J. Plant Biol. (2010) 53:417–424
DOI 10.1007/s12374-010-9130-y

ORIGINAL RESEARCH

The Pathway and Regulation of Salicylic Acid Biosynthesis in Probenazole-Treated *Arabidopsis*

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Received: 21 July 2010 / Revised: 23 September 2010 / Accepted: 29 September 2010 / Published online: 15 October 2010

Abstract Probenazole (PBZ; 3-allyloxy-1,2-benzisothiazole-1,1-dioxide) is a highly effective chemical inducer of systemic-acquired resistance (SAR). It has been used widely to protect rice plants against the rice blast fungus *Magnaporthe grisea*. Previous studies have shown that PBZ induces SAR through enhanced accumulation of salicylic acid (SA). Plants synthesize SA by either a pathway that uses phenylalanine as substrate or another that involves isochorismate. To clarify how SA is produced in PBZ-treated *Arabidopsis*, we examined the expression patterns and enzyme activities of phenylalanine ammonia lyase (PAL) and isochorismate synthase (ICS), which are the main components of the phenylalanine and isochorismate pathways, respectively. PBZ exposure significantly improved the accumulation of SA and increased ICS activity. In the *sid2–2* mutant, which has a defect in *ICS1*, PBZ had no effect on the level of endogenous SA or activity of ICS. In contrast, PAL activity and the expression of most PAL genes were down-regulated by such treatment in wild-type plants. These results suggest that SA is mainly synthesized via the ICS-mediated pathway in *Arabidopsis*.

Keywords *Arabidopsis thaliana* · Isochorismate synthase · Phenylalanine ammonia lyase · Probenazole · Salicylic acid

Abbreviations

CM	Chorismate mutase
ICS	Isochorismate synthase
PAL	Phenylalanine ammonia lyase
PBZ	Probenazole
SA	Salicylic acid

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Introduction

Plants have several defense mechanisms that protect them from pathogen attack. One common mechanism is the hypersensitive response (HR), which is characterized by the formation of necrotic lesions in the infected area to either restrict or kill pathogens. Another response, systemic-acquired resistance (SAR), is activated quickly in whole plants after the formation of HR lesions, thereby enabling the plant to defend against a following attack (Ryals et al. 1996).

Studies of various types of SAR-deficient *Arabidopsis* mutants have shown that SAR is mediated by a salicylic acid (SA)-dependent process (Cao et al. 1994; Gaffney et al. 1993; Wildermuth et al. 2001). For investigating the molecular mechanisms underlying SAR in plants, the chemical probes capable of inducing or inhibiting its activation are useful. For example, exogenous application of SA, which is endogenously produced and functions in SAR development, can significantly increase plant resistance against pathogens and prompt the expression of a set of defense genes (Delaney et al. 1994). Two other synthesis compounds—benzo(1,2,3)thiadiazole-7-carbothioic acid *S-methyl* ester (BTH) and methyl–2,6-dichloroisonicotinic acid (INA)—also enhance disease resistance and can induce SAR marker genes in tobacco, *Arabidopsis*, and wheat (Gorlach et al. 1996; Lawton et al. 1996; Ward et al. 1991; White 1979). These two compounds also induce SAR in NahG transgenic plants, which are unable to accumulate

SA due to over-expression of the SA-degrading enzyme salicylate hydroxylase (Gaffney et al. 1993; Lawton et al. 1996). Research with *Arabidopsis* mutants has indicated that these two chemicals induce SAR either through stimulation downstream of SA accumulation or by acting as SA analogs in signal transduction for SAR development (Lawton et al. 1996; Vernooij et al. 1995).

In contrast, probenazole (PBZ; 3-allyloxy-1, 2-Benzisothiazole-1, 1-dioxide) is a distinct SAR-inducer (Yoshioka et al. 2001). It was initially developed by Meiji Seika Kaisha Ltd. in Asia to control rice blast disease, which is caused by *Magnaporthe grisea*. This chemical can also protect rice from other diseases, including bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* (Shimura et al. 1981; Watanabe et al. 1979). More importantly, PBZ can prevent pathogen infections in *Arabidopsis* and tobacco (Koganezawa et al. 1998; Yoshioka et al. 2001). Extensive studies have demonstrated that PBZ and its active metabolite, 1,2-benzisothiazole-1,1-dioxide (BIT), induce SAR in *Arabidopsis* by stimulating the site upstream of SA accumulation during signal transduction (Yoshioka et al. 2001).

SA had been thought to be synthesized only from phenylalanine via *t*-cinnamic acid and benzoic acid in tobacco, potato, and *Arabidopsis* (Coquoz et al. 1998; Leon et al. 1993; Mauch-Mani and Slusarenko 1996; Ribnicky et al. 1998; Yalpani et al. 1993). In fact, suppressing the expression of one phenylalanine ammonia lyase (PAL) gene in transgenic tobacco plants leads to a decrease in SA accumulation in response to inoculation with tobacco mosaic virus (TMV) (Pallas et al. 1996). Exogenous application of 2-aminoindan-2-phosphonic acid, which is an inhibitor of PAL, blocks SA accumulation in pathogen-infected *Arabidopsis* and elicitor-treated potato (Coquoz et al. 1998; Mauch-Mani and Slusarenko 1996). These reports suggest that PALs play important roles in the pathway of SA synthesis. In addition, benzoic acid 2-hydroxylase (BA2H) catalyzes the formation of SA from benzoic acid; this activity is increased in TMV-inoculated tobacco (Leon et al. 1993). Pan et al. (2006) have found that SA synthesized by BA2H participates in the development of thermotolerance in pea plants (Pan et al. 2006). These results clearly indicate that BA2H is also involved in SA synthesis (Pan et al. 2006). However, Wildermuth et al. (2001) have shown that *SA induction-deficient 2 (sid2)* mutants, which are defective in isochorismate synthase 1 (ICS1), produce less SA than do wild-type (WT) *Arabidopsis* plants following pathogen infection. *Pseudomonas aeruginosa* possesses this pathway, for which isochorismate synthase (ICS) is the rate-limiting enzyme (Gaille et al. 2003; Serino et al. 1995). This newly found synthesis pathway also exists in *Nicotiana benthamiana* (Catinot et al. 2008). Thus, both *Arabidopsis* and tobacco plants contain a second pathway for SA synthesis via isochorismate in response to pathogen attack.

Among the synthesis chemicals identified as SAR-activators, only PBZ can induce SA synthesis during SAR development. Although those two SA-synthesis pathways have been clarified (Fig. 1), the one found in PBZ-treated *Arabidopsis* has not been elucidated. Therefore, our objectives were to investigate transcript levels and the activities of enzymes associated with SA synthesis, as well as to monitor the metabolic effect of synthesized SA in PBZ-treated *Arabidopsis*.

Materials and Methods

Plant Materials and PBZ Treatment

Seeds of *Arabidopsis thaliana* (L.) "Columbia" ("Col-0" wild type) and the mutant *sid2–2* were stratified at 4°C for 2 days. The germinants were grown in square pots (10-cm sides) containing a mixture of peat/vermiculite/perlite (3.0:9.0:0.5, *v*/*v*/*v*; Shanghai Institute of Landscape Science) that was pre-soaked with a MS (Murashige–Skoog) solution. All plants were placed in a growth room under an 8:16 h light/dark regimen (photosynthetic photon flux density of approximately 100 μmol m^{-2} s^{-1}), at 22±2°C and 60% humidity.

Four-week-old seedlings were treated by drenching the roots and spraying the leaves with either plain water or

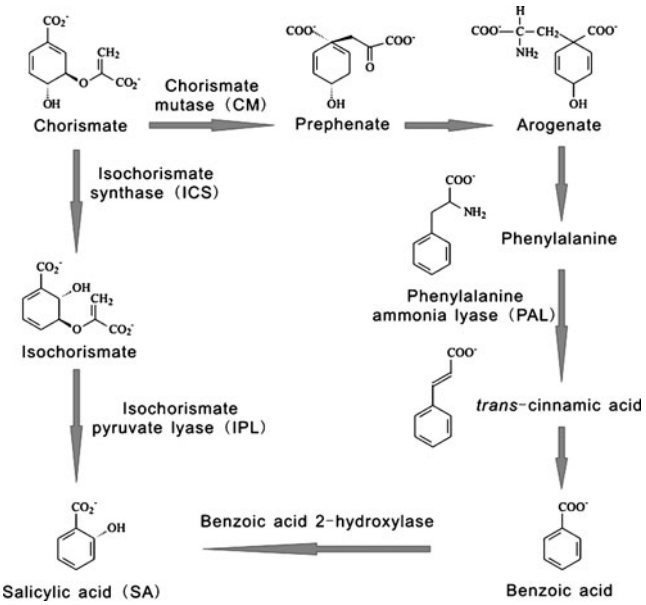


Fig. 1 Proposed pathways for SA biosynthesis

the agricultural chemical Oryzemate (Meijiseika), which contains 10% (wt/wt) probenazole. The efficient concentration used here was 0.2 mmol L^{-1}. After treatment, the leaves were harvested at indicated times.

Extraction and Measurements of Free and Total Salicylic Acid

Harvested samples (~1 g) were homogenized briefly and extracted with 3 ml of 90% methanol. After centrifugation, the pellet was re-extracted with another 3 ml of 100% methanol. These two supernatants were combined and then dried in a speed vacuum at 40°C. The dried residue was re-suspended in 4 ml of water and incubated at 80°C for 10 min. One-milliliter aliquots were used for analyzing both free SA and total SA (free SA plus SA β-glucoside, or SAG). One aliquot was re-suspended in 2.5 mL of 1:1 ethylacetate/cyclohexane after the addition of 50 μL of concentrated hydrochloride (HCL). The organic phase containing free SA was dried under nitrogen. This residue was dissolved in 0.5 mL of the high-performance liquid chromatography (HPLC) mobile phase (20% methanol in 20-mM sodium acetate buffer, pH 5.0), then filtered and applied to HPLC as the free SA sample.

Total SA was quantified as follows: 1 mL of β-glucosidase solution (3U mL^{-1}) was added to the second aliquot of water extract and incubated for 8 h at 37°C. This was extracted with 2.5 mL of 1:1 ethylacetate/cyclohexane after the addition of 50 μL of concentrated HCL. The sample was then prepared as for free SA. HPLC was performed on a C18 column running at 40°C, with 20% methanol in a 20 mM sodium acetate buffer (pH 5.0) and at a flow rate of 0.8 ml min^{-1}. SA was detected and quantified fluorometrically (295 nm excitation and 370 nm emission).

RNA Extraction and Real-time PCR Analysis

Total RNA was extracted from the leaves of water-treated or probenazole-treated WT and mutant *Arabidopsis*, using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). This was followed by chloroform-extraction and isopropanol-precipitation. RNA samples were digested with Rnase-free Dnase (Promega, Madison, WI, USA) and quantified by spectrophotometer. Afterward, 5 μg of total RNA was reverse-transcribed with Superscript reverse transcriptase (Stratagene, La Jolla, CA, USA). The product was subsequently used as template. Real-time polymerase chain reaction (PCR) was performed with SYBR Green I (TOYOBO Co., Osaka, Japan) on an iCycler (Bio-Rad, Hercules, CA, USA) according to the manufacturer's suggestions. The *Actin2* (*ACT2*) gene served as our reference. Specific primers are listed in Table 1.

Measurement of Isochorismate Synthase Activity

Isochorismate synthase (ICS) was extracted and measured according to the method of Ogawa et al. (2007), with minor modifications. Plant materials were ground with mortar and pestle in liquid nitrogen. Afterward, 0.25 g of powder was transferred to a tube to which 1 ml of extraction buffer [0.1 M Tris–HCl (pH 7.5), 10% glycerol, 1 mM EDTA, 2% PVP, and 1 mM DTT] was added. After stirring, the homogenate was centrifuged at 10,000×*g* for 30 min. The supernatant was de-salted in a Sephadex G-25 column (NAP-10; particle size, 20~50 μm; volume, 3.6 ml; GE Healthcare Life Sciences) equilibrated with 0.1 M Tris–HCl (pH 7.5) containing 10% glycerol, 1 mM EDTA, and 1 mM DTT. All steps were performed at 4°C. The incubation mixture contained 250 μl of 0.1 M Tris–HCl (pH 7.5), 3 mM barium chorismate (Sigma, St Louis, MO, USA), 15 mM $MgCl_2$, and a 250-μl sample of this de-salted solution. After 1 h of incubation at 30°C, isochorismate was quantified according to the method described (Young and Gibson 1969). The amount of protein was determined with a BCA protein assay kit (Shanghai Generay Biotech Co., Ltd., China).

Extraction and Measurement of Phenylalanine Ammonia Lyase Activity

Leaf material (approximately 0.2 g) was harvested and placed on ice. Extraction and quantitative determination of the PAL enzyme were performed as previously (Ogawa et al. 2007).

Affymetrix ATH1 GeneChip Experiment

Wild-type plants were grown on soil for 4 weeks under a 8:16 h light/dark regimen at 22±2°C with 60% humidity. Total RNA was prepared using TRIzol (Invitrogen, Carlsbad, CA, USA) with RNeasy mini kit (Qiagen) and multiple isopropanol precipitations. Affymetrix experiments were performed as described in the Affymetrix technical manual. Total RNA was used for cDNA and cRNA synthesis with the Affymetrix GeneChip One-Cycle Target Labeling Kit. Hybridizations, washing, staining and scanning were performed in GeneTech Biotechnology Co., Ltd. Fold change for each gene was calculated by dividing the expression level of a PBZ-treated 72 h sample by the expression level of a PBZ-treated 0 h sample. A twofold difference in expression level between PBZ-treated 72 and 0 h samples were set as the threshold for considering a gene to be PBZ inducible. This experiment was repeated two times with two pairs of independent samples.

Table 1 Oligonucleotide primers used in real-time reverse transcription (RT)-PCR

Gene	Froward primer(5′–3′)	Reverse primer (5′–3′)
ACT2	GGCTCCTCTTAACCCAAAGGC	CACACCATCACCAGAATCCAG
PR-1	GTTCACAACCAGGCACGAGGAGC	CCAGACAAGTCACCGCTACCCCAG
PR-2	CGTTCAGAGCTACAGAGATGGTG	CGGAGGAGACGTATCAGTGGTGGT
PR-5	CCTGCAAGAGTGCCTGTGAGAG	CGTCATAAGCGTAGCTATAGGCGT
ICS1	CCCGCAAGAAGTATGAGTCATGT	AGTTCAGAGACGGCGGAGATTAG
ICS2	CATTCTCCTCCGGCATTGTC	GAAGAAGATCTGGACGGCCA
PAL1	TCTCCTACATCGCCGGACTT	GACCTTCCTTAGGCTGGAGATCA
PAL2	GAACTGCGCCGATTCCTAAC	TCCTCTCCCGGAGACACAAC
PAL3	CGGTACTGTTCGATGCCAACA	CTGACCAGGATGGTGCTTGA
PAL4	TTCCCGTCTAGCCATTGCTT	GCGCCTTTAAACCCGTAATCA
CM1	GGTGTTGCATTTTGCTGCTGA	AGGCAGATAGCGTCACAGACAG
CM2	CATCGGCTTGCACCCTAAG	AGGCGAGATCACTAGACGCAGTT
CM3	CCCACAATGCTTGCCTGAA	TACCGTCATCCCCTGGCTT

Results

PBZ Increases the Levels of Free and Total Salicylic Acid in Wild-type *Arabidopsis*

BIT, an active metabolite of probenazole (Uchiyama et al. 1973), can increase the level of SAG in *Arabidopsis* plants (Yoshioka et al. 2001). Therefore, we investigated here how PBZ itself regulates the amounts of both free SA and total SA (free SA plus SAG), and how the former is synthesized in WT *Arabidopsis*. After treating the plants with PBZ, we first examined time-dependent changes in the levels of the two forms. Both began to accumulate after 2 days of treatment, with total SA peaking at day 6 before decreasing. Control plants treated only with water showed no significant rise in the level of either free or total SA at any sampling point (Fig. 2a, b).

The Mutation in *ICS1* Blocks SA Synthesis that is Induced by PBZ

Two SA-synthesis pathways can function in pathogen-infected plants—one regulated by PAL, BA2H, and chorismate mutase (CM); the other by ICS and isochorismate pyruvate lyase (IPL) (Fig. 1). The *Arabidopsis* genome contains two *ICS* genes, *ICS1* and *ICS2*. Wildermuth et al. (2001) have found that, upon pathogen attack, *SA induction-deficient 2* (*sid2*) mutants, defective in isochorismate synthase 1 (ICS1), produce less SA and show lower expression of pathogen-related (PR) genes compared with the WT.

To investigate whether this *ICS1* mutation also causes a defect in PBZ-induced SA synthesis, we measured amounts of free and total SA in PBZ-treated *sid2–2*. Mutants showed no rise in either form, such that those contents remained at a basal level similar to that measured in the water-treated control (Fig. 3a, b). We also examined the patterns of expression for *PR-1*, *PR-2*, and *PR-5* in both the WT and *sid2–2* mutants after PBZ exposure. As expected, the former showed an obvious increase in expression of *PR-1*, reaching a maximum at 4-day post-treatment. A similar response was observed with *PR-2* and *PR-5* (Fig. 3c). In sharp contrast, expression by those three PR genes was not changed in the mutant at any time after PBZ treatment by using *t* test analysis (Fig. 3d). Therefore, our results demonstrate that PBZ-induced SA synthesis and expression of its downstream PR genes are significantly blocked by the mutation in *ICS1*.

PBZ Treatment Dramatically Improves ICS Activity

To further clarify how PBZ regulates the SA-synthesis pathway when mediated by *ICS1* in WT *Arabidopsis*, we analyzed the time-dependent expression pattern of genes encoding isochorismate synthase (*ICS1* and *ICS2*) as well as changes in enzyme activity during PBZ treatment. Real-time PCR was used to quantify transcript abundance. Levels of ICS1 transcript were greatly increased after 2 days of treatment, peaking at day 4 before gradually declining about eightfold by day 8. However, PBZ had no effect on expression of *ICS2* at any sampling point (Fig. 4a). We then monitored ICS activity within WT and *sid2–2* mutant plants during treatment. Activities in the former increased after 2 days, reaching the highest level at day 6 before declining, whereas levels in the latter showed no change (Fig. 4b). These results suggest that PBZ-induced SA synthesis is mainly through a rise in the transcriptional level of *ICS1* and its enzyme activity.

PBZ-induced SA Synthesis is Independent of the PAL-mediated Synthesis Pathway

SA is synthesized by the PAL pathway in many plant species. To examine the contribution of the PAL-mediated

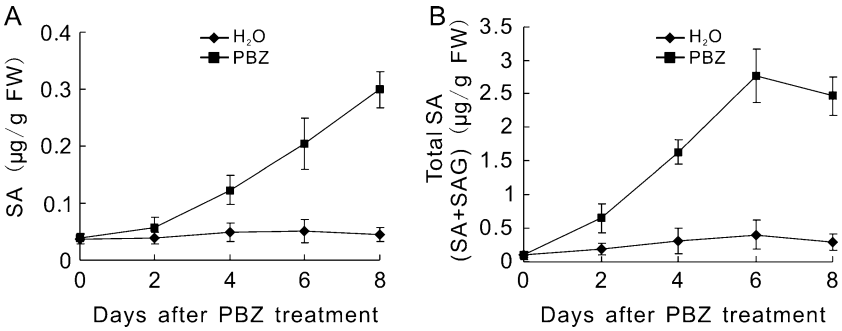


Fig. 2 Accumulations of free and total salicylic acid (free SA plus SAG) in WT ("Col-0") *Arabidopsis* plants treated with PBZ. **a**, **b** Leaves were harvested at indicated times after treatment with 0.2 mM PBZ or H_2O. SA levels were quantified by HPLC

pathway in PBZ-induced SA synthesis in *Arabidopsis*, we analyzed the expression pattern for genes encoding chorismate mutase (*CM1*, *CM2*, and *CM3*) and PAL (*PAL1*, *PAL2*, *PAL3*, and *PAL4*). Of the three CM genes, only mRNA from *CM2* rose slightly after 2 days of treatment whereas transcripts of *CM3* remained unaffected and *CM1* showed a slight decrease (Fig. 5a). In contrast, the expression of *PAL1*, *PAL2*, and *PAL4* was down-regulated after 2 days, while no significant change in *PAL3* transcripts was detected during the treatment period (Fig. 5b). Through microarrays, we analyzed patterns of global expression over 72 h and determined the fold changes for CM and PAL genes from two independent experiments (Fig. 5c). These microarray data were quite consistent with those data obtained via quantitative real-time PCR.

We also monitored the effect of PBZ on PAL, and found that activity in the WT decreased gradually after treatment. A similar trend was observed with our *sid2–2* mutants (Fig. 5d), suggesting the PAL-mediated synthesis pathway may be not involved in SA biosynthesis that is PBZ-induced.

Discussion

Salicylate is an important signal molecule that plays a major role in numerous physiological and defense responses. However, some aspects of its biosynthesis remain unclear in several plant species. As evaluated here, the expression level of *ICS1* and the activity of ICS were greatly increased after probenazole treatment. Moreover, the levels of free SA and total SA were increased in treated WT plants but not in the *sid2–2* mutant, which has a defect in *ICS1*. These results suggest that the ICS-mediated synthesis pathway is important for SA biosynthesis in PBZ-treated *Arabidopsis*.

The PAL-mediated pathway is also essential to normal SA synthesis in plants such as potato, tobacco, and *Arabidopsis*, or as a reaction to stress (Coquoz et al. 1998; Leon et al. 1993; Mauch-Mani and Slusarenko 1996; Ribnicky et al. 1998; Yalpani et al. 1993). For instance, SA levels are diminished in response to TMV inoculation in transgenic tobacco plants where expression of endogenous *PAL* mRNA is suppressed (Pallas et al. 1996). Application

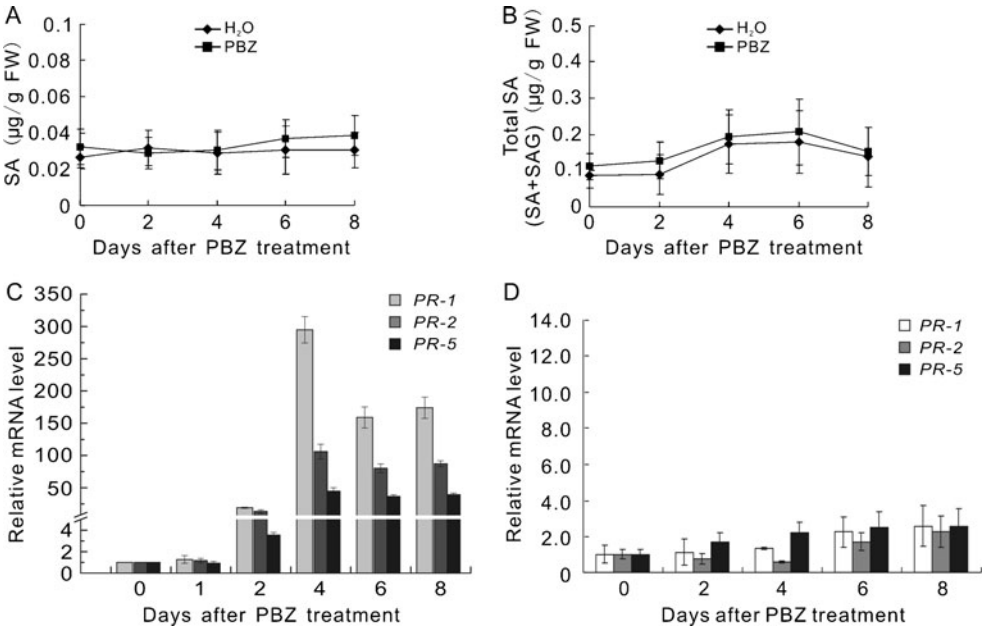


Fig. 3 PBZ-induced accumulations of free and total SA and PR gene expression were abolished in *sid2–2* mutant plants. **a**, **b** Levels of SA in PBZ- or H_2O-treated mutant; **c**, **d** time-course analysis of *PR-1*, *PR-2*, and *PR-5* expression in leaves of WT or mutant treated with 0.2 mM PBZ and harvested at indicated times. Transcript levels were quantified by real-time RT-PCR, with *ACT2* as reference. Expression levels of *PR-1*, *PR-2*, and *PR-5* in plants treated for 0 day were normalized separately to "1". All data points are mean values (n=3) ± SD

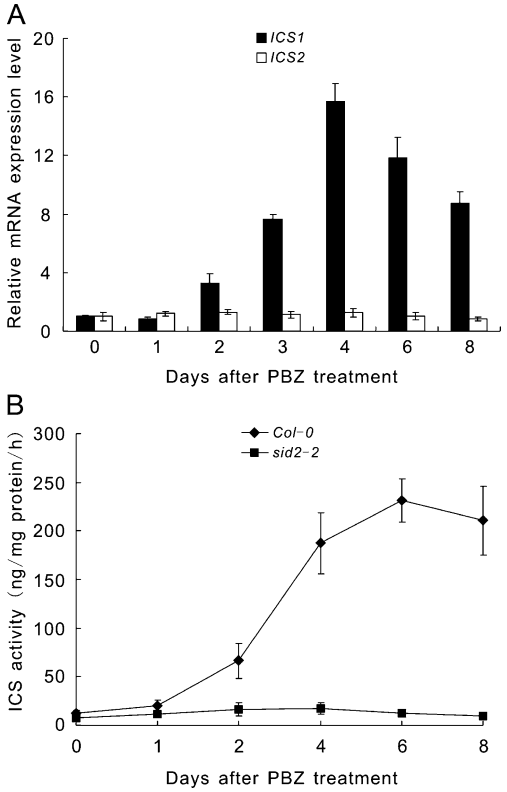


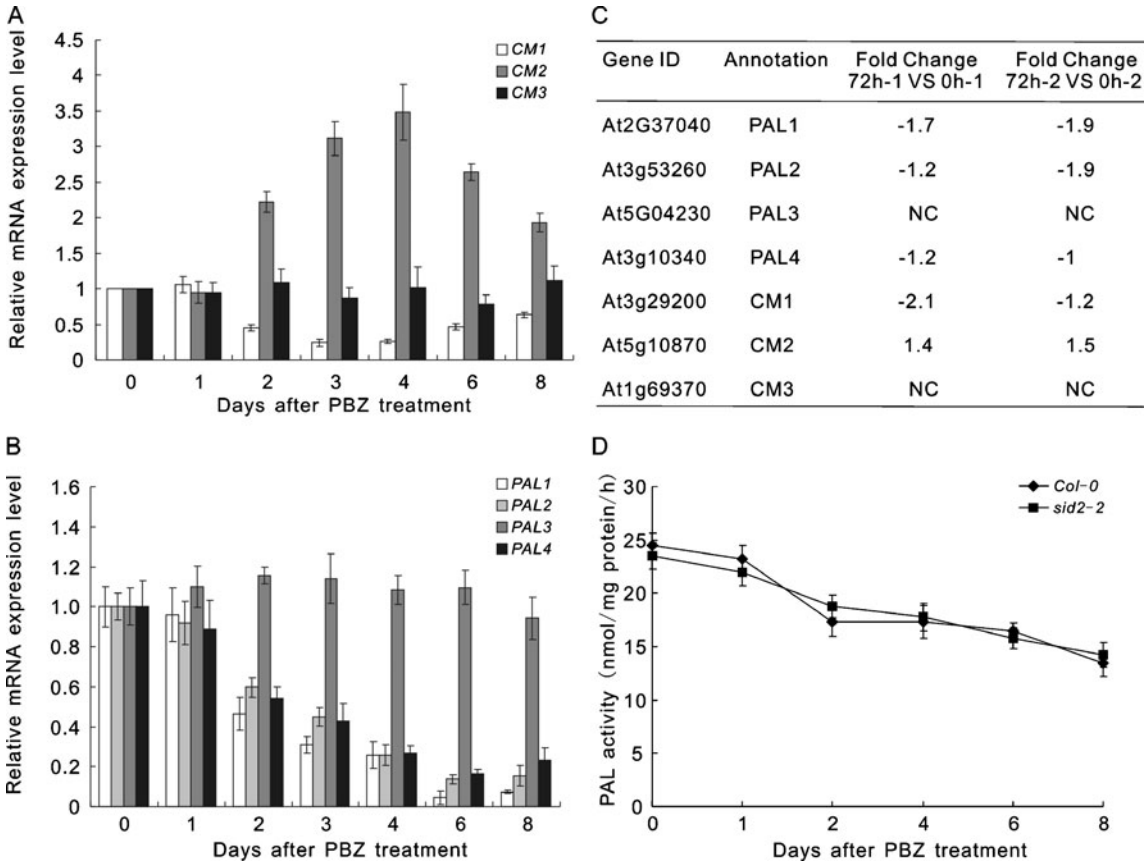
Fig. 4 PBZ treatment increased ICS activity and mRNA level of *ICS1*. **a** Time-course analysis of *ICS1* and *ICS2* expression in PBZ-treated WT plants. Leaves were harvested at indicated times after treatment. Transcript levels were quantified by real-time RT-PCR, with *ACT2* as reference. Expression levels of *ICS1* and *ICS2* in plants treated for 0 day were normalized to "1". **b** Dynamic changes in ICS activity by WT and *sid2–2* mutant plants after treatment. Numbers along *x*-axis indicate days after treatment. Data points are means of three independent experiments, and *vertical bars* show ± SD

of 2-aminoindan-2-phosphonic acid, which is an inhibitor of PAL, blocks SA accumulations in both pathogen-infected *Arabidopsis* and elicitor-treated potato (Coquoz et al. 1998; Mauch-Mani and Slusarenko 1996). However, Wildermuth et al. (2001) have described a new SA-synthesis pathway that is mediated by *ICS1* in *Arabidopsis*. Furthermore, Ogawa et al. (2005, 2007) have reported that essentially all SA is synthesized by the PAL-mediated pathway in O_3-exposed tobacco plants, but is mainly synthesized by the ICS-mediated pathway in O_3-treated *Arabidopsis*. *Nicotiana benthamiana* plants with silenced *ICS* expression also provide evidence for the existence of an ICS-mediated pathway (Catinot et al. 2008). Therefore, it seems likely that two SA-synthesis pathways operate simultaneously in many species, and that each pathway is induced by specific stimuli.

We showed here that *PAL* expression and PAL enzyme activity are decreased in response to PBZ. This implies that the PAL-mediated pathway does not contribute substantially to SA biosynthesis in treated *Arabidopsis*. Iwai et al. (2007) have found that PBZ increases the level of endogenous SA in rice (*Oryza sativa*) only at the 8-leaf developmental stage even though SA is present at very high levels under normal conditions. PBZ treatment can also significantly enhance PAL activity in rice (Iwata et al. 1980). Based on these results, we can conclude that the effect of PBZ on PAL activity differs completely between *Arabidopsis* and rice. Only one *ICS* gene has been found in japonica rice, but it has not yet been studied. Other research with that species has proven that SA is mainly synthesized within a PAL-dependent pathway (Sawada et al. 2006; Silverman et al. 1995). Such contrasting results, therefore, imply that the pathway involved in PBZ-induced SA synthesis in rice is regulated by developmental signals and is quite different from that in *Arabidopsis*. Further studies are needed to elucidate why these divergent pathways are induced by probenazole.

The existence of two ICS genes in *Arabidopsis* raises the issue of their specificity and the extent of their redundancy. Using *ics1*, *ics2*, and *ics1 ics2* mutants, Garcion et al. (2008) have demonstrated that *ICS2* contributes to SA accumulations but in limited amounts, detectable only when *ICS1* is lacking. Moreover, the occurrence of SA in the *ics1 ics2* double mutant provides genetic evidence for the presence of an ICS-independent SA-biosynthesis pathway in *Arabidopsis*. Our examination of the pattern for *ICS2* showed that PBZ treatment had no effect on its transcripts. However, specific stimuli have previously induced the expression of *ICS2*, with or without *ICS1* induction (Zimmermann et al. 2005), suggesting that the former has a yet undiscovered, but important, function.

This ICS-mediated pathway was first described in bacteria, where SA is synthesized from chorismate via the rate-limiting enzymes ICS and IPL (Verberne et al. 1999). Transgenic tobacco plants that over-express bacterial monofunctional ICS and IPL targeted to the plastid exhibit an SA-overproduction functional phenotype (Verberne et al. 2000). In *Arabidopsis*, a true SA over-expression phenotype has been observed that expresses constitutively an SA synthase PchBA fusion protein with functions of both ICS and IPL in the plastid compartment (Mauch et al. 2001). However, the gene encoding a protein with IPL activity similar to PchB in *Pseudomonas aeruginosa* (Pae PchB) has not yet been cloned in *Arabidopsis*. Pae PchB encodes a small 101-amino acid protein that appears to have evolved from a chorismate mutase; it retains residual CM activity with tenfold less affinity for chorismate than for isochorismate (Gaille et al. 2002). The *Arabidopsis* genome contains three confirmed CM genes (*AtCM1* At3g29200, *AtCM2* At5g10870, and *AtCM3* At1g69370) (Eberhard et al. 1996; Mobley et al. 1999). Among them, only *CM2* transcripts were induced after our PBZ treatment, suggesting the possibility that it is an IPL candidate in *Arabidopsis*.



Gene ID	Annotation	Fold Change 72h-1 VS 0h-1	Fold Change 72h-2 VS 0h-2
At2G37040	PAL1	-1.7	-1.9
At3g53260	PAL2	-1.2	-1.9
At5G04230	PAL3	NC	NC
At3g10340	PAL4	-1.2	-1
At3g29200	CM1	-2.1	-1.2
At5g10870	CM2	1.4	1.5
At1g69370	CM3	NC	NC

Fig. 5 Transcript levels of *CM* and *PALs*, and PAL activity in PBZ-treated *Arabidopsis*. **a** Real-time PCR analysis of expression for *CM1*, *CM2*, and *CM3*. **b** Real-time PCR analysis of expression for *PAL1*, *PAL2*, *PAL3*, and *PAL4*. Total RNAs were obtained from leaves harvested at indicated days after treatment. Transcript levels were quantified by real-time RT-PCR, with *ACT2* as reference. Expression level of each gene in plants treated for 0 day was normalized to "1". **c** Microarray analysis of *CM* and *PAL* expression. Data from two independent replicates are presented, i.e., 72 h-1 versus 0 h-1 and 72 h-2 versus 0 h-2. RNA samples were compared between *Arabidopsis* plants treated with PBZ for 72 h and 0 h. Note that fold-change values are log-base 2 transformed. *NC* no altered expression. **d** Dynamic changes in PAL activity by WT and *sid2–2* mutant plants. Numbers along *x*-axis indicate days after treatment. *Vertical bars* show SD obtained from three replicates

Pathogen infection can significantly induce expression of *ICS1* and many PR genes in *Arabidopsis* (Wildermuth et al. 2001). Based on earlier reports and current results, we speculate that the action mode for PBZ might be the same as for pathogens, at least in *Arabidopsis*. An examination of the *ICS1* promoter region has revealed many W-box elements that are recognized by various WRKY transcription factors as generally involved in regulating pathogen responses (Eulgem et al. 2000). Using expression microarrays, we previously demonstrated that 15 WRKY family genes are up-regulated after PBZ exposure (Jin et al. 2010). At least one of them may play an important role in the positive regulation of SA synthesis that is mediated by *ICS1* during pathogen infection or PBZ treatment.

In conclusion, this is the first report that SA is mainly synthesized from the ICS-mediated pathway rather than the PAL-mediated pathway in PBZ-treated *Arabidopsis*. Further study will focus on identifying the factors that regulate *ICS1* expression, and determining the kind of synthesis pathway that is involved in other plant species, e.g., tobacco and rice, during probenazole treatment. This may provide a key to understanding the molecular basis for modulating SA synthesis and could help researchers draw a universal SA-synthesis map among different species.

Acknowledgements We are grateful to Professor X. Dong for providing the *sid2–2* mutant. We also gratefully acknowledge the skillful technical assistance of Miss Z. Huang during the HPLC analysis. This work was supported by grants from the National Program of Transgenic Variety Development of China (2008ZX08009-004, 2009ZX08009-061B) and from the Science and Technology Committee of the Shanghai Municipal Government (Grant No. 08DZ2270800).

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