A stress-inducible sulphotransferase sulphonates salicylic acid and confers pathogen resistance in *Arabidopsis*

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ABSTRACT

Sulphonation of small molecules by cytosolic sulphotransferases in mammals is an important process in which endogenous molecules are modified for inactivation/activation of their biological effects. Plants possess large numbers of sulphotransferase genes, but their biological functions are largely unknown. Here, we present a functional analysis of the *Arabidopsis* sulphotransferase AtSOT12 (At2g03760). AtSOT12 gene expression is strongly induced by salt, and osmotic stress and hormone treatments. The T-DNA knock-out mutant *sot12* exhibited hypersensitivity to NaCl and ABA in seed germination, and to salicylic acid (SA) in seedling growth. In *vitro* enzyme activity assay revealed that AtSOT12 sulphonates SA, and endogenous SA levels suggested that sulphonation of SA positively regulates SA production. Upon challenging with the pathogen *Pseudomonas syringae*, *sot12* mutant and *AtSOT12* over-expressing lines accumulate less and more SA, respectively, when compared with wild type. Consistent with the changes in SA levels, the *sot12* mutant was more susceptible, while *AtSOT12* over-expressing plants are more resistant to pathogen infection. Moreover, pathogen-induced PR gene expression in systemic leaves was significantly enhanced in *AtSOT12* over-expressing plants. The role of sulphonation of SA in SA production, mobile signalling and acquired systemic resistance is discussed.

Key-words: pathogen resistance; salicylic acid; sulphotransferase; systemic response.

INTRODUCTION

Sulphonation is an important reaction involved in the biotransformation of various endogenous molecules and xenobiotics. This biological phenomenon is present in various organisms and has a crucial role in cell growth, development and defence (Strott 2002). The sulphonation reaction is catalysed by sulphotransferases in living organisms. The reaction involves transfer of a sulpharyl group (SO₃⁻) from the universal sulphonate donor 3′-phosphoadenosine 5′-phosphosulphate (PAPS) to an acceptor substrate. Acceptor molecules include wide array of substances, from endogenous molecules, such as hormones, neurotransmitters and peptides, to exogenous chemicals such as drugs, xenobiotics and toxins.

Sulphonation of small molecules can either activate or inactivate a biological response. Sulphonation of steroid and thyroid hormones can lead to inactivation of their biological activities because sulphonated hormones are incapable of binding to their cognate receptors (Strott 2002). In general, sulphoconjugation converts drugs and xenobiotics into more soluble and less toxic metabolites; therefore, aiding their excretion from the body. Thus, sulphonation can be considered to be a cellular mechanism against toxic chemicals, and SULTs are often referred to as enzymes of chemical defence (Negishi et al. 2001). In some cases, however, SULTs can activate hormones and change certain exogenous compounds into mutagenic and carcinogenic metabolites (Banoglu 2000; Glatt 2000). For example, sulphonation of the hormone pregnenolone leads to the formation of a potent neuroexcitatory agent pregnenolone sulphate (Paul & Purdy 1992); sulphonylation of the drug minoxidil leads to the formation of the active form minoxidil sulphate (Buhl et al. 1990). Sulphonation is also an important factor in the regulation of steroid biosynthesis, because the sulphated dehydroepiandrosterone (DHEA) and estrone are the precursors of androgen and estrogen biosyntheses (Falany et al. 1994; Falany & Falany 1997).

Like mammals, plants contain large numbers of sulphotransferase genes (designated as SOTs to avoid nomenclature confusion with ST broadly used as the abbreviation of sulphate transporter; Klein & Papenbrock 2004). Sequence analysis has identified 18 cytosolic sulphotransferases in *Arabidopsis* and 35 in rice. However, the biological functions of SOTs in plants are still largely unknown. Varin et al. (1992) isolated, for the first time, two cDNA clones coding for flavonol 3- and 4′-sulphotransferases from *Flaveria chloraeofolia*. These two SOTs exhibit strict substrate and hydroxyl position specificity (Varin et al. 1992; Varin, Marsolais & Brisson 1995). Choline-O-sulphate accumulates in the halophytic genus *Limonium* and other Plumbaginaceae under saline conditions. The formation of choline-O-sulphate is catalysed by a choline sulphotransferase whose activity is induced by salt stress in these halophyte species (Rivoal & Hanson 1994), suggesting that this
SOT has a role in tolerance against salt stress by producing osmoprotectant choline-ß-sulphate. In 
Brassica napus, a SOT protein was shown to sulphonate 24-epibrassinolide and abolish its biological activity, implicating this SOT in brassinosteroid hormone homeostasis in plants (Rouleau et al. 1999).

Arabidopsis SOTs (designated as AtSOTs) can be divided into seven subfamilies according to sequence similarity (Klein & Papenbrock 2004). Of the 18 AtSOTs, several members have been characterized at a biochemical and/or molecular level to various degrees. The first identified Arabidopsis SOT RaR047 (At2g03760, SOT12) by Lacomme & Roby (1996) has been recently characterized (Marsolais et al. 2007). AtSOT12 was shown to be stereospecific for 24-epibrassinosteroids, with a substrate preference for the metabolic precursor 24-epicathasterone, and has catalytic activity with hydroxysteroids and estrogens. Comparable activity profiles of AtSOT12 with human SULT2A1 suggest architecture similarities for the acceptor-binding site between the two enzymes, and may reflect a common ability to conjugate certain xenobiotics (Marsolais et al. 2007). Another Arabidopsis SOT, AtSOT10/At2g14920 sharing 44% in amino acid sequence with AtSOT12 was also characterized as a brassinosteroid sulphotransferase with the capacity to sulphonate brassinosteroids with diverse side chain structures, including 24-epibrassinosteroids and the naturally occurring (22R, 23R)-28-homobrassinosteroids (Marsolais et al. 2007). However, two other members, AtSOT8/At1g13420 and AtSOT9/At1g13430, were not catalytically active for brassinosteroids, although these two SOTs have high sequence identity with AtSOT10. The AtSOT5/At3g45070 was shown to be a flavonoid 7-sulphotransferase (Gidda & Varin 2006). Three AtSOT members, AtSOT16/At1g74100, AtSOT17/At1g18590 and AtSOT18/At1g74090, could catalyse the sulphonation of desulphoglucosinolates (Piotrowski et al. 2004; Klein et al. 2006). SOT15/At5g07010 sulphonates hydroxyjasmonate, suggesting that this sulphotransferase plays a role in plant JA inactivation (Gidda et al. 2003).

Salicylic acid (SA) is an important signal molecule in plant defence. The role of SA in local defence and in systemic acquired resistance (SAR) has been extensively studied (Vlot, Dempsey & Klessig 2009). Although SA production is required for SAR, a number of experiments argue against SA being the mobile signal molecule for SAR (Durrant & Dong 2004). In tobacco, methyl salicylate (MeSA) appears to serve as a SAR signal (Park et al. 2007); however, MeSA production does not appear to be essential for SAR in Arabidopsis (Attaran et al. 2009). Here, we present molecular evidence that SA can be sulphonated by the Arabidopsis sulphotransferase AtSOT12 (At2g03760) and that this derivatization impacts pathogen resistance and systemic signalling in Arabidopsis. To our knowledge, there are no reports as to whether SA derivatization by sulphonation may play a role in SAR signalling. Sulphonated SA in vivo can trigger SA production as evidenced by both loss-and-gain-of-function analyses. Sulphonated SA is also proposed to serve as a mobile signal for Arabidopsis SAR.

MATERIALS AND METHODS

Plant materials and growth

Arabidopsis thaliana ecotypes Col-0 and WS-0, T-DNA knock-out mutant sot12 and transgenic plants were grown in 16 h photoperiod at 23 °C in 50–60% humidity. sot12 T-DNA insertion mutant in WS-0 background was obtained from INRA (FLAG_340D07). Homozygous knock-out mutant plants were identified by PCR amplification using the T-DNA-specific primer and a pair of gene-specific primers flanking the T-DNA insertion. The gene-specific primers used are: forward, 5'-CGTG|TACTAGTGCTAGTGGCC-3' and reverse, 5'-CTTCTGGTCAACC|AACCC ACACC-3'. Arabidopsis transgenic plants for promoter analysis, AtSOT12 over-expression and sot12 complementation were generated by transfer the constructs described below into Arabidopsis using Agrobacterium-mediated floral dip method. AtSOT12 over-expression construct was made by insertion of the AtSOT12 complete open reading frame (ORF) into BamHI/Sall sites of the plasmid vector pCAMBIA 99-1, which generates an AtSOT12 over-expression construct driven by the 35S promoter. The primers used to amplify AtSOT12 ORF are: 5'-ATC|GGATCC|TCTAGGTTTCTTTCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTTTCCTTTT
MS agar media with different concentrations of chemicals. Germination (emergence of radicals) was scored daily for 10 d. Root growth on different media was assayed by transferring 4-day-old seedlings onto 1/2 MS medium with 1.2% agar supplemented with various concentrations of different chemicals, and the plates were placed vertically. New root elongation was measured at designated time intervals. To test sensitivity of sot12 mutant and AtSOT12 over-expressing plants to pathogen, the bacterial pathogen Pst DC3000 was grown with empty vector (pVSP61) or vector containing avrRps4 at 28 °C on King’s medium supplemented with 50 μg mL⁻¹ rifampicin and 50 μg mL⁻¹ kanamycin. Bacterial cells were resuspended in 10 mm MgCl₂ and pressure-infiltrated into leaves using a needleless syringe. Three lower leaves of a plant were first infiltrated with various bacterial suspensions and a 10 mm MgCl₂ control solution. Two days after primary infiltration, non-treated upper leaves of the plant were inoculated with virulent Pst DC3000. Three days after infection, leaf discs of the upper leaves (systemic leaves) were combined and then ground in 10 mL of cold LiCl, and by sorbitol treatment, and weakly induced by treatments with different salts, including NaCl, KCl and LiCl, and remained high up to 24 h (Fig. 1b). The expression of AtSOT12 is also induced by auxins such as 2,4-D and IAA, as well as cytokinin such as 6-BA and kinetin. AtSOT12 is slightly induced by MeJA and SA, but not induced by ethylene treatment (Fig. 1c).

Sulphotransferase activity assay

The complete ORF of AtSOT12 gene was inserted in-frame into the pGEX-2T vector (Amersham Biosciences, Uppsala, Sweden), resulting a translational fusion protein of GST-SOT12. The construct was verified by sequencing and introduced into the Escherichia coli strain Rosetta 2 (Novagen, Madison, WI, USA). Expression of GST-SOT12 fusion protein was induced by the application of 0.5 mm IPTG for 18 h at 25 °C. Cells were harvested, resuspended in 1× GST binding/washing buffer (4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.3) and incubated on ice for 20 min. The suspended cells were then lysed by French Pressure Cell Press (Thermo Electron Corporation, Waltham, MA, USA) and centrifuged at 12,000 rpm at 4 °C for 30 min. The supernatant was added into GST binding resin (Novagen) and mixed gently by shaking at room temperature. After 30 min, the resin was collected by passing the mixture through a column and washed with 10 mL of 1× GST binding/washing buffer for three times. After resuspended in 1 mL of 1XPBS buffer, the resin PBS solution was transferred into a new-tube, and thrombin protease was added and mixed. The mixture was incubated at room temperature by gentle shaking for 16 h. The mixture was applied to a column, and the pass-through solution containing SOT12 proteins was collected for enzyme activity assay. The sulphotransferase activity of SOT12 protein was measured by using radio-HPLC method. [³⁵S]-phosphoadenosine phosphosulphate (PAPS, PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) was used as the sulphate donor to maximize detection sensitivity. Briefly, reaction in a mixture (100 μL) containing 10 μL of [³⁵S]-PAPS, 10 μg of purified SOT12 protein and 1 mm substrate 50 mm MES buffer, pH 5.5 was allowed to proceed for 30 min at 30 °C and then stopped by adding 3 volumes of cold methanol and incubated at −80 °C for 2 h. After centrifuging at 12,000 g for 10 min to precipitate proteins, the supernatant was transferred to a new tube and dried by using speed vacuum. The remnant was dissolved in ice-cold 50% methanol and subjected to HPLC fractionation (Shimadzu 10Avp module system, Agilent Technologies ZORBAX Extend-C18 RP column, mobile phase: methanol with 1% acetic acid, 10–90% methanol gradient within 60 min). Then, 60 fractions (1 fraction per minute) were collected by using Gilson FC204 fraction collector. The radioactivity in each fraction was measured by liquid scintillation counting (Beckman, LS 6500). For enzyme kinetic analysis, eight SA concentrations ranging between 0.5 and 5 mm were used. The kinetic parameters were obtained by fitting data to the Michaelis-Menten equation.

SA measurement

Local and systemic leaves were collected from bacteria-inoculated plants, weighed and frozen in liquid nitrogen. Extraction and quantification of SA were carried out according to Lee et al. (2007). Briefly, frozen tissue was extracted in 500 μL ice-cold methanol at 4 °C for 24 h, and then 300 μL ice-cold water and 250 μL chloroform with 5 mm of a 3,4,5-trimethoxy-trans-cinnamic acid internal standard were added and mixed by vortexing. The samples were kept at 4 °C for 12 h. The supernatants were dried in a speed vacuum. The residue was suspended in 50 μL cold water/methanol (1:1 v/v) solution and analysed by using HPLC as described above.

RESULTS

AtSOT12 is induced by salt stress and hormone treatments

AtSOT12 gene is differentially expressed in different tissues, with moderate expression levels in root and leaf, higher in flower, but non-detectable in stem and silique (Fig. 1a). The expression of AtSOT12 is strongly induced by treatments with different salts, including NaCl, KCl and LiCl, and by sorbitol treatment, and weakly induced by cold, ABA, but not induced by desiccation (Fig. 1a). NaCl-induced AtSOT12 expression is concentration and time-dependent. With 100 mm NaCl treatment, the AtSOT12 transcript accumulation peaked at 12 h after treatment and remained high up to 24 h (Fig. 1b). The expression of AtSOT12 is also induced by auxins such as 2,4-D and IAA, as well as cytokinin such as 6-BA and kinetin. AtSOT12 is slightly induced by MeJA and SA, but not induced by ethylene treatment (Fig. 1c).
Mutation in AtSOT12 gene resulted in hypersensitivity of the mutant to SA

A T-DNA insertion mutant of AtSOT12 (designated as sot12) was obtained from INRA. Extensive phenotyping was performed by examining the sensitivity of this mutant to different environmental stresses including cold, freezing, drought, salt and osmotic stress, and to different hormones including ABA, GA, MeJA, auxin and SA. Among all treatments tested, sot12 mutant was found to be hypersensitive to SA during seedling growth (Fig. 2a,b). The seed germination of sot12 was also more inhibited by NaCl and ABA comparing with that of wild type (Fig. 2c). Hypersensitivity of sot12 mutant to SA was restored by introducing the wild-type AtSOT12 gene into the mutant plants (Fig. 2a), indicating that the mutation in sot12 mutant is indeed responsible for these phenotypes. Although AtSOT12 enzyme has been shown to sulphonate brassinosteroids in vitro (Marsolais et al. 2007), sot12 mutant did not show significant difference in response to brassinolide and brassinosteroid biosynthesis inhibitor brassinazole (data not shown). Thus, our results suggest that AtSOT12 plays a role in the modification of the defence hormone SA, as well as in stress responses.

AtSOT12 sulphonates SA

Sensitive phenotype of sot12 mutant to SA implied that AtSOT12 might sulphonate SA to reduce its toxicity to plant cells. To determine whether SA is a direct substrate of AtSOT12, an in vitro enzymatic assay was performed. AtSOT12 proteins fused with GST were expressed in E. coli and purified to near homogeneity (Fig. 3a). The purified AtSOT12 proteins were used for enzymatic reactions. Initial attempts using regular reversed-phase (RP)-HPLC to analyse the reactions with non-radioactive PAPS (Sigma-Aldrich, cat no. A1651), and different compounds including SA as substrates were unsuccessful, probably because of the impurity of PAPS which contains about 40% PAP, a product of the enzymatic reaction (Klein et al. 2006). Therefore, a more sensitive detection method using sulfur-35 radio-labelled PAPS as sulphuryl donor in the reaction in combination with RP-HPLC fractionation, and liquid scintillation counting was employed to test whether AtSOT12 could sulphonate SA. If the substrate could be sulphonated by AtSOT12, the S-35 labelled sulphur group from S-35 PAPS should be transferred to the substrate and an S-35 labelled product in the reaction would be detected. In all reactions, a strong radioactive peak was detected around 3 min, representing the remaining S-35 labelled PAPS in the reaction products (Fig. 3b). The retention time of this peak is consistent with the retention time of PAPS standard (2.7 min) using the same HPLC conditions. When SA was used as a substrate in the reaction, an additional radio-labeled peak with retention time of 7 min representing sulphonated SA was detected (Fig. 3b). This peak elutes at the same retention time as the sulphonated SA standard (salicyl sulfate disodium salt, Sigma-Aldrich, Cat No. UC301) (Fig. 3b, insert), confirming that this peak is indeed sulphonated SA at its 2-OH position. Analysis of the enzyme kinetics of AtSOT12 revealed that the $K_m$ for SA is 0.44 mM and $V_{max}$ is 2.67 pkat/ per mg protein (Fig. 3c).
Figure 2. Characterization of sot12 mutant. (a) sot12 mutant is hypersensitive to salicylic acid (SA) in root growth. Four-day-old seedlings grown on 1/2 MS medium with 1.2% agar were transferred to the same medium supplemented with 10 µM SA. Pictures were taken 7 d after transfer. (b) Quantitative measurement of new root growth of wild type (WS-0), sot12 mutant and two homozygous complementation lines (1–4 and 4–4) grown on 1/2 MS medium (1.2% agar) supplemented with different concentrations of SA. Values are average ± SD (n = 6). (c) Seed germination rate of sot12 mutant and wild type in response to 100 mM NaCl (up panel) or 0.5 mM ABA (low panel). Values are average of three replicates ± SD.

Figure 3. In vitro enzyme activity assay. (a) Expression and purification of AtSOT12 proteins from Escherichia coli. Shown is an SDS–PAGE photograph with proteins stained by Coomassie brilliant blue. 1, proteins before addition of IPTG; 2, proteins after induction of AtSOT12 expression by IPTG; 3, purified GST–AtSOT12 fusion protein; 4, purified AtSOT12 protein without GST tag. Upper arrow indicates GST–AtSOT12 fusion proteins; middle arrow shows AtSOT12 proteins without GST tag; lower arrow shows GST proteins. (b) Radio-HPLC detection of sulphonation of salicylic acid (SA). The first radioactivity peak is S-35 labelled PAPS. The peak at about 7 min is sulphonated SA. Up panel of the insert shows HPLC detection of sulphonated SA standard by UV detector using the same HPLC condition as radio-HPLC. Y axis is the intensity of UV absorbance. Low panel of the insert shows the sulphonated SA peak only. Y axis is the intensity of radioactivity of the fractions. Note that the retention time of the standard is essentially same as the peak detected by radio-HPLC. (c) Determination of $V_{\text{max}}$, $K_a$ and $K_{\text{cat}}$ of AtSOT12 for SA.

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AtSOT12 promotes internal SA accumulation

Sulphonation of SA in plants is a previously unknown biochemical process. Its physiological role could be simply to reduce toxic level of SA accumulated during plant stress response by modifying SA into less toxic sulphonated form. If this were the case, SA content should be higher in sot12 mutant, but lower in AtSOT12 over-expressing plants than in wild type after pathogen challenging. Although the detoxification role of AtSOT12 is well supported by the hypersensitive phenotype of sot12 mutant to SA, measurements of internal SA levels in wild type, sot12 mutant and AtSOT12 over-expressing plants indicated otherwise. After challenging with pathogen P. syringae, SA level in wild type was increased in both local leaves and systemic leaves (Fig. 4) as previously shown by numerous studies. Unexpectedly, SA levels in both local and systemic leaves of sot12 mutant were not elevated after pathogen challenging (Fig. 4), which suggests that AtSOT12 sulphotransferase does not simply function as a detoxification enzyme for increased endogenous SA, but instead is required for SA accumulation during plant response to pathogen attacks. This notion is further supported by the observation that AtSOT12 over-expressing plants accumulated more SA in both local and systemic leaves after pathogen infection (Fig. 4). Thus, sulphonated SA may be a signal molecule that triggers SA production for pathogen defence. However, alternative modes by which SOT12 sulphonates other endogenous molecules thus indirectly modulates SA accumulation could also be possible.

AtSOT12 enhances local and systemic resistance to pathogen

Because sulphonation of SA by AtSOT12 modulated internal SA levels in both local and systemic leaves during pathogen response, it is likely that this biochemical process is important for pathogen resistance. As shown in Fig. 5a, after infiltrated with virulent Pst DC3000, sot12 mutant displayed more sensitive phenotype than wild type. Trypan blue staining of individual leaves inoculated with Pst DC3000 revealed more cell death in sot12 mutant, but less cell death in AtSOT12 over-expressing leaves when compared with wild type (Fig. 5b). Measurements of bacterial growth in local leaves after inoculation with Pst DC3000 also revealed that sot12 mutant plants were more sensitive, but AtSOT12 over-expressing plants were more resistant to pathogen infection (Fig. 5c).

To determine the role of AtSOT12 in SAR, local leaves were inoculated with avirulent strain Pst DC3000 avrRps4. After 2 d of inoculation, systemic leaves were infiltrated with virulent Pst DC3000, and bacterial growth in the systemic leaves was measured. Figure 5d shows that systemic leaves of wild type acquired resistance in response to infection of local leaves with avirulent Pst DC3000 avrRps4. Interestingly, sot12 mutant exhibited complete loss of SAR in response to local infection with Pst DC3000 avrRps4. Furthermore, AtSOT12 over-expressing plants displayed enhanced SAR (Fig. 5d). The role of AtSOT12 in pathogen response and SAR was further determined by analyzing the expression of pathogen-related (PR) genes in local and systemic leaves of AtSOT12 over-expression plants after infection of local leaves with virulent and avirulent strains (Fig. 5e). In local leaves, the PR1 gene expression is strongly induced by infection with virulent strain DC3000, but only slightly induced by infection with avirulent strain DC3000 avrRpm1 and avrRps4 (Fig. 5e, upper panel). The expression levels of PR2 and PR5 genes were rather low and did not show induced expression by pathogen infection (data not shown). In systemic leaves, the expression levels of PR1, PR2 and PR5 genes in AtSOT12 over-expressing plants were substantially higher than that in the wild type after infection of local leaves with avirulent strains (Fig. 5e; lower panel). However, without infection of the local leaves (MgCl2 treatment), the transcript levels of PR genes in the systemic leaves of AtSOT12 over-expressing and wild-type plants were similar, which indicates that the enhanced induction of PR genes in systemic leaves of AtSOT12 over-expressing plants was not caused by over-expression of AtSOT12 in the systemic leaves per se, but instead, because of over-expression of AtSOT12 in the local leaves resulting in more signal molecules that were mobile to the systemic

![Figure 4](image-url)
leaves causing stronger response. These results suggest that AtSOT12, presumably through sulphonation of SA, enhances plant response to pathogen infection and contributes to long distance signalling in SAR.

**DISCUSSION**

*Arabidopsis* possesses 18 predicted cytosolic SOTs, but their physiological and biochemical functions are still quite obscure. Here, we presented a detailed study of one of the *Arabidopsis* SOTs, AtSOT12, by employing genetic, physiological, molecular and biochemical approaches. We showed that AtSOT12 enzymatically sulphonates SA *in vitro* and mutation in AtSOT12 gene rendered the mutant hypersensitive to SA. Among 18 SOTs, AtSOT12 gene is clustered with and encodes a protein with high sequence similarity to AtSOT11 (At2g03750) and AtSOT13 (At2g03770). However, mutation in AtSOT11 and AtSOT13 did not cause a more sensitive phenotype of the mutants to SA (data not shown), suggesting unique and non-overlapping functions of AtSOT12 in SA modification.

AtSOT12 gene expression is highly induced by abiotic stresses and hormone treatments (Fig. 1), which suggests a role of sulphonation of small molecules in stress and hormone response. Consistent with this observation, sot12 mutant is hypersensitive to salt stress and ABA in seed germination (Fig. 2c). Greater seed germination inhibition for the sot12 mutant line under stress conditions could be attributed to deficiency of SA sulphonation in the mutant. Besides SA’s essential role in plant resistance to pathogen, this signalling molecule has also been shown to play a role in plant response to abiotic stresses (Yuan & Lin 2008). Borsani, Valpuesta & Botella (2001) provided evidence supporting the hypothesis that SA potentiates the generation of reactive oxygen species (ROS) in photosynthetic tissues during salt and osmotic stresses. More recently, Alonso-Ramirez et al. (2009) found that GA3 is able to reverse the inhibitory effect of salt, oxidative and heat stresses in seed germination and seedling establishment by increase of SA biosynthesis. In sot12 mutant seeds, deficiency in SA sulfonation may block the positive feedback regulation for SA production, thus reducing SA content in seeds to below the optimal levels that are required for plant growth and development.
for promotion of germination under stress conditions. SA-mediated signalling has also been shown to antagonistically crosstalk with ABA-mediated signalling of environmental stress response (Yasuda et al. 2008). Perhaps, reduced SA in sot12 mutant seeds influences ABA biosynthesis or signalling, resulting in hypersensitivity of the mutant seeds to ABA.

Most interesting and important finding in this study is that AtSOT12 sulphonates SA (Fig. 3). Sulphonation of SA is a previously unknown modification of this defence hormone. The known modifications of SA in plants are glucosylation and methylation. Glucosylation of SA results in the formation of non-toxic SA 2-O-β-d-glucoside that is sequestered in vacuoles where it presumably forms a readily available hydrolyzable source of SA (Dean, Mohammed & Fitzpatrick 2005). Thus, glucosylation of SA seems to be a mechanism of SA homeostasis and detoxification. Methylation of SA generates MeSA, a volatile ester that is normally absent in plants, but dramatically induced upon pathogen infection. Formation of MeSA could also be a detoxification mechanism for SA by simply diffusion of this volatile from plant cells into air and/or MeSA could function as a volatile signalling molecule for systemic response (Loake & Grant 2007; Heil & Ton 2008). SA amino acid conjugation has recently been proposed to be a possible modification of SA (Jagadeeswaran et al. 2007; Nobuta et al. 2007). However, the biochemical basis of SA amino acid conjugates in SA metabolism and signalling is unclear.

Based on the analysis of enzyme kinetics, we speculate SA to be the endogenous substrate of AtSOT12, and the conversion rate of SA to sulphonated SA is low in the cell. The $K_m$ of AtSOT12 to SA at the high micromolar range (0.44 mm, Fig. 3c) suggests that sulphonation of SA occurs mainly when SA level is significantly increased after pathogen infection or under other stress conditions. Given that the concentration of SA is about 20–40 μm in cells after pathogen infection (estimated from Fig. 4), the AtSOT12 catalysed sulphonation of SA at this concentration range would be a first-order reaction, and the reaction is SA concentration dependent. Thus, production of sulphonated SA could reach to a significant level when SA concentration is increased in response to pathogen attack or other stress conditions.

What is the biological significance of sulphon conjugation of SA? It seems clear that sulphonated SA is less toxic to plant cells than non-sulphonated form because loss of function of AtSOT12 resulted in hypersensitivity of the mutant to SA (Fig. 2). However, measurements of SA contents showing in Fig. 4 indicated that sulphonation of SA by AtSOT12 is not simply to reduce the elevated and possible toxic level of SA, but seems to be required for SA accumulation upon pathogen challenging. Reduced SA accumulation in sot12 mutant after pathogen infection is consistent with the phenotype of sot12 being more sensitive to pathogen. Similarly, increased SA accumulation in AtSOT12 over-expressing plants after pathogen infection may account for its increased resistance (Fig. 5). Our results suggest that sulphonated SA may function as a signalling molecule for both local and systemic response rather than metabolic inactivation of SA. Although we could not exclude the possibility that SOT12 sulphonates other endogenous molecules, thus indirectly regulates SA levels, it is likely that sulphonation of SA triggers both local and systemic SA accumulation because the sulphonated SA is more water soluble, thus more mobile in the water-based sap of the vascular transport system during translocation of this putative signalling molecule from local leaf to systemic leaves. However, this hypothesis needs to be experimentally validated. Nevertheless, increased PR gene expression in the systemic leaves of AtSOT12 over-expressing plants suggested that sulphonation of SA indeed contributes, at least at some extent, to long distance signalling.

In addition to SA, AtSOT12 might also sulphonate other endogenous compounds in Arabidopsis. As an attempt to identify additional endogenous substrates of AtSOT12, we performed a metabolite profiling analysis by using capillary electrophoresis (CE)–time of flight (TOF)–mass spectrometer (MS) (Supporting Information Appendix S1). Approximately 1200 metabolites were detected from sot12 mutant and wild-type seedlings with or without NaCl treatment. Supporting Information Tables S1 and S2 listed metabolites with m/z value, retention time and changes in abundance of the metabolites between sot12 mutant and wild type. Because of the limitation of metabolite databases, comprehensive identification of the listed compounds was not possible. Regardless, these results provide valuable information about the changes in endogenous molecules that could be AtSOT12 substrates or products. These data sets could also be useful for identification of NaCl-induced changes in metabolites.

In summary, we have identified a novel pathway for SA modification that plays an important role in pathogen resistance and SAR. We have proposed that sulphonated SA could be a mobile signal molecule for both local and systemic response. Further study to identify endogenous sulphonated SA and its role in mobile signalling will provide important insights of this new SA metabolic pathway in SAR and pathogen resistance.

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REFERENCES


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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Anionic metabolite changes.
Table S2. Cationic metabolite changes.

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