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Original Paper

Biotic and abiotic stresses induce *AbSAMT1*, encoding *S*-adenosyl-L-methionine: salicylic acid carboxyl methyltransferase, in *Atropa belladonna*

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Abstract We characterized the expression pattern of *Atropa belladonna* salicylic acid (SA) carboxyl methyltransferase gene, *AbSAMT1*, encoding *S*-adenosyl-L-methionine (SAM): SA carboxyl methyltransferase (SAMT) in *A. belladonna*, after the application of biotic and abiotic stresses to plants. *AbSAMT1* was expressed after treatments of exogenous SA and also 1,2-benzisothiazole-1,1-dioxide (BIT), which is a chemical inducer related to the SA-dependent response. Expression of the *AbSAMT1* gene was also induced by infection of *A. belladonna* plants with *Pseudomonas syringae* pv. *tabaci* (*Pst*), when distinctive disease resistance symptoms were observed. Moreover, it was confirmed that the expression of the *AbSAMT1* gene was also induced by physical wounding and methyl jasmonate (MeJA), as well as the disease resistance response. These results suggest that AbSAMT1 may play a dual regulation role of distinct signaling in *A. belladonna* plants, namely the signaling pathway of the SA-dependent response, and also a jasmonic acid (JA) dependent response in local regions.

Key words: *Atropa belladonna*, methyltransferase, salicylic acid, stress response, systemic acquired resistance.

Plants have a number of volatile secondary metabolites and many of them are either common ingredients of floral scent to attract pollinators (Kundsen et al. 1993) or defensive metabolites to protect plants against herbivores and pathogens (Shulaev et al. 1997; Arimura et al. 2000; Chen et al. 2003; Xu et al. 2006; Koo et al. 2007). One of these metabolites, methyl salicylate (MeSA) has been found in many floral scents (Kundsen et al. 1993), and it is emitted during infection by pathogens or attack by insects or parasites (Shulaev et al. 1997; Van Poecke et al. 2001; Chen al et. 2003; Ament et al. 2004). It has been shown that MeSA is emitted from tobacco leaves infected with tobacco mosaic virus (TMV) and absorbed from the air by non-infected leaves, as an airborne signal. It is then converted to salicylic acid (SA) by tobacco SA-binding protein 2 (SABP2), found to involve MeSA-esterase activity (Forouhar et al. 2005), and induces SA-mediated defense responses such as systemic acquired resistance (SAR) (Shulaev et al. 1997).

MeSA is synthesized from SA via a reaction catalyzed by *S*-adenosyl-L-methionine (SAM): salicylic acid carboxyl methyltransferase (SAMT) with a methyl group donated by the molecule SAM. Since *Clarkia breweri* SAMT (*CbSAMT*) was first isolated from flowers, leaves and other parts of *C. breweri* (Dudareva et al. 1998, Ross et al. 1999), SAMTs have been identified and purified in several other plants.

The carboxyl methyltransferases family can be divided into two groups based on substrate specificity, namely SAMT and BSMT/BAMT [SAM: benzoic acid (BA) SA methyltransferase (BSMT)/SAM: BA carboxyl methyltransferase (BAMT)] (D'Auria et al. 2003). SAMT activity, which is greater with SA than BA, has been characterized in plants such as *C. breweri*, *Stephanotis floribunda* and *Antirrhinum majus* (Ross et al. 1999, Pott et al. 2002, Negre et al. 2002). BSMT, which has similar activity for both SA and BA, has been characterized in *Arabidopsis thaliana*, *Arabidopsis lyrata*, *Petunia hybrida* and *Nicotiana suaveolens* (Chen et al. 2003, Negre et al. 2003, Pott et al. 2004). *A. majus* BAMT (*AmBAMT*) has only shown activity for BA (Murfitt et al. 2000).

Abbreviations: AbSAMT, *Atropa belladonna S*-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase; BIT, 1,2-benzisothiazole-1,1-dioxide; BTH, benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester; CMPA, 3-chloro-1-methyl-1*H*-pyrazole-5-carboxylic acid; JA, jasmonic acid; MeJA, methyl jasmonate; MeSA, methyl salicylate; PR, pathogenesis related protein; *Pst*, *Pseudomonas syringae* pv. *tabaci*; SA, salicylic acid; SAR, systemic acquired resistance

This article can be found at http://www.jspcmb.jp/

It has been shown that some SAMT genes are induced in defense responses under biotic and/or abiotic stresses (Fukami et al. 2002; Negre et al. 2002; Chen et al. 2003; Ament et al. 2004; Xu et al. 2006; Koo et al. 2007). The *A. thaliana* BSMT1 (*AtBSMT1*) gene was induced in leaves by treatment with alamethicin, a channel-forming peptide that mimics the effect of pathogen attack, *Plutella sylostella* herbivory, uprooting, physical wounding and methyl jasmonate (MeJA), but it was not induced by SA (Chen et al. 2003). The expression of the *Oryza sativa* L. benzothiadiazole induced SAMT1 (*OsBISAMT1*) gene in rice was a response to blast disease caused by *Magnaporthe grsiea* and physical wounding. However, the *OsBISAMT1* gene was also expressed when rice was treated with benzothiadiazole or with SA, which is the inducer of SAR in rice (Xu et al. 2006). This is distinct from the expression of the *AtBSMT1* gene, which is not induced by treatment with SA.

Recently, tobacco SABP2 was found to be involved in MeSA-esterase activity that converts MeSA into SA (Forouhar et al. 2005). SAR was not induced in *SABP2*-silenced plants (Kumar et al. 2003) or in *SAMT*-silenced plants (Park et al. 2007) suggesting that SAMT may play a role in the biosynthesis of MeSA as an endogenous signal.

Previous research isolated and identified the SAMT gene in the cultured hairy roots of *A. belladonna*. *A. belladonna* SAMT1 (*AbSAMT1*) (GenBank accession number: AB049752) showed that the expression of the *AbSAMT1* gene was activated when exogenous SA was added to a medium containing cultured hairy roots of *A. belladonna* (Fukami et al. 2002). In the present study, *AbSAMT1* was expressed when *A. belladonna* was treated with exogenous SA, pathogens or 1,2-benzisothiazole-1,1-dioxide (BIT), by activating SAR as the chemical inducer. In addition, we also showed that MeJA or wounding treatments affected the induction of *AbSAMT1* gene expression. In this study we investigated the theory that AbSAMT1 may have two functional roles function in *A. belladonna*, as exo- and endo-genous signaling of the SA-dependent response, and also to deplete SA storage as a result of the induction of the jasmonate (JA) -dependent response.

Materials and methods

Plant materials and treatments

A. belladonna plant material was collected from the Research Center for Medicinal Plant Resources, National Institute of Biomedical Innovation. *A. belladonna* plants were grown on sterilized soil (Kureha, Tokyo, Japan) in pots at 25°C under 16 hours light/8 hours dark for 4-6 weeks. After the 4-6 weeks of cultivation, when the plants had developed 4-6 leaves, the plants were treated with different concentrations of chemical inducers or with water. The pots of plants were drenched with SA, BIT, benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) and 3-chloro-1-methyl-1*H*-pyrazole-5-carboxylic acid (CMPA). The CMPA was a gift from Nissan Chemical Industries, Ltd. Different concentrations of MeJA were applied by vaporizing 1 mM, 5 mM or 10 mM MeJA in 1 ml of ethanol in a closed container containing *A. belladonna* plants. Leaf samples were collected at set times after the treatments, and analyzed by northern blot.

Wounding of *A. belladonna* leaves, to analyse expression of *AbSAMT1* genes in wounded leaves, was performed by removing two discs from a leaf of *A. belladonna* using a 6 mm hole punch. Leaf samples were harvested at different times after wounding treatment.

Pathogen infection assays

Culturing *Pseudomonas syringae* pv. *tabaci* (*Pst*) was performed in nutrient broth medium containing 20 μg ml^{-1} rifampicin for 24 hours at 28°C and 150 r.p.m. *Pst* was suspended in 10 mM $MgCl_2$ (approximately 1×10^6 colony-forming unit (cfu) ml^{-1}). The infection of *Pst* was performed by infiltration of the bacterial suspension into a 1 cm^2 area on the back of a leaf using a 1 ml syringe without a needle. At regular intervals after inoculation two leaf sections were harvested and homogenized in 10 mM $MgCl_2$. The number of bacteria (cfu) was determined by growth on nutrient broth agar plates after dilution.

Northern blot analysis

Leaves were collected from *A. belladonna* plants at regular intervals after treatment and frozen in liquid nitrogen. Total RNA was extracted from each leaf sample using Sepasol RNA I super (NACALAI TESQUE Inc., Kyoto, Japan). DNA probes for *AbSAMT1* (0.5 μg), *pathogenesis related* protein 1 (*PR1*) and *pathogenesis related* protein *2* (*PR2*) were synthesized with Psoralen-Biotin, following the protocol provided by BrightStar Psoralen-Biotin non-isotopic labeling kit (Ambion Inc., Austin, TX). Five micrograms of mRNA was denatured in formamide, separated on a 1% agarose gel containing formaldehyde and then transferred to a positively charged nylon membrane (Hybond-N$^+$, GE Healthcare Ltd., Buckinghamshire HP7 9NA, England). After hybridization with a Psoralen-Biotin labeled probe, membranes were treated with the BrightStar BioDetect kit (Ambion Inc.). Detection of the bands was conducted by fluorescence image using BioMax XAR films (Kodak Industrie, Chalon-sur-Saône, Cedex, France).

Quantification of SA and MeSA

SA and SA ß-glucoside (SAG) were extracted and quantified as described by Bowlling et al. (1994) and Seskar et al. (1998). Leaf samples (1 g fresh weight) were frozen in liquid nitrogen, then homogenized to fine powder and extracted with 10 ml of 90% methanol. After centrifugation, the pellet was re-extracted with 10 ml of 100% methanol. Two ml of the combined methanol extracts were dried under nitrogen on a 40°C heat block. The residue was re-suspended with 1 ml of water on an 80°C heat block, and filtered through a 0.45 μm pore filter. The extracted

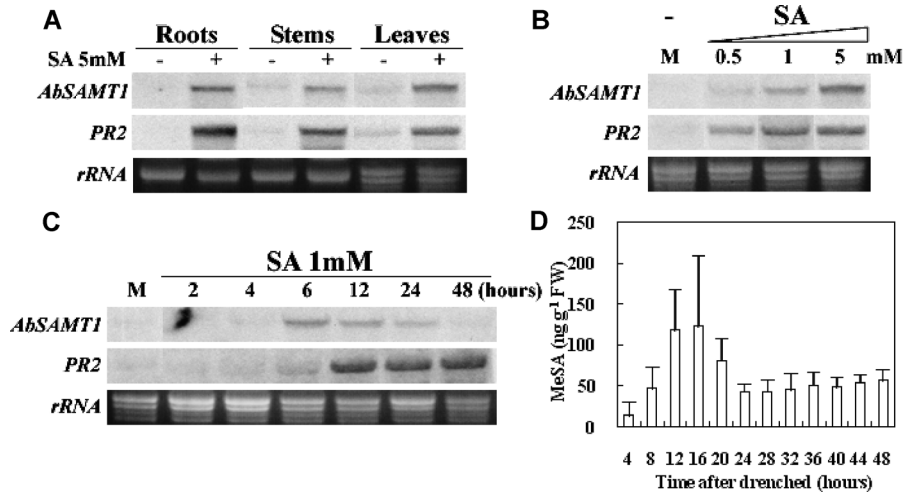

Figure 1. Analysis of *AbSAMT1* gene expression after SA was exogenously administered to *A. belladonna* plants. (A) Organ-specific expression of *AbSAMT1* and *PR2* genes at 12 hours after administration of 5 mM SA (+) or water (−) to real organs. (B) Expression of *AbSAMT1* and *PR2* genes in *A. belladoona* plant at 12 hours after administration of 10 ml pot^{-1} of 0.5 mM, 1 mM and 5 mM SA. (C) Time course of *AbSAMT1* and *PR2* gene expression after 1 mM SA treatment. (D) Time course of MeSA levels after 1 mM SA treatment. The expression *AbSAMT1* and *PR2* genes and MeSA levels were analyzed by Northern blot and GC-MS. Values±SD are averages of three treated samples. Mock (M) is drenching with water. FW, fresh weight.

SA solution was then divided into 1 ml of free SA and 1 ml of total SA (free SA and SAG) solutions. Each solution was mixed with 1 ml of 0.1 M sodium acetate buffer (pH 5.0) or 1 ml of 0.1 M sodium acetate buffer (pH 5.0) containing 36 units mg^{-1} fresh weight ß-glucosidase (Wako, Osaka, Japan). Following enzymatic hydrolysis (6 hours at 37°C), the reaction was stopped with 50 μl of 1 M HCl. The free SA was separated from the solutions by organic extraction with 2 ml of ethylacetate : cyclohexane (50 : 50). The organic phase containing the free SA was then dried under nitrogen. The dried extract was suspended in 1 ml of 20% methanol and filtered. HPLC was performed on a C_{18} reverse-phase column (4.6 mm×250 mm×5 μm), TSK-Gel ODS-120T (TOSOH, Tokyo, Japan) maintained at 40°C, and equilibrated in methanol : 20 mM sodium acetate butter (pH 5.0) (20 : 80) with a flow rate of 0.5 ml min^{-1}. The free SA was determined by fluorescence (excitation 295 nm, emission 370 nm).

Volatile MeSA released from the pretreated plants was continuously collected using charcoal traps (5 mg of charcoal, GHP Acrodisc filter, PALL, NY, USA) over a 48 hour period using air circulation as described by Grob et al. (1976) and Engelberth et al. (2001). Every 4 hours the volatile compounds adsorbed by the charcoal traps were desorbed with dichloromethane (2×35 μl), and the extracts were directly analyzed by gas chromatography and mass spectrometry (GC-MS) with DB-1 (15 m×0.25 mm, Agilent Technologies, CA, USA). The compounds were separated under programmed conditions; at 30°C for 2 minutes and then the temperature was increased at a rate of 5°C minute^{-1} up to 230°C. Helium was used as the carrier gas (1.7 ml minute^{-1}). GC-MS analysis was performed with the GC interface at 230°C and a scan range of 50 to 320 at ionization voltage 70 eV. The compounds were quantified on the internal standard (nonyl acetate).

Results

Expression of AbSAMT1 gene in plants by exogenous SA

We examined whether the expression of *AbSAMT1* gene was induced by an increase in SA levels. In order to confirm a SA-response we analyzed the expression of the PR gene in a SA-dependent response, after SA was applied (Delaney et al. 1993; Delaney et al. 1994). We tested the effect of exogenous SA in organs of roots, stems and leaves of *A. belladonna* plants (Figure 1A). The SA-dependent response, which induced *AbSAMT1* and *PR2* expression, was observed in the organs of roots, stems and leaves as the exogenous SA spread through the plants. The induced expression of *AbSAMT1* and *PR2* genes increased in relation to both SA concentration and time (Figure 1B, C). The expression of *AbSAMT1* gene induction increased dramatically for 6 hours after treatment with 1 mM SA, and thereafter slowly decreased (Figure 1C). MeSA levels were also associated with an initial increase followed by a decrease in the expression of the *AbSAMT1* gene (Figure 1D). After 24 hours MeSA levels remained fairly constant. These results suggest that the increase of SA level in *A. belladonna* plants is enough to induce the expression of *AbSAMT1* gene.

Expression of AbSAMT1 gene by chemical inducer

We investigated whether the accumulation of SA was necessary for the expression of the *AbSAMT1* gene. BIT induced SAR by activating the SA biosynthesis in *A. thaliana* (Yoshioka et al. 2001) and tobacco

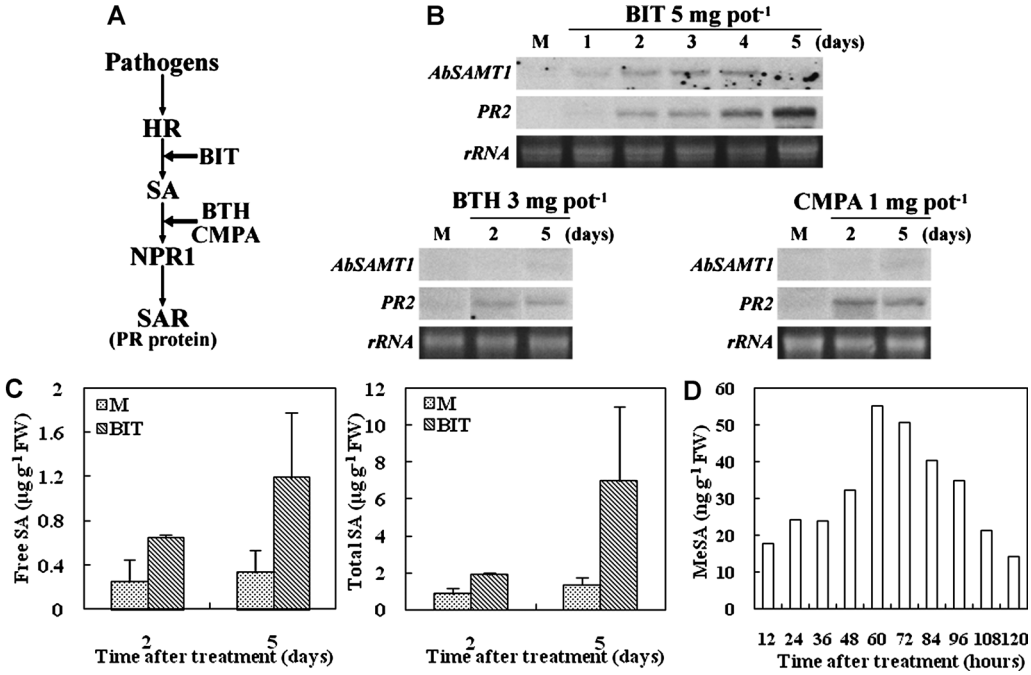

Figure 2. Expression of *AbSAMT1* and *PR2* genes in leaves of *A. belladonna* after treatment with chemical inducers. (A) SA signaling pathways in *Arabidopsis*. BIT is the upstream chemical inducer of SA; BTH and CMPA are the downstream chemical inducers of SA in SAR. (B) Expression of *AbSAMT1* and *PR2* genes by BIT, BTH and CMPA. The leaf samples were regularly harvested after *A. belladonna* was drenched with either 5 mg pot^{-1} BIT, 3 mg pot^{-1} BTH or 1 mg pot^{-1} CMPA. Analysis was by northern blot. M=drenching with water. (C) Free SA and total SA and (D) MeSA levels in *A. belladonna* after BIT or water (M) treatments of *A. belladonna* plants. Values±SD are averages of three treated samples. FW, fresh weight.

(Nakashita et al. 2002). However, BTH and CMPA were effective in inducing the expression of SAR genes in these plants without accumulating SA (Friedrich et al. 1996; Lawton et al. 1996; Yasuda et al. 2003) (Figure 2A). The expression of the *PR2* gene was examined in plants treated with BIT, BTH or CMPA as a molecular marker for SAR. BIT induced the expression of *AbSAMT1* and *PR2* genes at 2 days after 5 mg pot^{-1} treatment (Figure 2B). The levels of endogenous free and total SA in BIT-induced *A. belladonna* plants were approximately three- and four-fold higher, respectively, than those in the control plants at five days post treatments (Figure 2C), but there was no increase in BTH- or CMPA-induced plants (data not shown). These results showed that the biosynthesis of SA was only stimulated by the treatment of BIT. The increased MeSA levels in the BIT-treated *A. belladonna* plants, detected by GC-MS (Figure 2D), were caused by induced *AbSAMT1* gene expression when endogenous SA levels were increased in tissues. In contrast, the treatments with BTH and CMPA did not induce the expression of *AbSAMT1* gene, but instead activated the expression of *PR2* gene (Figure 2B). Therefore these results showed that an increase of SA level was probably necessary for the expression of the *AbSAMT1* gene.

Expression of AbSAMT1 gene by an avirulent bacterium

Since SA plays an important role in disease resistance, we investigated the relationship between *AbSAMT1* gene expression and disease resistance response. Infection with *Pst* resulted in hypersensitive response (HR) within 24 hours (Figure 3A). No further bacterial growth was detected in plant tissues after 24 hours post inoculation (Figure 3B) indicating that *A. belladonna* presumably has a resistance gene specific to *Pst*. During the resistance response to *Pst*, the expression of *AbSAMT1* and *PR2* was induced (Figure 3C). We also assessed the concentration of endogenous free SA and total SA after infection. By 8 hours after inoculation with *Pst* the levels of endogenous free SA and total SA in *A. belladonna* had both increased one hundred fold to 5 $\mu g\,g^{-1}$ fresh weight and twenty five fold to 7 $\mu g\,g^{-1}$ fresh weight, respectively (Figure 3D). MeSA levels also increased up to 12 hours post inoculation (Figure 3E), associated with the increased expression of *AbSAMT1* gene. These results suggest that the upregulation of SA levels during the disease resistance response induced the expression of *AbSAMT1* and the resulting MeSA production.

Expression of AbSAMT1 gene by wounding

In *A. thaliana* and *O. sativa* plants, the expressions of the respective *AtBSMT1* and *OsBISAMT1* genes were induced by physical wounding (Chen et al. 2003; Xu

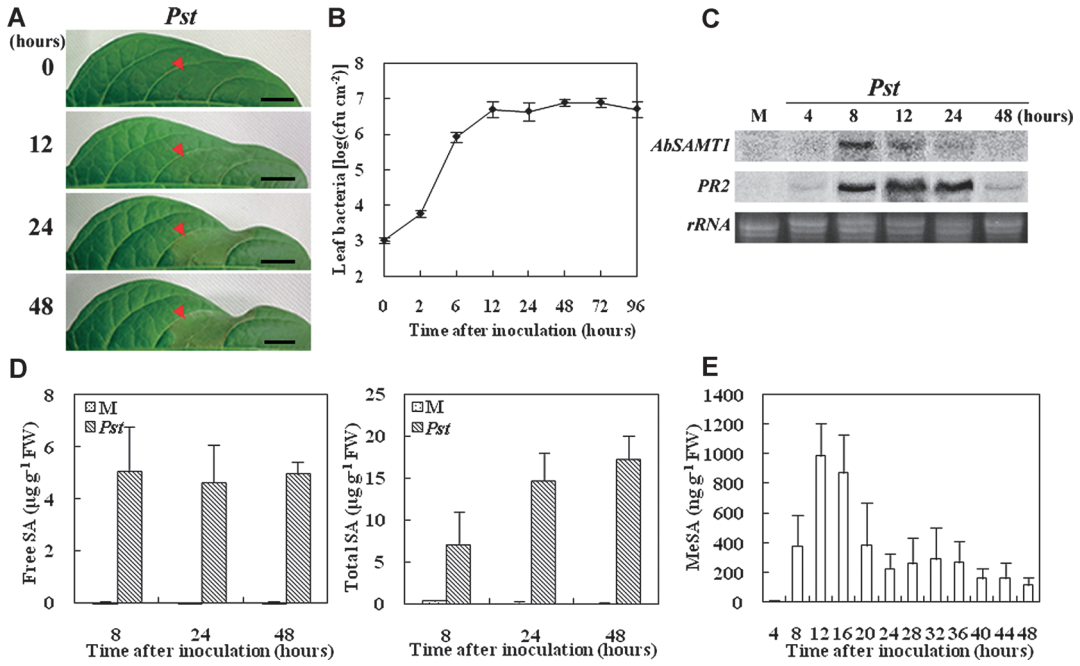

Figure 3. Expression of *AbSAMT1* and *PR2* genes in leaves of *A. belladonna* after infection with the virulent bacterial pathogen *Pst*. (A) Disease susceptibility of *A.belladonna* after inoculation with *Pst*. Infected leaves were photographed 0, 12, 24 and 48 hours after inoculation; Red arrows = inoculation regions, Bar=1 cm. (B) Growth curve of *Pst* in *A. belladonna* leaf tissues. Values are the mean of 9 samples (three leaf samples from each of three replicate plants)±SD. (C) Expression of *AbSAMT1* and *PR2* genes after *Pst* inoculation. *A. belladonna* plants were inoculated with approximately 1×10^6 cfu ml^{-1} *Pst* or 10 mM $MgCl_2$ solution (M). Northern blot analysis was conducted 4, 8, 12, 24 and 48 hours after inoculation and repeated three times with similar results. (D) Free SA and total SA and (E) MeSA levels in *A. belladonna* after *Pst* or $MgCl_2$ solution (M) inoculation of *A. belladonna* plants. Values±SD are averages of three infected samples. FW, fresh weight.

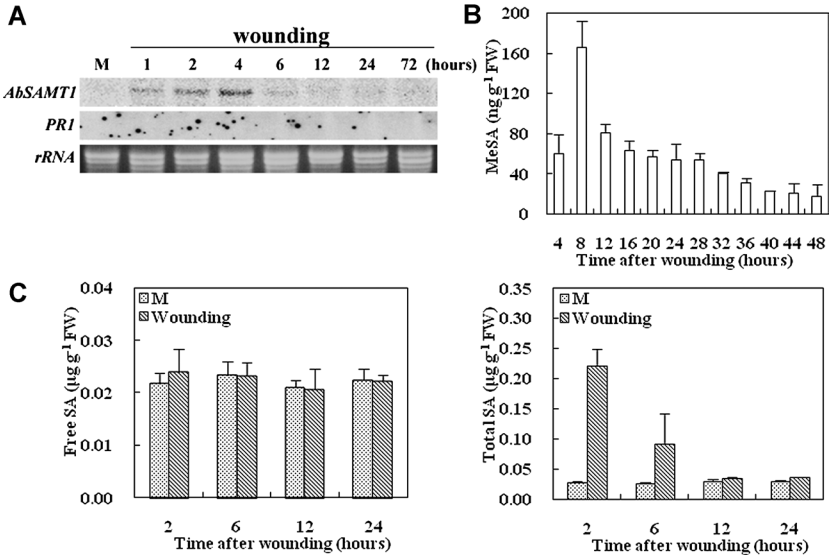

Figure 4. Expression of *AbSAMT1* gene in leaves of *A. belladonna* after wounding. (A) Expression of *AbSAMT1*, *PR1* and *PR2* in leaves of *A. belladonna* after wounding. Two discs were removed from a leaf by 6 mM hole-punch before each sampling time; no discs were removed for mock (M). Analysis was by northern blot. (B) MeSA, (C) free SA and total SA levels in wounded or unwounded (M) leaves, analyzed by GC-MS and HPLC. Values±SD are averages of three wounded samples. FW, fresh weight.

et al. 2006). We analyzed the expression of *AbSAMT1* to determine the induction of gene expression in wounded leaves. As shown in Figure 4A, *AbSAMT1* mRNA accumulated quickly in the first hour after leaf wounding before declining by 12 hours after wounding, but in unwounded leaves no expression was detected. Wounding also increased MeSA levels rapidly in the first 8 hours after treatment before then decreasing (Figure 4B). Furthermore, there was no difference in endogenous free SA levels in *A. belladonna* after wounding, compared

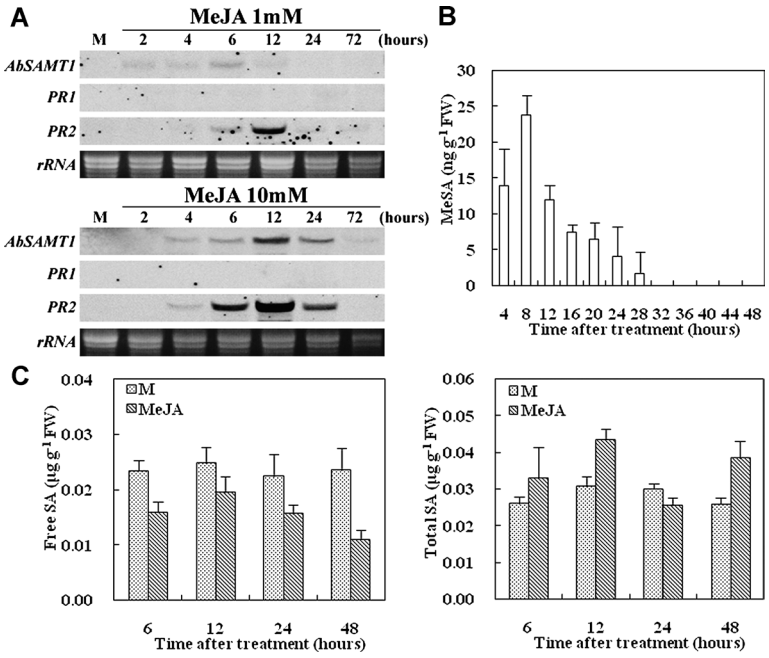

Figure 5. Expression of *AbSAMT1* gene in leaves of *A. belladonna* after exposure to vaporized MeJA in a closed container. (A) Expression of *AbSAMT1*, *PR1* and *PR2* in leaves, analyzed by northern blot; M received no MeJA treatment. (B) MeSA, (C) free SA and total SA levels in leaves exposed to vaporized 1 mM MeJA or untreated (M). Samples were analyzed by GC-MS and HPLC. Values±SD are averages of three treated samples. FW, fresh weight.

with the control plants (Figure 4C). This result demonstrated that the expression of *PR1* gene was not induced by wounding. However, total SA levels did increase to about 8 fold higher than control plants during the 2 hours after wounding, before sequentially decreasing. The increase in volatile MeSA levels after wounding appeared to be caused by the conversion of total SA to MeSA, but the exact mechanism is yet to be clarified. These results confirm that physical wounding induced *AbSAMT1* gene expression and MeSA production.

Expression of AbSAMT1 gene by MeJA

Since JA signaling is part of the wounding stress response, we examined the ability of MeJA to induce *AbSAMT1* expression. The *AbSAMT1* and *PR2* genes were induced in leaves of *A. belladonna* after they were treated with 1 mM and 10 mM MeJA, but the *PR1* gene was not expressed (Figure 5A). Expression levels of *AbSAMT1* rapidly increased at 4 hours after treatment, consistent with the response after wounding. MeSA production also increased after the induction of *AbSAMT1* gene expression in the 1 mM MeJA treatment (Figure 5B), but were about 7 fold lower than those caused by wounding (Figure 4B) because there were insufficient levels of endogenous free SA and total SA (Figure 5C). Free SA levels were lower than in control plants over the MeJA treatment period, but total SA increased during the first 12 hours after MeJA treatment, then temporarily decreasing after 24 hours (Figure 5C). It was assumed that endogenous SA was converted into volatile MeSA. The results indicated that MeJA treatment also induced the expression of the *AbSAMT1* gene.

Expression of AbSAMT1 gene by cross talk between SA and MeJA

Cross talk between SA and JA has been reported to play an important role in the regulation of stress responses. SAR is inhibited by the JA-dependent response in tobacco and *A. thaliana* (Niki et al. 1998; Kunkel et al. 2002). In contrast, SA-dependent response represses the JA-dependent response in *A. thaliana* (Spoel et al. 2003). In order to determine whether *AbSAMT1* plays a role in cross talk between SA- and JA-dependent responses in *A. belladonna*, we examined the expression of *AbSAMT1*, *PR1* and *PR2* genes in leaves of *A. belladonna* that were treated with 5 mM or 10 mM SA followed by 5 mM MeJA (Figure 6). *PR1* was induced by treatment with SA, but not by treatment with MeJA. The combined treatments with 5 mM SA and 5 mM MeJA showed that *PR1* was suppressed by the regulation of antagonistic interaction between SA- and JA-dependent responses. However, at 6 hours and 12 hours after the combined application of 5 mM SA and 5 mM MeJA, the expression of the *AbSAMT1* gene was not suppressed, in contrast to *PR1* gene expression. *PR2* exhibited a similar expression pattern to the *AbSAMT1* gene. These results indicated that the expression of *AbSAMT1* and *PR2* was induced by either SA or MeJA, and was not affected by the cross talk between SA- and MeJA-mediated signaling pathways.

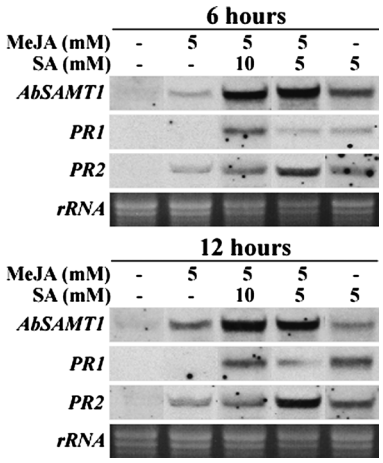

Figure 6. Expression of *AbSAMT1* gene in leaves of *A. belladonna* after sequential treatments of MeJA and SA. The leaves of *A. belladonna* were treated with 5 mM or 10 mM SA by soil drench, followed by exposure to vaporized 5 mM MeJA. Expression of *AbSAMT1*, *PR1* and *PR2* genes in leaves was analyzed by northern blot at 6 and 12 hours after treatments.

Discussion

The *A. belladonna* SAMT gene was isolated and characterized in the cultured hairy roots of *A. belladonna* after exogenous SA had been administered (Fukami et al. 2002). In the present study, *AbSAMT1* was induced by exogenous SA in *A. belladonna* plants as well as in cultured hairy roots (Figure 1). The continuous presence of exogenous SA in the pots of *A. belladonna* plants increased the amount of volatile MeSA levels emitted from the plants to a sustained level of about 50 $\mu g\, g^{-1}$ fresh weight (Figure 1D). Furthermore, we studied whether the expression of *AbSAMT1* gene was related to stimulation of SA biosynthesis. The expression of *AbSAMT1* gene was induced by BIT, the upstream regulator of SA in SAR, but not by the downstream regulators BTH and CMPA that do not need SA for the induction of SAR. However, the *PR2* gene was induced by all three chemical inducers (Figure 2B). These results show that the expression of the *AbSAMT1* gene is related to the accumulation of SA in *A. belladonna*. The expression of *OsBISAMT1* and *A. majus* SAMT (*AmSAMT*) genes has also been detected in SA treatments but not *O. sativa* BSMT1 (*OsBSMT1*) or *AtBSMT1* genes (Negre et al. 2002; Chen et al. 2003; Xu et al. 2006; Koo et al. 2007).

AbSAMT1 is activated by treatments of exogenous SA and also by BIT, which is a chemical inducer related to SAR. Indeed, we verified the expression of the *AbSAMT1* gene when the disease resistance was elicited by pathogenic infection in *A. belladonna*. In studies on tobacco it has been reported that TMV caused an increase of endogenous SA levels and induced PR protein, a marker gene of SA-dependent response, in leaves of tobacco plants (Nakashita et al. 2002; Yasuda et al. 2003). In our study, when *A. belladonna* plants were inoculated with the pathogenic bacteria *Pst* the plants showed distinctive HR to pathogen infection (Figure 3A), and the HR stopped the growth of *Pst* in the inoculated leaves (Figure 3B). The increase in MeSA levels after *Pst* infection (Figure 3E) appeared to be associated with the expression of the *AbSAMT1* gene (Figure 3C). However, it is not yet apparent exactly what biological role the expression of the SAMTs gene plays against pathogen infection in various plants. Some pathogen resistance studies reported that the function of MeSA, synthesized by SAMTs, was an airborne signal emitted by plants that were infected by pathogens or attacked by insects or parasites (Shulaev et al. 1997; Van Poecke et al. 2001; Chen et al. 2003; Ament et al. 2004). Furthermore, in studies on *SABP2* and *SAMT*-silenced plants, it was suggested that MeSA was essential for an SA-dependent response, as an endogenous signaling pathway (Kumar et al. 2003; Park et al. 2007). Therefore, these results imply that the role of SAMTs in plants is to regulate the exo- and endo-genous signaling of SA-dependent response.

The SA and JA signaling pathways have antagonistic functions to each other in plants, but it is not clear how these cross talk mechanisms lead to a significant interaction. Recently, SAMTs studies on various plants have reported that *AmSAMT* and *OsBISAMT1* were activated by SA and JA/MeJA, but *AtBSMT1* and *Solanum licopersicon* (*Lycoperscon esculentum*) *SAMT* (*LeSAMT*) were only induced by MeJA (Negre et al. 2002; Chen et al. 2003; Ament et al. 2004; Xu et al. 2006). In our study the expression of the *AbSAMT1* gene in *A. belladonna* was also rapidly induced and MeSA levels increased after wounding and treatment with vaporized MeJA (Figure 4, 5).

Our study shows that the increase in intracellular SA, as a result of pathogen infection, was sufficient to induce the expression of the *AbSAMT1* gene in *A. belladonna*. Furthermore, JA/MeJA, which negatively affects the SA-dependent response, also induced the expression of the *AbSAMT1* gene. Therefore, in conclusion these results suggest that there is dual regulation of distinct signaling pathways by *AbSAMT1* activity in *A. belladonna* plants. The first type of regulation is to produce the exogenous (Shulaev et al. 1997) and endogenous (Forouhar et al. 2005; Park et al. 2007) MeSA signaling pathways following the SA accumulation in response to biotic and abiotic stresses. The second is the depletion of stored SA, triggered by the JA-mediated signaling pathway, to allow the effective induction of the JA-dependent response in local regions.

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References

Ament K, Kant MR, Sabelis MW, Haring MA, Schuurink RC (2004) Jasmonic acid is a key regulator of spider mite-induced volatile terpenoid and methyl salicylate emission in tomato. *Plant Physiol* 135: 2025–2037

Arimura GI, Ozawa R, Shimoda T, Nishioka T, Boland W, Takabayashi J (2000) Herbivory-induced volatiles elicit defence genes in lima bean leaves. *Nature* 406: 512–515

Bowling SA, Guo A, Cao H, Gordon AS, Klessig DF, Dong X (1994) A mutation in *Arabidopsis* that leads to constitutive expression of systemic acquired resistance. *Plant Cell* 6: 1845–1857

Chen F, D'Auria JC, Tholl D, Ross JR, Gershenzon J, Noel JP, Pichersky E (2003) An *Arabidopsis thaliana* gene for methylsalicylate biosynthesis, identified by a biochemical genomics approach, has a role in defense. *Plant J* 36: 577–588

D'Auria JC, Chen F, Pichersky E (2003) The SABATH family of MTs in *Arabidopsis thaliana* and other plant species. In: Romeo JT (ed) *Recent Advances in Phytochemistry*, vol 37, Elsevier Science & Technology, Oxford, pp 253–283

Delaney TP, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H, Ryals J (1993) Requrement of salicylic acid for the induction of systemic acquired resistance. *Science* 261: 754–756

Delaney TP, Uknes S, Vernooij B, Friedrich L, Weymann K, Negrotto D, Gaffney T, Gut-Rella M, Kessmann H, Ward E, Ryals J (1994) A central role of salicylic acid in plant disease resistance. *Science* 266: 1247–1250

Dudareva N, Raguso RA, Wang J, Ross JR, Pichersky E (1998) Floral scent production in *Clarkia breweri*. III. Enzymatic systhesis and emission of benzenoid esters. *Plant Physiol* 116: 599–604

Dudareva N, Murfitt LM, Mann CJ, Gorenstein N, Kolosova N, Kish CM, Bonham C, Wood K (2000) Developmental regulation of methyl benzoate biosynthesis and emission in Snapdragon flowers. *Plant Cell* 12: 949–691

Effmert U, Saschenbrecker S, Ross J, Negre F, Fraser CM, Noel JP, Dudareva N, Piechulla B (2005) Floral benzenoid carboxyl methyltransferases: from in vitro to in planta fuction. *Phytochemistry* 66: 1211–1230

Farmer EE (1994) Fatty acid signaling in plants and their associated microorganisms. *Plant Mol Biol* 26: 1423–1437

Forouhar F, Yang Y, Kumar D, Chen Y, Fridman E, Park SW, Chiang Y, Acton TB, Montelione GT, Pichersky E, Klessig DF, Tong L (2005) Structural and biochemical studied identify tobacco SABP2 as a methyl salicylate esterase and implicate it in plant innate immunity. *Proc Natl Acad Sci USA* 102: 1773–1778

Friedrich L, Lawton K, Ruess W, Masner P, Specker N, Gut-Rella M, Meier B, Dincher S, Staub T, Uknes S, Métraux JP, Kessmann H, Ryals J (1996) A benzothiadiazole derivative induces systemic acquired resistance in tobacco. *Plant J* 10: 61–70

Fukami H, Asakura T, Hirano H, Abe K, Shimomura K, Yamakawa T (2002) Salicylic acid carboxyl methyltransferase induced in hairy root cultures of *Atropa belladonna* after treatment with exogenously added salicylic acid. *Plant Cell Physiol* 43: 1054–1058

Grob K, Zürcher F (1976) Stripping of trace organic substances from water equipment and procedure. *J Chromatogr* 117: 285–294

Knudsen JT, Tollsten L, Bergstrom G. (1993) Floral scents a checklist of volatile compounds isolated by head-space techniques. *Phytochemistry* 33: 253–280

Koch T, Krumm T, Jung V, Engelberth J, Boland W (1999) Differential induction of plant volatile biosynthesis in the lima bean by early and late intermediates of the octadecanoid-signaling pathway. *Plant Physiol* 121: 153–162

Koo YJ, Kim MA, Kim EH, Song JT, Jung CK, Moon JK, Kim JH, Seo HS, Song SI, Kim JK, Lee JS, Cheong JJ, Choi YD (2007) Overexpression of salicylic acid carboxyl methyltransferase reduces salicylic acid-mediate pathogen resistance in *Arabidopsis thaliana*. *Plant Mol Biol* 64: 1–15

Kumar D, Klessig DF (2003) High-affinity salicylic acid-binding protein 2 in required for plant innate immunity and has salicylic acid-stimulated lipase activity. *Proc Natl Acad Sci USA* 100: 16101–16106

Kunkel BN, Brooks DM (2002) Cross talk between signaling pathways in pathogen defense. *Curr Opin Plant Biol* 5: 325–331

Lawton K, Friedrich L, Hunt M, Weymann K, Dalaney T, Kessmann H, Staub T, Ryals J (1996) Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. *Plant J* 10: 71–82

Malany J, Carr JP, Klessig DF, Raskin I (1990) salicylic acid: A likely endogenous signal in the resistance response of Tobacco to viral infection. *Science* 250: 1002–1004

McConn M, Creelman RA, Bell E, Mullet JE, Browse J (1997) Jasmonate is essential for insect defense in Arabidopsis. *Proc Natl Acad Sci USA* 94: 5473–5477

Métraux JP, Signer H, Ryals J, Ward E, Wyss-Benz M, Gaudin J, Raschdorf K, Schmid E, Blum W, Inverardi B (1990) Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science* 250: 1004–1006

Murfitt LM, Kolosova N, Mann CJ, Dudareva N (2000) Purification and characterization of *S*-adenosyl-L-methionine: benzoic acid carboxyl methyltransferase, the enzyme responsible for biosynthesis of the volatile ester methyl benzoate in flowers of *Antirrhinum majus*. *Arch Biochem Biophys* 382: 145–151

Nakashita H, Yasuda M, Nshioka M, Hasegawa S, Arai Y, Uramoto M, Yoshida S, Yamaguchi I (2002) Chloroisonicotinamide derivative induces a broad range of disease resistance in rice and tobacco. *Plant Cell Physiol* 43: 823–831

Nakashita H, Yoshioka K, Yasuda M, Nitta T, Arai Y, Yoshida S, Yamaguchi I (2002) Probenazole induces systemic acquired resistance in tobacco through salicylic acid accumulation. *Physiol Mol Plant Pathol* 61: 197–203

Negre F, Kolosova N, Knoll J, Kish CM, Dudareva N (2002) Novel *S*-adenosyl-L-methionine: salicylic acid carboxyl methyltransferase, an enzyme responsible for biosynthesis of methyl salicylate and methyl benzoate, is not involved in floral scent production in Snapdragon flowers. *Arch Biochem Biophys* 406: 261–270

Niki T, Nitsuhara I, Seo S, Ohtsubo N, Ohashi Y (1998) Antagonistic effect of salicylic acid and jasmonic acid on the expression of pathogenesis-realted(PR) protein genes in wounded mature Tobacco leaves. *Plant Cell Physiol* 39: 500–

507

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: 113–115

Pott MB, Pichersky E, Piechulla B (2002) Evening specific oscillations of scent emission, SAMT enzyme activity, and SAMT mRNA in flowers of *Stephanotis floribunda*. *Plant Physiol* 159: 925–934

Pott MB, Hippauf F, Saschenbrecker S, Chen F, Ross J, Kiefer I, Slusarenko A, Noel JP Pichersky E, Effmert U, Piechulla B (2004) Biochemical and structural characterization of benzenoid carboxyl methyltransferases involved in floral scent production in *Stephanotis floribunda* and *Nicotiana suaveolens*. *Plant Physiol* 135: 1946–1955

Ross JR, Nam KH, D'Auria JC, Pichersky E (1999) *S*-adenosyl-L-methionine: salicylic acid carboxyl methyltransferase, an enzyme involved in floral scent production and plant defense, represents an new class of plant methyltransferases. *Arch Biochem Biophys* 367: 9–16

Seskar M, Shulaev V, Raskin I (1998) Endogenous methyl salicylate in pathogen-inoculated tobacco plants. *Plant Physiol* 116: 387–392

Shulaev V, Silverman P, Raskin I (1997) Airborne signaling by methyl salicylate in plant pathogen resistance. *Nature* 385: 718–721

Spoel SH, Koornneef A, Claessens SMC, Korzelius JP, Van Pelt JA, Mueller MJ, Buchala AJ, Métraux JP, Brown R, Kazan K, Van Loon LC, Dong X, Pieterse CMJ (2003) N*PR1* modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. *Plant Cell* 15: 760–770

Van Poecke RM, Posthumus MA, Dicke M (2001) Herbivore-induced volatile production by *Arabidopsis thaliana* leads to attraction of the parasitoid *Cotesia rubecula*: chemical, behavioral, and gene-expression analysis. *J Chem Ecol* 27: 1911–1928

Weber H, Vick BA, Farmer EE (1997) Dinor-oxo-phytodienoic acid: a new hexadecanoid signal in the jasmonate family. *Proc Natl Acad Sci USA* 94: 10473–10478

Xu R, Song F, Zheng Z (2006) *OsBISAMT1*, a gene encoding *S*-adenosyl-L-methionine: salicylic acid carboxyl methyltransferase, is differentially expression in rice defense responses. *Mol Biol Rep* 33: 223–231