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Manipulation of salicylate content in *Arabidopsis thaliana* by the expression of an engineered bacterial salicylate synthase

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Summary

Salicylic acid (SA) plays a central role as a signalling molecule involved in plant defense against microbial attack. Genetic manipulation of SA biosynthesis may therefore help to generate plants that are more disease-resistant. By fusing the two bacterial genes *pchA* and *pchB* from *Pseudomonas aeruginosa,* which encode isochorismate synthase and isochorismate pyruvate-lyase, respectively, we have engineered a novel hybrid enzyme with salicylate synthase (SAS) activity. The *pchB-A* fusion was expressed in *Arabidopsis thaliana* under the control of the constitutive cauliflower mosaic virus (CaMV) *35S* promoter, with targeting of the gene product either to the cytosol (c-SAS plants) or to the chloroplast (p-SAS plants). In p-SAS plants, the amount of free and conjugated SA was increased more than 20-fold above wild type (WT) level, indicating that SAS is functional in Arabidopsis. P-SAS plants showed a strongly dwarfed phenotype and produced very few seeds. Dwarfism could be caused by the high SA levels *per se* or, perhaps more likely, by a depletion of the chorismate or isochorismate pools of the chloroplast. Targeting of SAS to the cytosol caused a slight increase in free SA and a significant threefold increase in conjugated SA, probably reflecting limited chorismate availability in this compartment. Although this modest increase in total SA content did not strongly induce the resistance marker *PR-1,* it resulted nevertheless in enhanced disease resistance towards a virulent isolate of *Peronospora parasitica*. Increased resistance of c-SAS lines was paralleled with reduced seed production. Taken together, these results illustrate that SAS is a potent tool for the manipulation of SA levels in plants.

Keywords: salicylate synthase, chorismate, metabolic engineering, bifunctional enzyme, disease resistance, Arabidopsis.

Introduction

In plants, salicylate (SA) has many different functions (Raskin, 1995), but it is probably known best for its role as a key signalling compound in the activation of certain defense responses against invading pathogens (for a recent review see Dempsey *et al.*, 1999). In some plants, such as tobacco and cucumber, exogenous application of SA induces systemic acquired resistance (SAR), a mechanism by which the entire plant becomes more resistant to subsequent viral, bacterial, or fungal infection. Malamy *et al.* (1990) showed that SA is also an endogenous signal molecule which accumulates to high levels at an infection site (locally) and to a lesser extent also in uninfected tissues (systemically). Although SA is mobile and can be found in the phloem of pathogen-infected tobacco and cucumber (Métraux *et al.*, 1990; Rasmussen *et al.*, 1991; Smith *et al.*, 1991), its role as a long distance SAR signal is still a matter of debate (Dempsey *et al.*, 1999). The importance of SA in plant defense has been demonstrated in transgenic tobacco and Arabidopsis plants expressing the *Pseudomonas putida nahG* gene, which encodes the SA metabolizing enzyme salicylate hydroxylase. *NahG* plants are unable to accumulate high levels of SA upon pathogen infection and fail to establish SAR (Delaney *et al.*, 1994; Gaffney *et al.*, 1993; Vernooij *et al.*, 1994). Furthermore, it

has been shown that several Arabidopsis mutants which constitutively display an SAR phenotype accumulate high levels of SA (Bowling *et al.*, 1994; Bowling *et al.*, 1997; Clarke *et al.*, 1998; Dietrich *et al.*, 1994; Greenberg *et al.*, 1994; Klessig *et al.*, 1996; Weymann *et al.*, 1995; Yu *et al.*, 1998). Genetic manipulation of SA levels may therefore help to engineer crops that have improved disease resistance. However, the biosynthetic pathway leading to SA is only partly understood in plants (for recent reviews see Raskin, 1995; Verberne *et al.*, 1999). Tracer studies have shown that in healthy and virus-inoculated tobacco SA is synthesized from *trans*-cinnamate via benzoate (Yalpani *et al.*, 1993).

SA is also made by certain bacteria belonging to the genera *Azospirillum, Pseudomonas, Mycobacterium and Yersinia* where this compound is involved in iron acquisition. SA is part of different siderophores such as pyochelin in *Pseudomonas* spp., carboxy mycobactin in *Mycobacterium* spp. and yersiniabactin in *Yersinia* spp. (Adilakshmi *et al.*, 2000; Ankenbauer and Cox, 1988; Drechsel *et al.*, 1995) which complex Fe^{3+} in the environment and deliver it to the cytoplasm via specific membrane receptors. In the absence of inorganic phosphate, SA may also act as a siderophore in its own right (Meyer *et al.*, 1992; Ratledge *et al.*, 1974; Saxena *et al.*, 1986; Visca *et al.*, 1993). When produced by root colonizing strains of *Pseudomonas aeruginosa* or *P. fluorescens*, SA was shown to be important for the induction of systemic resistance in bean and tobacco leaves (De Meyer and Höfte, 1997; De Meyer *et al.*, 1999; Maurhofer *et al.*, 1998). Marshall and Ratledge (1972) showed that, in *Mycobacterium smegmatis,* SA is formed from chorismate via isochorismate. The same pathway occurs in *P. aeruginosa*, where the biosynthetic genes *pchA* for isochorismate synthase (ICS) and *pchB* for isochorismate pyruvate-lyase (IPL) have been isolated (Serino *et al.*, 1995). These genes are located in the *pchDCBA* operon which, together with the closely linked *pchEFG* genes, is required for the formation of the siderophore pyochelin (Figure 1; Quadri *et al.*, 1999; Reimmann *et al.*, 1998; Serino *et al.*, 1997; our unpublished results).

Here we show that the SA biosynthetic genes *pchBA* of *P. aeruginosa* can be used to manipulate SA production in plants. We report the genetic engineering of a *pchB-A* gene fusion encoding a novel bifunctional enzyme with salicylate synthase (SAS) activity and we demonstrate the use of this enzyme in the model plant *Arabidopsis thaliana*.

Results

Engineering of a bacterial salicylate synthase by gene fusion

In *P. aeruginosa* the *pchA* gene (encoding ICS) is expressed only when the *pchB* gene (encoding ICL) is present *in cis* (Serino *et al.*, 1995). We therefore did not envisage to engineer Arabidopsis for constitutive SA production by trying to express these genes separately. Instead, we constructed a bifunctional ICS/ICL, by fusing the *pchA* and *pchB* genes via a linker region of various sizes, following a strategy illustrated in Figure 2 and described in Experimental procedures. As a linker region we chose the polylinker of the cloning vector pBluescript KS. Briefly, the *pchB* coding sequence without its stop codon was PCR-amplified and cloned into pBluescript KS to give pME6141. Similarly, the 5′ part of *pchA* was PCR-amplified and reassembled with the downstream part of *pchA* in pUC18 to give pME6150. The coding region of *pchB* was then joined at different restriction sites located in the polylinker (*Hin*cII, *Eco*RV, or *Sma*I, respectively) with the coding sequence of *pchA* (at *Sma*I), resulting in three different *pchB-A* gene fusions, with linker regions of 2 (in pME6145), 9 (in pME6146) and 15 codons (in pME6140).

SA and pyochelin formation by P. aeruginosa *derivatives carrying* pchB-A *gene fusions*

To test which of the *pchB-A* fusion proteins was the most active, we compared the formation of SA and pyochelin in the wild type strain PAO1 with that in PAO1 derivatives carrying either of the three *pchB-A* gene fusions obtained above. The three derivatives PAO6309 (2-codon linker), PAO6307 (9-codon linker) and PAO6308 (15-codon linker) were constructed by exchanging the chromosomal *pchBA* genes of PAO1 against a single copy of each of the fusions (see Experimental procedures). The presence of a PchB-A fusion protein in these strains was verified in a Western analysis using antibodies raised against purified PchA and

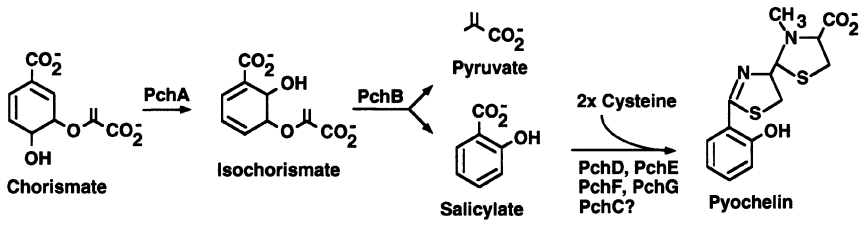

Figure 1. Biosynthetic pathway of SA and pyochelin in *P. aeruginosa.*

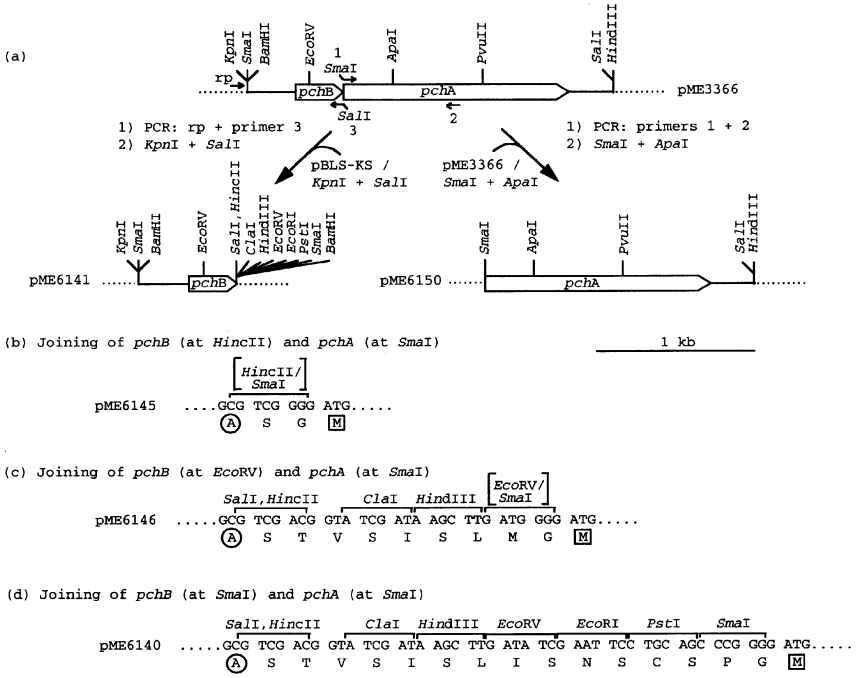

Figure 2. Construction of in-frame *pchB-A* gene fusions.
(a) The pUC18-derivative pME3366, which carries the *pchBA* genes, served as a template in two PCR reactions, which amplified *pchB* and the 5′ part of *pchA* to allow the construction of pME6141 and pME6150, respectively. Vector sequences (pUC18 or pBluescript KS) are indicated by dotted lines. (b–d) Nucleotide and derived amino acid sequences of the linker regions between the fused *pchB* and *pchA* genes present in pME6145, pME6146 and pME6140 are shown. The C-terminal amino acid alanine of *pchB* and the N-terminal amino acid methionine of PchA are highlighted.

Table 1. SA and pyochelin content in culture supernatants from *P. aeruginosa* strains carrying either the wild type *pchA* and *pchB* genes, or one of the three *pchB-A* gene fusions

Strain	*pchB-A* linker	SA (pmol ml^{-1})	Pyochelin (pmol ml^{-1})
PAO1	–	780 ± 80	9990 ± 1030
PAO6309	2 codons	480 ± 4	10570 ± 820
PAO6307	9 codons	260 ± 6	3370 ± 340
PAO6308	15 codons	510 ± 3	9230 ± 700

The values given represent the means ± standard deviations of three independent experiments.

purified PchB, respectively. Similar amounts of a 63-kDa protein, corresponding to the expected size of a PchB-A fusion, were detected with both antibodies in crude extracts of PAO6307, PAO6308, and PAO6309, but not in an extract prepared from strain PAO1. Furthermore, the individual PchA and PchB proteins, which were produced by the wild type strain under these iron-limiting growth conditions, were not detectable in PAO6307, PAO6308 and PAO6309 (data not shown).

The content of SA and pyochelin in culture supernatants of strain PAO1, PAO6307, PAO6308 and PAO6309 were determined by HPLC. As shown in Table 1, both compounds were made by PAO1 and by all three derivatives, indicating that each gene fusion produced an active bifunctional enzyme. The amounts of SA produced by the 2-and the 15-codon linker derivatives were similar and reached about 65% of the wild type level. In addition, comparable pyochelin concentrations were measured in supernatants of these derivatives and PAO1. Less SA and pyochelin were made by the 9-codon linker strain PAO6307, reflecting a lower activity of this hybrid enzyme. For the following plant experiments, the 2-codon linker construct was chosen and the corresponding hybrid enzyme was termed SAS for salicylate synthase.

Production of transgenic Arabidopsis expressing salicylate synthase (SAS)

Transgenic Arabidopsis plants were produced that express the chosen *pchB-A* gene fusion under the control of the constitutive CaMV *35S* promoter. To identify plants with an elevated SA content, 16 6-week-old primary (heterozygotic) transformants constitutively expressing SAS in the cytosol (c-SAS) were analysed for free and conjugated (glycosylated) SA. Compared to the SA levels measured in wild type plants (WT), the levels of free and conjugated SA were found to be maximally increased by a factor of 2 and 4, respectively (data not shown). Two lines with the highest levels of conjugated SA (> 2 μg g^{-1} fresh weight [FW]) produced only a few seeds. Therefore, eight transgenic lines with intermediate levels of conjugated SA (0.8–1.6 μg g^{-1} FW) were chosen for further experiments. These c-SAS lines were subjected to segregation analysis to identify those with a single insertion locus. Three such lines were propagated to the next generation to identify progeny homozygous for the *pchB-A* insertion. The

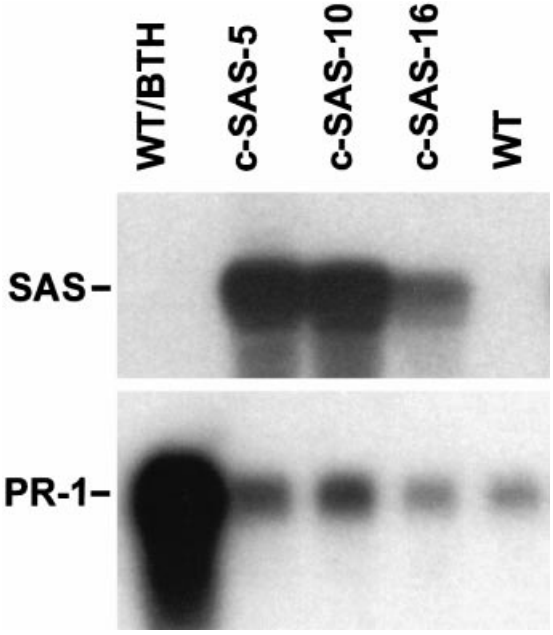

Figure 3. RNA blot analysis of *pchB-A* and *PR-1* expression in homozygous transgenic c-SAS plants.
Five μg of total RNA from WT Columbia plants and from three homozygous c-SAS lines were analysed for levels of *SAS* mRNA by hybridization to a radioactively labelled *pchB-A* probe or a radioactively labelled *PR-1* cDNA. RNA isolated from BTH treated Arabidopsis (WT/BTH) served as a positive control for *PR-1* gene expression.

phenotypes of these lines (c-SAS-5, c-SAS-10 and c-SAS-16) were further investigated.

First, expression of the *pchB-A* fusion was tested in a Northern analysis. As shown in Figure 3, the transgenic lines c-SAS-5 and c-SAS-10 expressed high levels of *pchB-A* RNA, whereas expression in c-SAS-16 was considerably lower. No RNA crosshybridizing with the *pchB-A* probe was detected in the WT. To test whether high *pchB-A* expression was correlated with increased SA content, we measured the amount of free and conjugated SA in 3-week-old transgenic and WT plants. As summarized in Table 2, the content of free SA was only slightly (and insignificantly) higher in the homozygous c-SAS plants compared to the WT, whereas conjugated SA was found to be significantly increased by a factor of 2.3 (in c-SAS-16) to 3.1 (in c-SAS-5). Thus, c-SAS plants continuously synthesize more SA than does the WT but as free SA is constantly conjugated, this modest increase in SA production manifests itself only by enhanced levels of conjugated SA.

The c-SAS-16 line with the lowest total SA content produced seeds in similar quantities as did the WT, whereas seed production by c-SAS-5 and c-SAS-10 plants was considerably reduced and reached only 26% and 65%, respectively (Table 2). Seeds of the c-SAS-5 line were smaller but remained fertile. Thus, the results obtained with these 3 homozygous c-SAS lines confirmed the previous analysis of 16 primary transformants where high SA content correlated with poor seed production. The threshold level of total SA content above which seed production is affected appears to be rather low and may be close to the level determined in line c-SAS-16. One line, c-SAS-5, showed a slight, statistically insignificant reduction in biomass (data not shown), whereas the two other lines had a normal growth phenotype (see Figure 4 for line c-SAS-10). None of the lines showed necrotic flecks and no microlesions were detected after staining with trypan blue.

Table 2. SA content and seed production of WT and transgenic SAS lines

Plant line	Free SA μg g^{-1} FW	Conjugated SA μg g^{-1} FW	Seed production mg $plant^{-1}$
WT	0.17 ± 0.10	0.85 ± 0.10	34 ± 9
c-SAS-5	0.26 ± 0.08	2.61 ± 0.36	9 ± 5
c-SAS-10	0.24 ± 0.09	2.43 ± 0.20	22 ± 11
c-SAS-16	0.20 ± 0.09	1.92 ± 0.41	35 ± 10
WT	0.11	0.92	nd
p-SAS-1	2.07	23.29	nd
p-SAS-2	3.67	20.56	nd

nd, not determined.

Targeting of SAS to chloroplasts

The modest increase in SA production observed in c-SAS plants may have two likely reasons. Either the synthetic SAS has limited catalytic activity in plants, or its substrate, chorismate, is not abundant in the cytoplasm. Chorismate is the substrate for aromatic amino acid biosynthesis, which occurs predominantly, if not exclusively, in the chloroplast (Schmid and Amrhein, 1995). Consequently, the main chorismate pool is expected to be present in the chloroplast rather than in the cytoplasm. Therefore, a second SAS-construct (p-SAS) was made which included a plastidial targeting signal from ferredoxin. This signal sequence was amplified by PCR from genomic Arabidopsis DNA and a fusion construct with the *pchB-A* sequence was made as described in Experimental procedures. The transgenic p-SAS plants thus generated showed a dramatic phenotype. All kanamycin resistant primary transformants were severely dwarfed. One representative of a less affected p-SAS line, which still managed to flower and produce a few seeds, is shown in Figure 4. Many primary transformants did not grow taller than a few millimetres and were not fertile. Because of their small size and their problems with seed production, SA content was not analysed on an individual basis but as pools of 12 primary transformants. As shown in Table 2, the levels of both free and conjugated SA were increased at least 20-fold over WT levels. The absolute amount of 3.67 μg of free SA g^{-1} FW measured in pool 2 is similar to the local level

found in Arabidopsis inoculated by bacterial or fungal pathogens (Nawrath and Métraux, 1999).

The high SA content of the p-SAS plants shows that the engineered SAS is highly active in Arabidopsis and that the low SA levels achieved in c-SAS lines are most likely caused by substrate limitation. The phenotype of p-SAS plants illustrates that a constitutive production of SAS targeted to the chloroplast is detrimental to plant development.

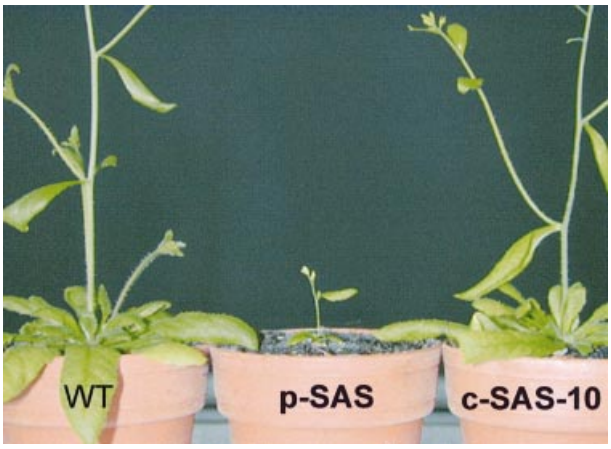

Figure 4. Growth phenotype of 6-week-old WT and transgenic p-SAS and c-SAS plants.

Disease resistance of c-SAS plants

To test whether transgenic plants with increased total SA content would be more resistant to pathogen attack, 3-week-old c-SAS plants of all three homozygous lines were challenged with an isolate of the biotrophic pathogen *Peronospora parasitica* to which the Columbia WT plants are susceptible. (The p-SAS plants were not tested for changes in disease resistance because their dwarfed phenotype would have made an interpretation of the results difficult.) Within a week following inoculation, *P. parasitica* completely colonized the leaf tissue of WT plants and produced asexual conidiospores as well as sexual oospores. A typical picture of a colonized WT plant is shown in Figure 5(a). All leaves present at the time of inoculation are heavily colonized as visible by an irregular network of trypan blue stained hyphae. A higher magnification (Figure 5b) shows the hyphal network and the presence of oospores. In contrast to WT plants, the development of *P. parasitica* was severely restricted in the c-SAS lines as illustrated by the examples given in Figure 5(c,d). The c-SAS lines reacted with a trailing necrosis response (Figure 5d), which was not observed in WT plants. The host cells in the vicinity of the pathogen showed an elevated membrane permeability, as revealed by their increased trypan blue staining. Note that, in these experiments, the youngest leaves were intensively stained

Figure 5. Disease resistance of WT and c-SAS plants.
Three-week-old seedlings were inoculated with isolate NOCO of *P. parasitica* to which the WT is susceptible. The samples were stained 1 week later with trypan blue to reveal pathogen structures and dead plant cells. Leaves of WT (a and b) are colonized with a dense network of hyphae (black arrowheads), which produce many oospores (white arrow heads). The transgenic lines c-SAS-5 (c) and c-SAS-16 (d) permit only limited growth of hyphae (black arrowheads) with little production of any form of spores. The host tissue of c-SAS plants reacts with a HR-like trailing necrosis response (d). Vascular elements are stained in WT and c-SAS lines.

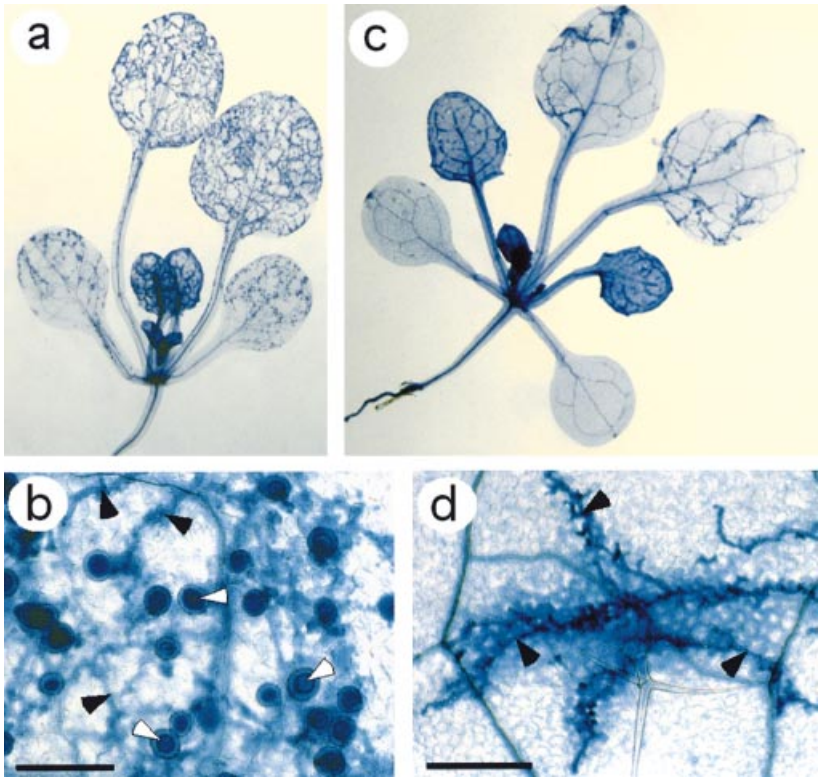

Table 3. Conidia production on WT and transgenic c-SAS lines infected with *P. parasitica* isolate NOCO

Plant line	Number of conidia per plant	% of WT value
WT	6762 ± 696	100
c-SAS-5	4292 ± 464	63
c-SAS-10	2784 ± 696	41
c-SAS-16	2436 ± 812	36

The values given represent the means ± standard deviations of two parallel experiments.

because of their high cytoplasmic content and the retention of the stain inside the abundant vessels. These young leaves were not infected because they were not yet expanded at the time of inoculation. Oospores were only detected occasionally in c-SAS plants, while they were abundantly produced in WT plants. To assess disease resistance quantitatively, conidia were washed off from 60 infected WT and transgenic c-SAS plants and counted. As shown in Table 3, production of conidia on all three c-SAS lines was significantly reduced compared with the production on WT plants. The degree of resistance observed in c-SAS transgenic lines is quantitatively comparable to the SAR response after biological induction with avirulent pathogens (Mauch-Mani and Slusarenko, 1994). Accumulation of pathogenesis related protein 1 (PR-1) is a typical marker for SAR (Uknes *et al.*, 1992; Uknes *et al.*, 1993). However, *PR-1* mRNA appeared not to be increased in line c-SAS-16 and was weakly increased in lines c-SAS-5 and c-SAS-10 compared with the WT (Figure 3), whereas much stronger induction was observed when plants were treated with the chemical SAR inducer BTH (Lawton *et al.*, 1996).

Discussion

We have engineered a novel bacterial enzyme, SAS, to manipulate the SA content in plants. SAS was expressed in Arabidopsis, with targeting of the gene product either to the cytoplasm (c-SAS plants) or to the chloroplast (p-SAS plants). In both cases, the total SA content of transgenic plants was higher than that in control plants, indicating that the enzyme was functional and that its substrate chorismate is available in both compartments, as observed earlier (Sommer and Heide, 1998). Whereas a modest (two- to threefold) increase of conjugated SA was observed in c-SAS plants, the increase in p-SAS plants was more pronounced (20-fold) for both free and conjugated SA, probably reflecting a better chorismate availability in the chloroplast. It appears unlikely that modification of the N-terminus in p-SAS could have improved the enzyme's catalytic activity.

SAS was created by fusion of the *pchA* and *pchB* genes encoding ICS and IPL, respectively, from *P. aeruginosa.* Bifunctional enzymes can prevent intermediates from entering competing metabolic pathways by a mechanism known as channeling, which allows direct transfer of an intermediate between the two active sites (Miles *et al.*, 1999). Whether SAS displays channeling of isochorismate has yet to be investigated.

Whilst this work was submitted for publication, Verberne *et al.* (2000) reported the manipulation of SA biosynthesis by using a similar approach. However, these authors obtained increased SA production by expressing an ICS from *E. coli* and an IPL from *P. fluorescens* as two separate polypeptides. When both enzymes were targeted to chloroplasts, transgenic tobacco plants accumulated up to 1000 times more free and conjugated SA than did control plants. Interestingly, this dramatic increase in SA content had no deleterious effect on plant growth and reproduction. This is in contrast to the results obtained here with Arabidopsis where a 20-fold increase in the SA content of transgenic p-SAS plants correlated with a strongly dwarfed phenotype (Figure 4) and even the modest increase observed in transgenic c-SAS plants was paralleled by a poor seed production. Since various Arabidopsis mutants having a 20- to 30-fold increased SA content grow better than do p-SAS plants and show good seed production (Bowling *et al.*, 1994; Bowling *et al.*, 1997; Clarke *et al.*, 1998; Yu *et al.*, 1998), it is unlikely that elevated SA concentrations *per se* could be responsible for the dwarf phenotype of the p-SAS plants. What else could be the reasons for the adverse effects of constitutive SAS expression in transgenic Arabidopsis? Chorismate is a substrate not only for SAS but also for plant enzymes such as chorismate mutase and anthranilate synthase, which are involved in the biosynthesis of aromatic amino acids in the chloroplast. In p-SAS plants, these enzymes have to compete with SAS for chorismate. If SAS depleted the plastidial chorismate pool, the biosynthesis of aromatic amino acids could be curtailed to the point of limiting plant growth. However, in transgenic tobacco expressing ICS and IPL this does not appear to be the case, as high amounts of SA are produced without causing a growth reduction (Verberne *et al.*, 2000). Thus, unless large differences in the size of the chorismate pools exist between tobacco and Arabidopsis, a depletion of this pool by p-SAS expression is not very likely.

Interestingly, transgenic tobacco plants expressing only IPL targeted to the chloroplasts show severely retarded growth (Verberne *et al.*, 2000). The authors suggested that expression of IPL in the absence of ICS may cause a shortage of isochorismate which could affect plant growth. In higher plants, isochorismate is the precursor of anthraquinone and phylloquinone and can also play a role in secondary metabolism (Ledüc *et al.*, 1991;

Simantiras and Leistner, 1991). In transgenic tobacco plants expressing both ICS and IPL, isochorismate generated by ICS may be equally available to IPL producing SA and to the anthraquinone and phylloquinone biosynthetic enzymes. In transgenic Arabidopsis expressing p-SAS, by contrast, the isochorismate made by SAS may remain enzyme-bound and be converted directly to SA without becoming available to other biosynthetic pathways. If, in addition, p-SAS consumed the endogenous isochorismate pool produced by plant ICS – a putative ICS cDNA clone has recently been isolated from Arabidopsis (Meng *et al.*, 1998) – a shortage of isochorismate might be the result.

In the c-SAS lines there was a marginal increase in total SA (Table 2); it is therefore not surprising that expression of *PR-1* was not strongly induced (Figure 3). Nevertheless, all three lines showed SAR-like resistance towards a virulent *P. parasitica* isolate. As shown previously, resistance can be induced by exogenous application of relatively small amounts of SA. Induction of *PR-1* occurs in a dose-dependent manner and requires significantly higher SA concentrations (Pieterse *et al.*, 1996; van Wees *et al.*, 1999). The SA content in c-SAS plants, although sufficient for resistance induction, may be too low for an efficient activation of the *PR-1* gene. Similar conclusions can be drawn from the results obtained with transgenic tobacco (Verberne *et al.*, 2000). When both ICS and IPL were targeted to the chloroplasts, large amounts of SA accumulated, the plants showed resistance to viral and fungal infections and the expression of *PR-1* was induced. When ICS, but not IPL, was targeted to the chloroplasts, much lower levels of SA accumulated which still induced some resistance but were too low to induce *PR-1* expression.

C-SAS Arabidopsis plants, but not WT plants, reacted to an attempted infection with a trailing necrosis, suggesting an HR-like response. As in SAR, c-SAS plants may be conditioned to react rapidly to pathogen ingress. Whether this HR-like response is the cause of resistance and what the underlying mechanisms are that trigger this response remain to be determined.

In conclusion, our results demonstrate that the engineered SAS is a powerful tool for the manipulation of plant SA content. The possibility to engineer plants for inducible or constitutive SA production in the absence of exogenous stress will be useful to study various aspects of signal transduction via the SA pathway and to learn more about SA-conjugate formation and transport.

Experimental procedures

Bacterial strains, plasmids and growth conditions

P. aeruginosa strains used in this study are derivatives of the wild type PAO1 (ATCC 15692). Plasmids pBluescript KS (Stratagene, La Jolla, CA, USA), pUC18 (Yanisch-Perron *et al.*, 1985), pCR2.1 (Invitrogen, Gröningen, The Netherlands), pSPORT (Gibco BRL, Paisley, UK), and pGEM7 (Promega, Madison, WI, USA) served as cloning vectors in *Escherichia coli* DH5α (Sambrook *et al.*, 1989). The *E. coli* strain S17–1 (Simon *et al.*, 1983) was used for mobilization of gene replacement plasmids constructed in the suicide vector pME3087 (Voisard *et al.*, 1994). *Agrobacterium tumefaciens* GV3101 (Koncz and Schell, 1986) carrying derivatives of the binary vector pBI121 (Clontech, Palo Alto, CA, USA) was used in plant transformation experiments. Bacteria were usually grown on nutrient agar and in nutrient yeast broth (Stanisich and Holloway, 1972) at 37°C. Iron-depleted Casamino acids medium (DCAA medium; Visca *et al.*, 1993) was used to study the production of salicylate and pyochelin by *P. aeruginosa* strains. Antibiotics, when required, were added to the growth media at the following concentrations: tetracycline, 25 μg ml^{-1} for *E. coli* and 100 μg ml^{-1} for *P. aeruginosa*; ampicillin, 100 μg ml^{-1} for *E. coli*; kanamycin 25 μg ml^{-1} for *E. coli* and 50 μg ml^{-1} for *A. tumefaciens*. To counterselect *E. coli* donor cells in matings with *P. aeruginosa*, chloramphenicol was used at 10 μg ml^{-1}.

DNA manipulation and sequencing

Standard DNA manipulations were carried out as described by Sambrook *et al.* (1989). Small scale preparations of plasmid DNA were obtained by the cetyl trimethyl ammonium bromide (CTAB) method (Del Sal *et al.*, 1988) and large scale preparations were performed using Qiagen-tip 100 columns (Qiagen Inc.). Chromosomal DNA was prepared according to Gamper *et al.* (1992). Restriction and DNA modifying enzymes were used following the instructions of the manufacturers. DNA fragments were purified from agarose gels with a Geneclean DNA extraction kit (Bio 101, La Jolla, CA, USA). Transformation of *E. coli*, *P. aeruginosa*, and *A. tumefaciens* was carried out by electroporation (Farinha and Kropinski, 1990). Constructs obtained by PCR were verified by sequence analysis using a Dye terminator kit (Perkin Elmer, no. 402080) and an ABI PRISM^{TM}373 sequencer.

Construction of pchB-A *gene fusions and gene replacement mutants in* P. aeruginosa

The strategy to obtain three different gene fusions between *pchB* and *pchA* is illustrated in Figure 2. A pUC18 derivative carrying the *pchBA* region from *P. aeruginosa* on a 2.3 kb *Xho*I–*Sal*I fragment (pME3366) was used as a DNA template to amplify the *pchB* coding region by PCR using reverse primer rp (5′-TTC-ACACAGGAAACAG-3′) and primer 3 (5′-TACCGTCGACGCGGCA-CCCCGTGTCTGGCG-3′). The PCR fragment was cleaved with *Kpn*I and *Sal*I and cloned into pBlue-Script-KS to give pME6141. Similarly, the 5′ part of *pchA* was amplified using primer 1 (5′-GCAGCC-CGGGGATGAGCCGGCTGGCGCCCCT-3′) and primer 2 (5′-GCC-AGCACGACCTTGCC-3′), cleaved with *Sma*I and *Apa*I and reassembled with the downstream part of *pchA* in pME3366 to obtain pME6150. In subsequent cloning steps the *Sma*I site immediately upstream of *pchA* in pME6150 was ligated to three different sites (*Hin*cII, *Eco*RV, *Sma*I) present in the polylinker downstream of *pchB* in pME6141 to generate *pchB-A* fusions in the pUC18-derivatives pME6145, pME6146 and pME6140, respectively (Figure 2).

The wild type *pchBA* genes in the chromosome of *P. aeruginosa* were replaced by the three different gene fusions using the marker exchange technique described previously (Reimmann *et al.*, 1998; Ye *et al.*, 1995). The suicide constructs pME6163 (2-codon *pchB-A* linker), pME6164 (9-codon *pchB-A* linker) and

pME6162 (15-codon *pchB-A* linker) were generated by cloning the 1.4 kb *Bam*HI–*Pvu*II fragments carrying the different *pchB-A* junction regions of pME6145, pME6146 and pME6140 (Figure 2), respectively, into the suicide vector pME3087. Plasmids pME6164 and pME6162 were mobilized from *E. coli* S17–1 to *P. aeruginosa* PAO1 and integrated via a first crossing-over event into the chomosome. In a second crossing-over event, the plasmids were excised and lost due to their inability to replicate in *P. aeruginosa*. In those cases where the two crossing-over events had occurred on both sides of the *pchB-A* junctions, the wild type *pchB* and *pchA* genes were replaced by the respective gene fusion. These mutants (PAO6307 carrying the 9-codon linker and PAO6308 carrying the 15-codon linker) were identified by Southern analysis (not shown) on the basis of acquired multiple restriction sites at the *pchB-A* junction. To construct the 2-codon linker mutant PAO6309, the suicide construct pME6163 was mobilized to strain PAO6307. This allowed identification by Southern analysis as the replacement of the 9-codon linker of PAO6307 by the 2-codon linker caused the loss of recognition sites for *Sal*I, *Cla*I and *Hin*dIII at the *pchB-A* junction.

Constructs for plant transformation

Attempts to amplify the whole insert of pME6145 by PCR were unsuccessful probably because of an inverted repeat structure downstream of the *pchB-A* stop codon. A strategy was therefore devised to remove this structure and to add restriction sites for cloning into the binary vector pBI121. To introduce an *Xba*I site upstream of the start codon, a fragment of 304 bp carrying the 5′ part of *pchB-A* was PCR-amplified from pME6145 with primer SAS1 (5′-GCTCTAGAAAATGATGAAAACTCCCGAAGACTGC-3′) and primer SAS2 (5′-TGTCTGGCGCCAGTACTTGATC-3′) and cloned into pCR2.1 using Invitrogen's TOPO TA cloning kit, to give pSAS304. The *pchB-A* sequence of pME6145 contains a *Bbs*I site at position 23 of the coding sequence and a second *Bbs*I site downstream of the stop codon, i.e. in the first half of the inverted repeat structure. This 1732 bp *Bbs*I fragment was isolated and ligated into pSAS304 digested with *Bbs*I and *Spe*I. After phenolization and ethanol precipitation the unjoined ends resulting from the second *Bbs*I site and the *Spe*I site were blunt-ended with Klenow polymerase and the product was religated to yield plasmid pSAS. An *Xba*I–*Sac*I fragment isolated from pSAS was then cloned into pBI121 and pSPORT to give plasmids pBI35S-SAS and pSPORT-SAS, respectively.

To add a plastid targeting signal to the SAS sequence, a signal sequence from ferredoxin (M35868) was amplified by PCR using primer FD1 (5′-GTCGACAAAATGGCTTCCACTGCTCTC-3′) with an introduced *Sal*I site at the 5′-end and FD2 (5′-GGATCC-GACCTTGTATGTAGCCATGGC-3′) with a *Bam*HI site. This 186 bp fragment was cloned into pCR2.1, resulting in plasmid pFD. To introduce a *Bgl*II site at the 5′ end of SAS, a 300 bp fragment was amplified from pME6145 with primer SAS3 (5′-AGATCTATGATGAAAACTCCCGAAGACTG-3′) and primer SAS2 and cloned into pCR2.1. A *Hin*dIII–*Sca*I fragment was isolated from the resulting plasmid and subcloned into pSPORT-SAS to generate a full length SAS sequence with a *Bgl*II site immediately upstream of the start codon. The complete SAS sequence was subcloned from pSPORT-SAS as a *Bgl*II–*Kpn*I fragment into pFD digested with *Bam*HI and *Kpn*I to yield plasmid pFD-SAS. The insert of pFD-SAS encodes a fusion protein with the plastid targeting signal and the first five amino acids (ATYKV) of the mature ferredoxin followed by two amino acids (GS) generated by the *Bam*HI/*Bgl*II-fusion which are joined to the complete SAS amino acid sequence. The FD-SAS insert was then subcloned via a pGEM7-derived linker on a *Xba*I–*Sac*I fragment into pBI121 to give pBI35S-FD-SAS. The correctness of all PCR products and of newly generated fusion junctions was verified by sequencing.

Identification of SA and pyochelin in culture supernatants of P. aeruginosa

DCAA medium (100 ml) was inoculated with 0.5 ml of a preculture from *P. aeruginosa* grown in the same medium and incubated with shaking at 220 r.p.m. for 24 h until the stationary phase was reached (OD_{600} 0.8–1.0, measured in a Pharmacia Ultrospec III spectrophotometer). For HPLC analysis, ethyl acetate extracts of 20 ml culture supernatants acidified to pH 1–2 were dried by evaporation and dissolved in 400 μl 70% (v/v) methanol and 10 mM H_3PO_4. An aliquot of 100 μl was injected into an HPLC system (Serino *et al.*, 1995). Compounds were identified by their retention times and UV spectra. SA was also quantified by fluorescence as described previously (Serino *et al.*, 1997).

Generation of SAS expressing plants

The binary vectors pBI35S-SAS and pBI35-FD-SAS were introduced into *A. tumefaciens* strain GV3101 via electroporation. Arabidopsis accession Columbia was transformed according to the *in planta* transformation protocol (Bechtold *et al.*, 1993). Transgenic plants constitutively expressing SAS in the cytoplasm were named c-SAS and those which constitutively express FD-SAS targeted to the plastids were named p-SAS. The surface sterilized seeds of the primary transformants were selected for antibiotic resistance on 0.8% agar plates containing 1/2 strenght MS-salts, 3% (w/v) sucrose, and 25 μg ml^{-1} kanamycin. The progeny of the kanamycin resistant c-SAS plants was analysed for kanamycin resistance segregation. Seeds of plants with a 3 : 1 segregation ratio were further cultivated and the resulting progeny was again analysed for segregation of kanamycin resistance to identify plants homozygous for the T-DNA insert. Three homozygous lines were selected for further analysis: c-SAS-5, c-SAS-10 and c-SAS-16. Because of problems related to the severely dwarfed phenotype, the p-SAS plants were analysed in the hemizygous stage.

RNA gel blot analysis

Total RNA was extracted from frozen plant material in a buffer containing 2 M Tris–HCl pH 8.0, 0.5 M EDTA pH 8.0, and 20% (w/v) SDS (1 : 2 : 1) with an equal volume of buffer-saturated phenol: chloroform:isoamyl alcohol (25 : 24 : 1) (Chirjwin *et al.*, 1979). After centrifugation, the aqueous phase was precipitated with 1 volume of 6 M LiCl at 4°C overnight. The RNA was then washed with 70% ethanol and resuspended in H_2O. Five μg of total RNA were separated by electrophoresis on formaldehyde/agarose gels (ZAP cDNA synthesis kit, Stratagene, La Jolla, CA, USA). Ethidium bromide was included in the loading buffer to allow direct confirmation of equal sample loading. The gels were blotted onto nylon membranes (Hybond-N, Amersham Pharmacia Biotech, Little Chalfont, UK) and hybridized to random-primed ^{32}P-labelled *pchB-A* and *PR-1* probes prepared according to standard procedures.

Determination of SA in plants and growth parameters

Free SA and SA conjugate levels were determined as described previously (Meuwly and Métraux, 1993; Mölders *et al.*, 1996). Primary transformants were analysed for SA content just before the bolting stage. The homozygous c-SAS plants were 3–4-weeks-old at the time of SA analysis and 4-weeks-old for the FW determination. The SA content of c-SAS plants given in Table 2 represents the mean value plus standard deviation of three independent experiments. The two independent pools of p-SAS plants consisted of 12 primary transformants that were 4-weeks-old at the time of analysis. Six to 10 plants were used for the determination of FW and seed production.

Analysis of disease resistance

Peronospora parasitica isolate NOCO was used as a pathogen. Inoculation of *Arabidopsis thaliana* accession Columbia with isolate NOCO results in a compatible interaction. Inoculation procedures and staining of the plant material with alcoholic trypan blue solution were performed as described previously (Mauch-Mani and Slusarenko, 1994). Arabidopsis plants were grown under a 16 h light phase at 20°C and 8 h dark at 17°C. The plants were 3-weeks-old at the time of the disease resistance tests. Quantitative analysis of conidia production was performed with 3-week-old plants that had been inoculated with *P. parasitica* isolate NOCO 1 week before. Sixty plants were collected in 7 ml of water. After shaking, the spore density was determined with a haemocytometer and the number of conidia produced per plant was calculated.

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