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

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Received 25 January 2000; accepted 9 April 2000

After a hypersensitive response to invading pathogens, plants show elevated accumulation of salicylic acid (SA), induced expression of plant defense genes, and systemic acquired resistance (SAR) to further infection by a broad range of pathogens. There is compelling evidence that SA plays a crucial role in triggering SAR. We have transformed tobacco with two bacterial genes coding for enzymes that convert chorismate into SA by a two-step process. When the two enzymes were targeted to the chloroplasts, the transgenic (CSA, constitutive SA biosynthesis) plants showed a 500- to 1,000-fold increased accumulation of SA and SA glucoside compared to control plants. Defense genes, particularly those encoding acidic pathogenesis-related (PR) proteins, were constitutively expressed in CSA plants. This expression did not affect the plant phenotype, but the CSA plants showed a resistance to viral and fungal infection resembling SAR in nontransgenic plants.

Keywords: salicylic acid, systemic acquired resistance, tobacco, Tobacco mosaic virus, *Oidium lycopersicon*, PR gene expression

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[1]. 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 pathogen attack[2–5]. After the formation of a necrotic lesion to restrict the spread of an invading pathogen, plants activate a pathway leading to systemic acquired resistance (SAR)[6]. 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[7]. Exogenous SA can induce SAR[8], and SA appears to be the endogenous signal that triggers SAR[9,10].

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[11]. 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[12].

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[7,13]. 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[14]. After biosynthesis, most SA is conjugated to SA 2-*O*-β-D-glucoside (SAG)[15,16]. 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[17].

Microorganisms, among them *Pseudomonas fluorescens* and *Escherichia coli*, produce SA and related compounds to serve as building blocks for iron-chelating siderophores[14]. 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[18]. Therefore, we fused the plastid targeting sequence (*ss*) of the small subunit of tobacco ribulose bisphosphate carboxylase precursor to the ICS coding sequence of the *E. coli entC* gene. Using the *pmsB* coding sequence from *P. fluorescens*, we constructed two types of IPL genes, one containing and the other lacking the plastid targeting sequence *ss* (Fig. 1). The resulting genes, cloned behind the constitutive 35S 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 $_{ss}$*entC* construct produced high levels of isochorismate in vitro, whereas extracts from healthy plants transformed with the $_{ss}$*entCpmsB* and $_{ss}$*entC*$_{ss}$*pmsB* constructs synthesized SA in vitro. This indicates that the *entC* and *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 $_{ss}$*entC*$_{ss}$*pmsB* transgenic plants, whereas the levels of free SA in the $_{ss}$*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 $_{ss}$*entC*$_{ss}$*pmsB* 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 SAG. As expected, the $_{ss}$*entC*$_{ss}$*pmsB* plants contain the highest levels with SAG to SA ratios of up to 100. The difference in SAG 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.

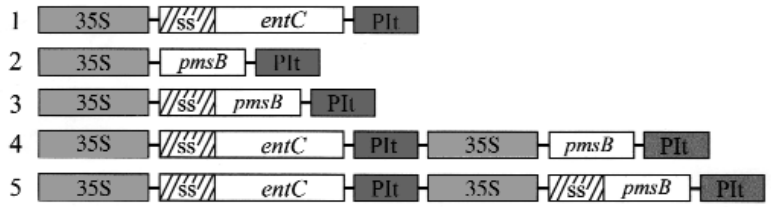

Figure 1. Schematic presentation of constructs used for plant transformation. Constructs 1–5 were used to generate plants $_{ss}$*entC* (1), *pmsB* (2), $_{ss}$*pmsB* (3), $_{ss}$*entCpmsB* (4), and $_{ss}$*entC*$_{ss}$*pmsB* (5). 35S, CaMV 35S promoter; PIt, proteinase inhibitor terminator; ss, chloroplast targeting signal; *entC*, ICS gene; *pmsB*, IPL gene.

The fact that transgenic plants other than $_{ss}$*entC*$_{ss}$*pmsB* plants have elevated levels of SAG indicates that SA is produced in these plants as well. Apparently, part of the overproduced isochorismate in the $_{ss}$*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 $_{ss}$*entC* plants can be explained by assuming that isochorismate produced by the transgenic ICS spontaneously converts to SA, as occurs in vitro[20].

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 phylloquinone synthesis. The growth of plants of several $_{ss}$*pmsB* lines was severely retarded. This could be

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

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

[a]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).

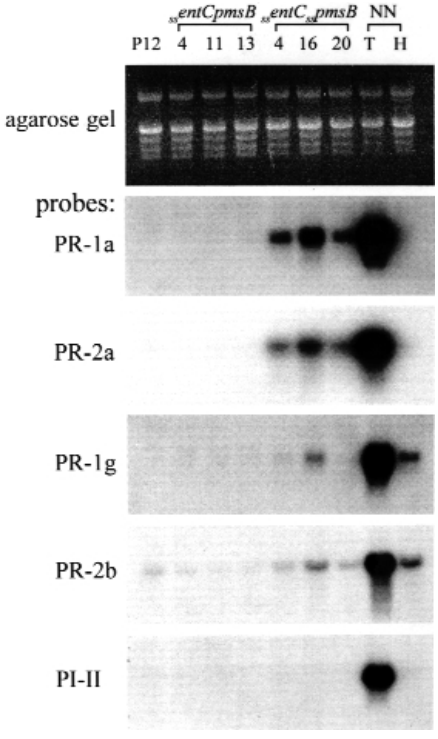

Figure 2. PR gene expression in plants expressing ICS and IPL enzymes. A northern blot was loaded with RNA from control plants (P12), $_{ss}$*entCpmsB* plants (lines 4, 11, 13), $_{ss}$*entC*$_{ss}$*pmsB* plants (lines 4, 16, 20), Samsun NN tobacco plants infected with TMV (T) and healthy tobacco plants (H). The blot was probed for genes encoding acidic proteins PR-1a and PR-2a, basic proteins PR-1g and PR-2b, and proteinase inhibitor II (PI-II).

due to a shortage of essential phylloquinones, 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 SA[21–23]. 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 ethylene[12]. 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 $_{ss}$*entC*$_{ss}$*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 $_{ss}$*entCpmsB* plants is too low to support a constitutive level of acidic PR protein gene expression. Also, the $_{ss}$*entC*, *pmsB* and $_{ss}$*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 lower[24,25].

Figure 3. Effect of constitutive SA synthesis in plants on TMV lesion size. A control P12 plant (left) and a $_{ss}$*entC*$_{ss}$*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 $_{ss}$*entCpmsB* 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 $_{ss}$*entC*$_{ss}$*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 lycopersicon*. Eight plants of each line were inoculated by spraying with a spore suspension, and each plant was

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

		Local			Systemic	
		T = 2	T = 4	T = 7	T = 3	T = 7
Control		**100**	**100**	**100**	**100**	**100**
$_{ss}$entC$_{ss}$pmsB	4	**57 (6)**	**45 (10)**	**46 (16)**	89 (15)	85 (15)
	16	**53 (3)**	**36 (6)**	**38 (9)**	100 (13)	97 (32)
	20	**57 (3)**	**41 (4)**	**52 (7)**	94 (9)	73 (9)
$_{ss}$entCpmsB	4	**94 (3)**	**94 (5)**	**90 (8)**	97 (9)	94 (14)
	11	**84 (4)**	**86 (3)**	**89 (5)**	106 (12)	117 (38)
	13	**91 (6)**	**88 (5)**	**89 (9)**	100 (11)	105 (14)
$_{ss}$entC	5	98 (3)	101 (7)	93 (5)	101 (18)	109 (37)
	8	**122 (4)**	**92 (5)**	**86 (4)**	112 (12)	117 (26)
	13	**110 (6)**	96 (4)	**93 (5)**	99 (13)	88 (20)
pmsB	5	**108 (3)**	100 (8)	93 (10)	101 (10)	112 (32)
	18	**105 (6)**	100 (3)	96 (7)	112 (14)	**135 (35)**
	1	**135 (6)**	**94 (5)**	**87 (6)**	98 (10)	87 (12)
$_{ss}$pmsB	16	97 (3)	**91 (6)**	97 (7)	106 (10)	91 (22)
	22	96 (4)	**84 (9)**	**79 (12)**	**81 (13)**	**75 (13)**
	10	97 (8)	**85 (6)**	**86 (15)**	**75 (6)**	**66 (8)**

[a]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.

Table 3. Growth of the fungus *O. lycopersicon* on CSA plant lines 4 and 16 and P12 control plants[a]

Line	Time after inoculation	Plant number 1	2	3	4	5	6	7	8
P12	14	+	++	+	+/–	++	+/–	+/–	+/–
$_{ss}$entC$_{ss}$pmsB4	14	–	–	–	–	–	–	–	–
$_{ss}$entC$_{ss}$pmsB16	14	–	–	–	–	–	–	–	–
P12	21	++	++	++	+	++	+	+/–	+
$_{ss}$entC$_{ss}$pmsB4	21	–	–	–	–	–	–	–	–
$_{ss}$entC$_{ss}$pmsB16	21	–	–	–	–	–	–	–	–

[a]Eight $_{ss}$*entC*$_{ss}$*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.

indexed for disease symptoms at 14 and 21 d.p.i. Table 3 shows that, while all of the P12 control plants became infected, none of the transgenic plants showed symptoms of infection. Previously, in vitro assays indicated that basic PR proteins rather than acidic PR proteins have antifungal activity[26]. Since the acidic PR proteins are constitutively expressed in CSA plants in particular, our results could indicate that in vivo these proteins do have antifungal activity. However, it is possible that constitutive biosynthesis of SA results in the expression of yet unidentified genes that are responsible for the enhanced resistance of the CSA plants to fungal infection.

In summary, we have shown that transgenic expression of bacterial ICS and IPL genes in tobacco results in constitutive accumulation of SA and an enhanced resistance of the plants to viral and fungal pathogens that is comparable to SAR. Although engineered resistance of tobacco to TMV or *O. lycopersicon* is not of commercial interest, the same strategy may be applied to other crops, and an enhanced resistance may be expected to pathogens sensitive to SA-mediated resistance responses. In addition, the CSA strategy may solve the problem of "thick roots" in hydrocultured plants that are now being treated by addition of SA to the culture medium. Since SAG and related phenolic glucosides play an important role in plant-herbivore interactions[27], it will be interesting to see if CSA plants show an enhanced protection against insect herbivores. When SAR is induced in nontransgenic plants, multiple signal transduction pathways are activated. CSA plants offer the advantage to study only those defense responses that are triggered by SA. Similar to *nahG* plants[7], CSA plants will be instrumental in the identification of components from the signal transduction pathway that act upstream or downstream of SA.

Experimental protocol

Construction of transformation vectors. The *entC* sequence from *E. coli*[28] was obtained by PCR on genomic DNA. By means of subcloning using intermediate vectors, the DNA was translationally fused to the chloroplast targeting sequence (*ss*) from the small subunit of ribulose bisphosphate carboxylase[28] and inserted between the CaMV 35S promoter and the proteinase inhibitor I terminator (PIt) from potato, and cloned in transformation vector pMOG800[29]. This vector contains the *npt*II selection marker. In the final construct (Fig. 1, construct 1), the first 36 nucleotides of the *entC* coding sequence were removed, but the resulting truncated ICS is fully active. Similarly, the *pmsB* sequence from *P. fluorescens* (to be published elsewhere) was cloned between the CaMV 35S promoter and PIt, and constructs with and without the chloroplast targeting sequence were cloned in pMOG800 (Fig. 1, constructs 3 and 2, respectively). Finally, transformation vectors containing both *entC* and *pmsB* constructs (Fig. 1, constructs 4 and 5) were obtained.

Plant transformation. *Nicotiana tabacum* cv. Samsun NN plants seven to nine weeks old were used to generate transgenic plants transformed with constructs 1–5 shown in Figure 1. Regenerated plants were self-pollinated, and T1 seeds were collected. As controls in the various experiments, P12 Samsun NN tobacco plants were used. These plants are transformed with the *P1* and *P2* genes of AMV[31].

Analysis of ICS and IPL activity in vitro. Enzyme extracts were made from transgenic plants[31], and ICS activity in the extracts was measured[17]. 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 305 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 [33,34] with minor modifications (Verberne et al., unpublished data). Salicylic acid was quantified by chromatography on a Phenomenex column, type LUNA 3µ C18 (2) 50 × 4.60 mm × 3 µm, with a SecurityGuard from Phenomenex (Metrohm, 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 305 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 Nuclear transfer membrane (Amersham, Arlington Heights, IL), and UV auto-crosslinked. The blots were hybridized with ^{32}P-labeled inserts from clones corresponding to PR-1a, PR-1g, PR-2a, PR-2b, and PI-II[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 $\alpha = 0.05$.

Inoculation with powdery mildew. Eight eight-week-old plants of $_{ss}$*entC*$_{ss}$*pmsB* lines 4 and 16, and eight control P12 plants were inoculated with *O. lycopersicon* by spraying a spore suspension of 3.0×10^3 spores ml^{-1} (total volume 400 ml). The conditions of the test room were 20°C, 16 h light period, and 80% relative humidity.

Acknowledgments

The authors would like to thank T. Luijendijk for assistance in SA-analysis and Dr. J. Hermans for his help with the statistical analysis of lesion sizes. Zeneca-Mogen is acknowledged for providing the fungus O. lycopersicon. This work was financially supported by the Ministry of Economic Affairs, the Ministry of Education, Culture and Science, and the Ministry of Agriculture, Nature Management and Fishery in the framework of a research program of the Association of Biotechnology Centres in the Netherlands (ABON), and by the van Leersum fund.

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