ruzzene M, Carimi F, Lo Schiavo F. 2007. Salicylic acid activates nitric oxide synthesis in Arabidopsis. *Journal of Experimental Botany* **58,** 1397–1405.