Overproduction of salicylic acid in plants by bacterial transgenes enhances pathogen resistance

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For centuries it was known that chewing the bark of the willow tree (Salix) relieved headaches and fevers, and in the 19th century salicylic acid (SA) was identified as the active component in the bark extract. Only recently it was found that in plants SA is involved in various physiological processes like stomatal closure, flower induction, and heat production, and that it plays a central role in defense against pathogens. The formation of a necrotic lesion to restrict the spread of an invading pathogen activates a pathway leading to systemic acquired resistance (SAR). Characteristics of SAR are resistance extending to plant tissues distant from the initial infection site, persistence for weeks to months, and plant protection against secondary infection by a broad spectrum of pathogens. Exogenous SA can induce SAR, and SA appears to be the endogenous signal that triggers SAR.

Systemic acquired resistance is accompanied by the induced expression of genes encoding pathogenesis-related (PR) proteins. Among these are antifungal chitinases, β-1,3-glucanases, and PR-1 and PR-5 proteins with anti-oomycete activity. PR proteins with a specific antiviral activity have not yet been characterized; however, SA has been implicated in development of virus resistance by modulation of the alternative oxidase pathway. In tobacco, subgroups of basic and acidic PR proteins accumulate in the vacuole and intercellular space, respectively. In particular, the genes encoding acidic PR proteins are systemically induced in leaves with acquired resistance.

Additional evidence for a role of SA in plant defense came from tobacco plants transformed with the nahG gene from Pseudomonas putida. The nahG gene product encodes salicylate hydroxylase, which converts SA to the biologically inactive catechol. In these plants SA can no longer accumulate, which results in the inability to induce SAR. Currently, genetic and biochemical data are accumulating on components of the signaling pathway acting upstream or downstream of SA.

In plants the biosynthesis of SA is thought to proceed by the phenylpropanoid pathway. After biosynthesis, most SA is conjugated to SA 2-O-β-D-glucoside (SAG). In addition, SAG may play a role in SAR, possibly serving as an inactive storage form that can be rapidly cleaved to release active SA at the site of infection. Microorganisms, among them Pseudomonas fluorescens and Escherichia coli, produce SA and related compounds to serve as building blocks for iron-chelating siderophores. For example, certain Pseudomonas species produce SA by means of a short biosynthetic pathway from chorismate, the general precursor of aromatic compounds. This substrate is converted by isochorismate synthase (ICS) to isochorismate, which is subsequently cleaved by isochorismate pyruvate lyase (IPL) to yield SA.

Here, we report the transformation of tobacco with bacterial ICS and IPL genes fused to a strong plant promoter. Overproduction of SA in the transgenic plants resulted in constitutive expression of defense genes and an enhanced resistance to various pathogens.

Results and discussion

Chorismic acid, the end product of the shikimate pathway, is mainly produced in the chloroplasts. Therefore, we fused the plastid targeting sequence (ss) of the small subunit of tobacco ribulose bisphosphate carboxylase to the ICS coding sequence of the E. coli entC gene. Using the pmsB coding sequence from P. fluorescens, we constructed two types of ICS genes, one containing and the other lacking the plastid targeting sequence ss (Fig. 1). The resulting genes, cloned behind the constitutive 3SS promoter from cauliflower mosaic virus (CaMV), were used to transform tobacco. For each construct three randomly chosen pri-
mary transformants were selfed and the T1 generation was used for further experiments.

**SA production in transgenic plants.** In contrast to leaf extracts from control tobacco plants, extracts from plants transformed with the \( \text{entC} \) construct produced high levels of isochorismate in vitro, whereas extracts from healthy plants transformed with the \( \text{entCpmsB} \) and \( \text{entCpmsB} \) constructs synthesized SA in vitro. This indicates that the \( \text{entC} \) and \( \text{pmsB} \) genes were expressed to yield active ICS and IPL, respectively (results not shown). Next, the transgenic plants were analyzed to check if the expression of the biosynthesis route had indeed resulted in elevated levels of SA in vivo. As can be seen in Table 1, free SA accumulated in the \( \text{entCpmsB} \) transgenic plants, whereas the levels of free SA in the \( \text{entCpmsB} \) plants and in the plants transformed with the single-gene constructs were not substantially different from those of uninfected wild-type or transgenic P12 plants (P12 tobacco plants are transformed with the P1 and P2 polymerase genes of alfalfa mosaic virus (AMV) and served as a control). This indicates that for efficient production of SA, both ICS and IPL need to be present in the chloroplast, where the highest chorismic acid levels are available. The \( \text{entCpmsB} \) plants are referred to as CSA plants (constitutive SA biosynthesis).

Salicylic acid is phytotoxic in concentrations >0.1 mM (ref. 19). In plants it is converted to nontoxic SAG and stored in the vacuole. Indeed, in tobacco mosaic virus (TMV)-infected plants most SA is glucosylated (SAG to SA ratio approximately 10, Table 1). Apparently, in the transgenic plants SA is also rapidly converted to SA, as occurs in vitro20.

Table 1. Accumulation of SA and SAG in plants expressing ICS and IPL enzymes, and in control plants

<table>
<thead>
<tr>
<th>Construct</th>
<th>Line number</th>
<th>Average SA levels (\mu g g^{-1} fresh weight)</th>
<th>SA</th>
<th>SAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>P12</td>
<td>0.01 (0.00)</td>
<td>0.01 (0.01)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NN control</td>
<td>0.01 (0.00)</td>
<td>0.02 (0.01)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMV local</td>
<td>0.68 (0.37)</td>
<td>9.49 (5.33)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMV systemic</td>
<td>0.03 (0.01)</td>
<td>0.48 (0.14)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{entCpmsB} )</td>
<td>4</td>
<td>0.10 (0.09)</td>
<td>12.82 (9.02)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>0.18 (0.10)</td>
<td>16.22 (5.74)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.06 (0.04)</td>
<td>7.15 (3.82)</td>
<td></td>
</tr>
<tr>
<td>( \text{entCpmsB} )</td>
<td>4</td>
<td>0.01 (0.01)</td>
<td>0.26 (0.13)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>0.02 (0.01)</td>
<td>0.05 (0.02)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>0.02 (0.01)</td>
<td>0.47 (0.46)</td>
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</tr>
<tr>
<td>( \text{entC} )</td>
<td>5</td>
<td>0.03 (0.01)</td>
<td>0.03 (0.01)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.02 (0.02)</td>
<td>0.06 (0.03)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>0.01 (0.01)</td>
<td>0.08 (0.02)</td>
<td></td>
</tr>
<tr>
<td>( \text{pmsB} )</td>
<td>5</td>
<td>0.05 (0.04)</td>
<td>0.15 (0.09)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>0.00 (0.00)</td>
<td>0.07 (0.02)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.08 (0.12)</td>
<td>0.20 (0.25)</td>
<td></td>
</tr>
<tr>
<td>( \text{pmsB} )</td>
<td>16</td>
<td>0.01 (0.01)</td>
<td>0.17 (0.08)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>0.03 (0.00)</td>
<td>0.05 (0.01)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.03 (0.01)</td>
<td>0.07 (0.03)</td>
<td></td>
</tr>
</tbody>
</table>

Four plants of each line were extracted in duplicate, and SA levels were measured before (column SA) and after (column SAG) conversion of SAG to SA by acid hydrolysis. Standard deviations are given in parentheses. The data are not corrected for the average extraction recovery of 81% (before hydrolysis) or 72% (after hydrolysis). Controls used are P12 tobacco (P12), nontransgenic tobacco (NN control), inoculated leaves of TMV-infected plants (TMV local), and noninoculated leaves of TMV-infected plants (TMV systemic).

The fact that transgenic plants other than \( \text{entCpmsB} \) plants have elevated levels of SA indicates that SA is produced in these plants as well. Apparently, part of the overproduced isochorismate in the \( \text{entCpmsB} \) plants is transported or diffuses to the cytosol, where it is converted by transgene-derived IPL into SA. The increased concentrations of SAG in \( \text{entC} \) plants can be explained by assuming that isochorismate produced by the transgenic ICS spontaneously converts to SA, as occurs in vitro20.

**Plants contain endogenous ICS activity.** In Catharanthus roseus the enzyme is encoded by a stress-inducible gene and is involved in secondary metabolism leading to 2,3-dihydroxybenzoic acid. Although tobacco ICS has not yet been identified, it is believed that such an activity exists because isochorismate is an expected intermediate in phyloquinone synthesis. The growth of plants of several \( \text{pmsB} \) lines was severely retarded. This could be accumulation between TMV-infected nontransgenic plants and noninfected transgenic CSA plants may be due to differences in regulation of SA metabolism, or it may be a reflection of the different time scales of SA production.

Figure 1. Schematic presentation of constructs used for plant transformation. Constructs 1-5 were used to generate plants \( \text{entC} \) (1), \( \text{pmsB} \) (2), \( \text{pmsB} \) (3), \( \text{entCpmsB} \) (4), and \( \text{entCpmsB} \) (5). 3SS, CaMV 3SS promoter; P1T, proteinase inhibitor terminator; ss, chloroplast targeting signal; entC, ICS gene; pmsB, IPL gene.
due to a shortage of essential phyloquinones, the synthesis of which would be hampered if the pmsB gene-encoded IPL had depleted the isochorismate produced by endogenous ICS. However, when the bacterial ICS and IPL were both targeted to the chloroplasts, the CSA plants showed fully normal development and appearance, and produced viable seeds. Apparently, in these plants the bacterial ICS provided sufficient substrate for SA biosynthesis by the IPL enzyme. For several plant species, lesion mimic mutants have been obtained that simulate a diseased phenotype and show disease resistance responses including the accumulation of SA21–23. Our observation that accumulation of SA has no detectable effect on the phenotype of the plant indicates that SA accumulation in lesion mimics is a result of the stress induced by the mutation but not the cause of the disease-simulating appearance of these plants.

**PR gene expression in SA-producing plants.** Exogenously applied SA is known to induce the tobacco genes encoding the acidic PR proteins of groups 1–5, whereas the basic PR proteins of these groups are highly induced by ethylene24. To determine if PR genes are also expressed in the CSA plants, northern blot experiments were performed with cDNA probes corresponding to acidic PR-1a and PR-2a, basic PR-1g and PR-2b, and ethylene-responsive proteinase inhibitor II (PI-II) from tobacco. The results are shown in Fig. 2. It is evident that only the entC,pmsB plants, which accumulate relatively high levels of SA and SAG, showed enhanced expression of the PR-1a and PR-2a genes. Apparently, the small quantity of SA produced in the entC,pmsB plants is too low to support a constitutive level of acidic PR protein gene expression. Also, the entC, pmsB and entC,pmsB plants did not show elevated levels of PR-1a or PR-2a gene expression (results not shown). As expected, genes encoding basic PR proteins and PI-II are not expressed at levels above background in the transgenic plants.

The level of acidic PR protein gene expression in the CSA plants is considerably lower than in the TMV-inoculated control. However, for the latter only the local infected leaves were sampled at three days after inoculation. Expression levels of the acidic PR protein genes in the noninoculated SAR leaves are much lower22,25.

**Table 2. Size of TMV-induced lesions on plants expressing ICS and IPL enzymes, expressed as a percentage of lesion size on control plants**

<table>
<thead>
<tr>
<th>Control</th>
<th>T = 2</th>
<th>Local T = 4</th>
<th>Systemic T = 3</th>
<th>T = 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>s(entC)pmsB</td>
<td>4</td>
<td>57 (6)</td>
<td>45 (10)</td>
<td>46 (16)</td>
</tr>
<tr>
<td>16</td>
<td>53 (3)</td>
<td>36 (6)</td>
<td>38 (9)</td>
<td>100 (13)</td>
</tr>
<tr>
<td>20</td>
<td>57 (3)</td>
<td>41 (4)</td>
<td>52 (7)</td>
<td>94 (9)</td>
</tr>
<tr>
<td>s(entC)pmsB</td>
<td>4</td>
<td>94 (3)</td>
<td>94 (5)</td>
<td>90 (8)</td>
</tr>
<tr>
<td>11</td>
<td>84 (4)</td>
<td>86 (3)</td>
<td>89 (5)</td>
<td>106 (12)</td>
</tr>
<tr>
<td>13</td>
<td>91 (6)</td>
<td>88 (5)</td>
<td>89 (9)</td>
<td>100 (11)</td>
</tr>
<tr>
<td>pmsB</td>
<td>4</td>
<td>98 (3)</td>
<td>101 (7)</td>
<td>93 (5)</td>
</tr>
<tr>
<td>11</td>
<td>122 (4)</td>
<td>92 (5)</td>
<td>86 (4)</td>
<td>112 (12)</td>
</tr>
<tr>
<td>13</td>
<td>110 (6)</td>
<td>96 (4)</td>
<td>93 (5)</td>
<td>99 (13)</td>
</tr>
<tr>
<td>pmsB</td>
<td>5</td>
<td>108 (3)</td>
<td>100 (8)</td>
<td>93 (10)</td>
</tr>
<tr>
<td>18</td>
<td>105 (6)</td>
<td>100 (3)</td>
<td>96 (7)</td>
<td>112 (14)</td>
</tr>
<tr>
<td>10</td>
<td>135 (6)</td>
<td>94 (5)</td>
<td>87 (6)</td>
<td>98 (10)</td>
</tr>
<tr>
<td>s(pmsB)</td>
<td>16</td>
<td>97 (3)</td>
<td>91 (6)</td>
<td>97 (7)</td>
</tr>
<tr>
<td>22</td>
<td>96 (4)</td>
<td>84 (9)</td>
<td>79 (12)</td>
<td>81 (13)</td>
</tr>
<tr>
<td>10</td>
<td>97 (8)</td>
<td>85 (6)</td>
<td>86 (15)</td>
<td>75 (6)</td>
</tr>
</tbody>
</table>

*Bold characters indicate results significantly different (P < 0.05) from control. Local refers to primary inoculated lower leaves of the plant. Systemic refers to secondary inoculated upper leaves of the plant. T, days after inoculation. Three lines of each type of transgenic plant were tested; line numbers are indicated in the first column.

**Figure 3. Effect of constitutive SA synthesis in plants on TMV lesion size.** A control P12 plant (left) and a s(entC)pmsB plant of line 16 (right) was inoculated with TMV. Lesions are shown four days after inoculation.

**CSA plants have elevated levels of resistance against viral infection.** In addition to the expression of PR genes, another characteristic of SAR is the reduction of the lesion size after inoculation with TMV. Therefore, CSA plants were inoculated with TMV and the lesion development was followed for seven days past inoculation (d.p.i.). The difference in lesion size development is already visible after four days (Fig. 3). To correct for variation over time, the mean of the controls is set to 100% at each day, whereas the other data are expressed as a percentage. Actual lesion diameters, averaged over 48 control plants, were 0.73 (±0.15 s.d.), 2.00 (±0.24 s.d.), and 2.74 (±0.45 s.d.) mm on primary inoculated lower leaves at two, four, and seven d.p.i., respectively, and 0.59 (±0.10 s.d.) and 0.81 (±0.27 s.d.) mm on secondary inoculated upper leaves at three and seven d.p.i., respectively. The assays on virus resistance and fungal resistance were done with T1 generation plants that had been germinated on selective medium containing kanamycin.

The results shown in Table 2 indicate that plants in which both ICS and IPL were targeted to the chloroplast and that accumulated SA and SAG had clearly reduced lesion sizes in comparison to control plants. At two d.p.i., the average lesion diameter was around half that of those on the P12 control plants. This difference in lesion size remained at four and seven d.p.i., indicating that the elevated SA production in the CSA plants resulted in a reduction of the lesion growth rate of about 50% or, conversely, in a doubling of resistance to TMV. The s(entC)pmsB plants and some of the single-gene transformants showed minor reductions of lesion sizes that were statistically significant (Table 2). However, these were much less prominent than the reductions observed with the s(entC)pmsB plants, and no clear correlation with the SA or SAG levels was observed (Table 1).

To see if inducing SAR could further increase the elevated resistance to TMV, lesion diameters were measured on the inoculated upper leaves of CSA and control plants of which the lower leaves had previously been inoculated with TMV. The data of Table 2 show that for most lines tested, there is no significant difference in lesion size between control, CSA, or other transgenic plants. Apparently, the resistance of the CSA plants cannot be further increased by TMV infection.

**CSA plants have elevated levels of resistance against fungal infection.** Two of the CSA tobacco lines were tested for resistance to infection with Oidium lycopersici. Eight plants of each line were inoculated by spraying with a spore suspension, and each plant was...
Table 3. Growth of the fungus O. lycopersicon on CSA plant lines 4 and 16 and P12 control plants.*

<table>
<thead>
<tr>
<th>Line</th>
<th>Time after inoculation</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>P12</td>
<td>14</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+/</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>sntC_pmsB4</td>
<td>14</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>sntC_pmsB16</td>
<td>14</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P12</td>
<td>21</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+/-</td>
</tr>
<tr>
<td>sntC_pmsB4</td>
<td>21</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>sntC_pmsB16</td>
<td>21</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Eight sntC_pmsB plants of lines 4 and 16 were inoculated with the fungus, and after 14 or 21 days the number of infection spots per leaf was counted. –, No growth of the fungus; +/-, one infection spot; +, two to four infection spots; ++, more than four infection spots.

Analysis of ICS and IPL activity in vitro. Enzyme extracts were made from transgenic plants and ICS activity in the extracts was measured. Conversion of isochorismate into SA by IPL in the plant extract was analyzed by HPLC connected to a fluorescence detector, using emission and excitation wavelengths of 407 nm and 353 nm, respectively.

Analysis of SA and SAG levels in plants. For each transgenic line, SA and SAG levels were determined in eight samples obtained by sampling four plants in duplicate. Extraction of free SA and hydrolysis of SAG to SA was done as described with minor modifications (Verbene et al., unpublished data).

Salicylic acid was quantified by chromatography on a Phenomenex column, type LUNA 3 µ C18 (2) 50 × 4.60 mm × 3 µm, with a SecurityGuard from Phenomenex (Metromoh, Herisau, Switzerland). The eluent of 90% 0.2 M sodium acetate, pH 5.5, and 10% methanol had a flow rate of 0.80 ml min⁻¹. Salicylic acid was detected with a Shimadzu RF-10Axl spectrofluorometric detector, using an emission wavelength of 407 nm and excitation wavelength of 353 nm.

RNA analysis. Equal amounts of leaf material from four T1 plants of each transgenic line were combined and used to extract RNA. About 10 µg RNA was denatured by glyoxal treatment and electrophoresed in a 1.5% agarose gel, blotted to a Hybond Transfer membrane (Amerham, Arlington Heights, IL), and UV auto-crosslinked. The blots were hybridized with 32P-labeled inserts from clones corresponding to PR-1a, PR-1g, PR-2a, PR-2b, and PI-1 [24,35].

Inoculation with TMV. Eight-week-old plants were inoculated with TMV (1 µg ml⁻¹), and lesion development was analyzed on three leaves per plant using eight plants per line. Two, four, and seven days after primary inoculation of the lower leaves of the plant, lesion size was measured with a magnifying glass containing a ruler calibrated to 0.1 mm divisions. The inoculated leaves were divided in four parts, and from each part two lesions were measured. Ten days after the primary inoculation, the upper leaves were subjected to a secondary inoculation and lesion size on these leaves was determined three and seven d.p.i., measuring four lesions per leaf, i.e. one from each quarter of the leaf. After collection of all data, a statistical analysis of the lesion size was performed. First the homogeneity of the lesions from three leaves of the same plant was examined. Subsequently, the data were pooled and the average lesion size per plant was calculated, resulting in eight means per line. These data were entered into a statistical program to compare at each of the three days separately the different lines using a one-way ANOVA and a LSD (Fisher) test with α = 0.05.

Inoculation with powdery mildew. Eight eight-week-old plants of sntC_pms lines 4 and 16, and eight control P12 plants were inoculated with O. lycopersicon by spraying a spore suspension of 3.0 × 10⁸ spores ml⁻¹ (total volume 400 ml). The conditions of the test room were 20°C, 16 h light period, and 80% relative humidity.

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