Inverse relationship between systemic resistance of plants to microorganisms and to insect herbivory

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Pre-inoculation of plants with a pathogen that induces necrosis leads to the development of systemic acquired resistance (SAR) to subsequent pathogen attack [1]. The phenylpropanoid-derived compound salicylic acid (SA) is necessary for the full expression of both local resistance and SAR [2,3]. A separate signaling pathway involving jasmonic acid (JA) is involved in systemic responses to wounding and insect herbivory [4,5]. There is evidence both supporting and opposing the idea of cross-protection against microbial pathogens and insect herbivores [6,7]. This is a controversial area because pharmacological experiments point to negative cross-talk between responses to systemic pathogens and responses to wounding [8–10], although this has not been demonstrated functionally *in vivo*. Here, we report that reducing phenylpropanoid biosynthesis by silencing the expression of phenylalanine ammonia-lyase (PAL) reduces SAR to tobacco mosaic virus (TMV), whereas overexpression of PAL enhances SAR. Tobacco plants with reduced SAR exhibited more effective grazing-induced systemic resistance to larvae of *Heliothis virescens*, but larval resistance was reduced in plants with elevated phenylpropanoid levels. Furthermore, genetic modification of components involved in phenylpropanoid synthesis revealed an inverse relationship between SA and JA levels. These results demonstrate phenylpropanoid-mediated cross-talk *in vivo* between microbially induced and herbivore-induced pathways of systemic resistance.

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Received: 3 November 1998
Revised: 27 January 1999
Accepted: 27 January 1999

Published: 15 March 1999

Current Biology 1999, 9:317–320
http://biomednet.com/elecref/0960982200900317

Results and discussion

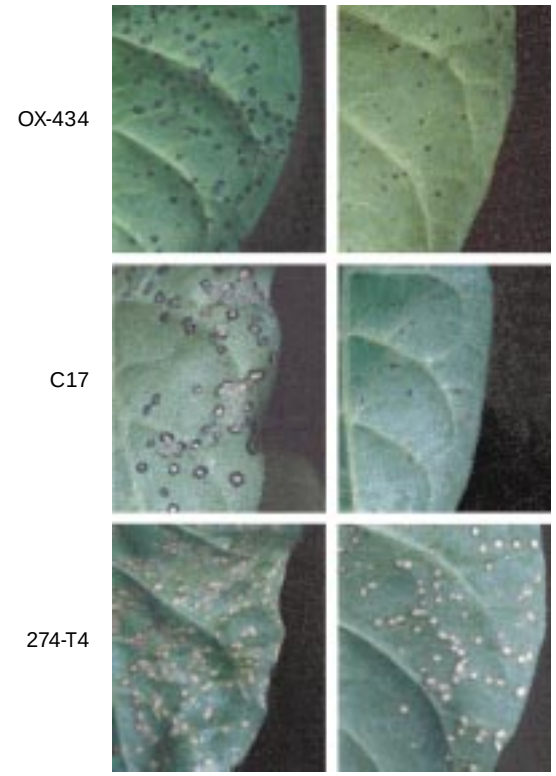
PAL catalyzes the first committed step in phenylpropanoid biosynthesis. Transgenic tobacco plants exhibiting gene silencing mediated by the bean *PAL2* transgene have reduced levels of phenylpropanoid compounds, have increased susceptibility to virulent fungal pathogens, and fail to express SAR following infection by TMV [11,12]. The lesions on systemic leaves of PAL-suppressed (274-T4) plants that had been pre-inoculated with TMV on a lower leaf 7 days previously were significantly larger and much lighter in color than those observed on systemic leaves of the corresponding C17 control line (Figure 1a,b). This increased lesion size — indicating a lack of SAR — may be due to reduced SA levels, because SAR is also eliminated in tobacco plants expressing bacterial SA hydroxylase (NahG), an enzyme that removes SA by converting it to catechol [2].

In some bean *PAL2* transgenic lines, gene silencing is lost following selfing, and the resultant progeny (for example, OX-434) exhibited severalfold higher PAL activity and correspondingly elevated levels of phenylpropanoid compounds, such as chlorogenic acid (CGA), compared with wild-type plants [13]. TMV-induced lesions on systemic leaves of PAL-overexpressing plants were very dark, and smaller than those produced on systemic leaves of control plants (Figure 1a,b). Increasing phenylpropanoid biosynthesis therefore enhances SAR. It is important to note that the elevated levels of phenylpropanoid compounds in the leaves of naïve (previously unchallenged) PAL-overexpressing tobacco plants do not significantly reduce the growth or the survival of *Manduca sexta* or *H. virescens* larvae [14]. This observation provides a control for the effects of preformed phenolic compounds in the following experiments that examined systemic resistance to insect herbivores.

Control (C17) tobacco plants that were pre-induced by larval grazing on a single lower leaf exhibited systemic induced resistance to larvae of *H. virescens*, as measured by reduced larval weight and increased larval mortality on systemic leaves, compared with non-pretreated C17 plants (Figure 2). As assessed by the above two criteria, PAL-suppressed plants exhibited increased systemic insect resistance, whereas induced insect resistance in plants overexpressing PAL was almost totally eliminated (Figure 2). Therefore, transgenic modification of phenylpropanoid biosynthesis has opposite effects on the ability of tobacco plants to develop SAR to TMV and to develop grazing-induced systemic insect resistance.

The leaves of naïve PAL-overexpressing plants had approximately twofold higher free SA levels than the

Figure 1



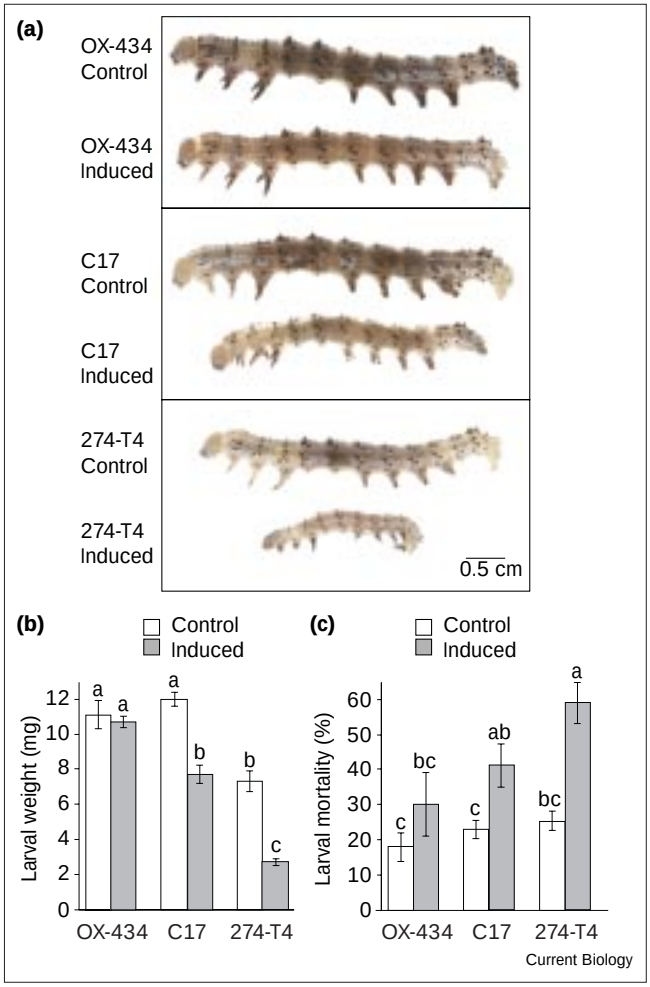
(b)

Line	Mean lesion diameter (mm)	
	Buffer	TMV
OX-434	1.90 ± 0.38	0.48 ± 0.28
C17	2.23 ± 0.32	0.99 ± 0.28
274-T4	2.00 ± 0.31	1.79 ± 0.29

Current Biology

(a) TMV-generated lesions on systemic leaves of control (C17), PAL-suppressed (274-T4) or PAL-overexpressing (OX-434) tobacco plants that had been pre-induced on a lower leaf with buffer or with U1 TMV. (b) Lesion sizes for the treatments described in (a). Each value represents the mean ± standard deviation of 30 lesions on three independent plants.

Figure 2

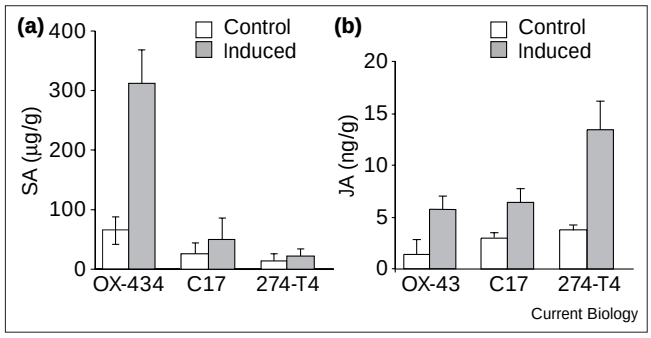


Insect resistance of systemic leaves of PAL-overexpressing (OX-434), PAL-suppressed (274-T4) or control (C17) tobacco plants that had been pre-induced with water on a lower leaf (control) or by grazing with *H. virescens* larvae (induced). (a) Typical appearance of larvae 5 days after feeding on systemic leaves. (b) Larval weight and (c) larval mortality after 5 days feeding on systemic leaves. (b,c) The mean ± standard error (SE) of four independent experiments is shown. Values for columns bearing different letters are significantly different at $P < 0.05$.

equivalent leaves from C17 control plants (Figure 3a), and the accumulation of SA in grazing-induced plants was much greater in the plants overexpressing PAL than in wild-type plants. Furthermore, PAL-suppressed plants had lower basal and induced free SA levels. To determine whether the increased insect resistance in PAL-suppressed plants was due to the decrease in phenylpropanoid compounds in general, or to a specific reduction in the levels of SA, we repeated the insect feeding experiments with transgenic tobacco harboring the bacterial *NahG* gene, and with its corresponding nontransformed control line. The systemic leaves of grazing-induced NahG tobacco plants, but not those of naïve NahG tobacco plants, were significantly more resistant to *H. virescens* than the equivalent leaves of the corresponding control line on the basis of larval mortality measurements, but not on the basis of larval weight measurements (Figure 4a,b). Thus, SA may contribute to the inverse relationship between the capacities for grazing-induced insect resistance and pathogen-induced SAR, although these data do not rule out the involvement of other phenylpropanoid compounds.

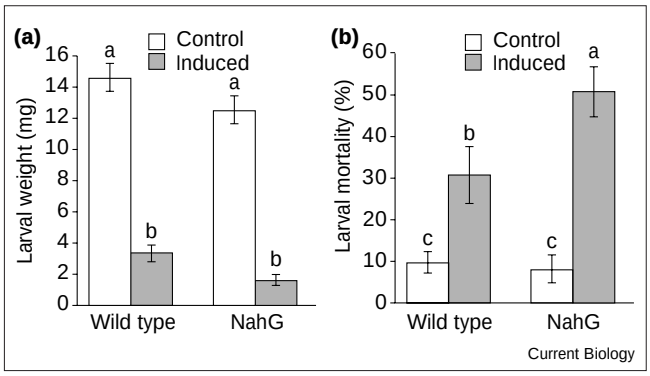
Exogenous application of SA or its derivatives, such as aspirin, blocks both JA biosynthesis and the action of JA in

Figure 3



Levels of (a) free SA and (b) JA in leaves of naïve (control) and wound-induced PAL-overexpressing (OX-434), PAL-suppressed (274-T4) or control (C17) tobacco plants. The levels of SA and JA are expressed as μg or ng per g fresh weight. The mean ± standard error (SE) of five (SA) and three (JA) independent experiments is shown.

Figure 4



Insect resistance of systemic leaves of NahG tobacco plants and the corresponding wild-type control plants following pre-induction of a lower leaf with water (control) or by grazing with *H. virescens* larvae (induced). (a) Larval weight and (b) larval mortality after 5 days feeding on the systemic leaves. The mean ± standard error (SE) of five independent experiments is shown. Values for columns bearing different letters are significantly different at $P < 0.05$.

wound signaling [8–10], suggesting a possible mechanism for the inverse relationship between grazing-induced insect resistance and pathogen-induced SAR revealed by the transgenic perturbation of phenylpropanoid metabolism. Consistent with this hypothesis, constitutive JA levels in the leaves of naïve PAL-modified plant lines (Figure 3b) correlated inversely with constitutive SA levels (Figure 3a). More strikingly, significantly higher levels of JA were induced by wounding in the PAL-suppressed line than in the PAL-overexpressing line (Figure 3b), the converse of the behavior of induced SA levels (Figure 3a). Likewise, the NahG-10 line, with dramatically reduced SA levels [2], had higher constitutive JA levels (10.8 ng per g fresh weight) than its corresponding wild-type control (3.7 ng per g fresh weight), although we consistently saw no induction of JA in NahG plants (data not shown). Finally, levels of transcripts encoding polyphenol oxidase (PPO) and 3-hydroxymethylglutaryl CoA reductase, and levels of the alkaloid nicotine, all of which are wound-inducible via the JA pathway [4,15,16], were highest in systemic leaves of grazing-induced PAL-suppressed plants (data not shown). Systemic leaves of PAL-suppressed plants had twice the nicotine levels as those of systemic leaves of PAL-overexpressing plants, which had levels of nicotine that were below those of the wild-type control. These data are all consistent with the insect-resistance phenotypes being mediated by SA–JA cross-talk.

On the basis of studies with artificial diets, some phenylpropanoid compounds have been proposed to act directly to prevent insects from feeding [17]. In the present system, however, there is no significant difference in the growth and the survival of *H. virescens* or *M. sexta* larvae on leaves of naïve transgenic tobacco plants either overexpressing or underexpressing PAL, although these differ by at least an order of magnitude in their CGA levels [14], and reducing phenylpropanoid compounds increases rather than decreases resistance to insect herbivory. In terms of insect defense, the effects of modifying phenylpropanoid expression are manifest only in relation to systemic resistance, and our results point to cross-talk *in vivo* between the phenylpropanoid pathway (potentially mediated by SA) and the pathway signaled by JA. We cannot formally rule out the possibility that the phenylpropanoid complement of systemic leaves of induced PAL-overexpressing plants is different from that of the corresponding leaves of naïve plants and contains a compound(s) that promotes insect survival. Other complementary evidence supports our conclusion that the effects reported here are mediated by SA–JA cross-talk, however. For example, exogenous JA inhibits SA-mediated induction of tobacco acidic pathogenesis-related (PR) proteins whereas exogenous SA inhibits JA-mediated induction of basic PR proteins [18], suggesting that pathway cross-talk may operate both in the direction established here, and also in the opposite direction.

It has been recently demonstrated that JA-induced responses, although benefiting plants under insect attack, are costly and result in reduced seed yield in plants not under insect pressure [19]. Impaired insect resistance could be a constraint against the constitutive expression of SA-inducible disease-resistance mechanisms. This negative cross-talk between pathways may play a role in prioritizing or channeling a plant's response to different biological stresses.

A corollary of SA-mediated negative cross-talk between SAR and systemic induced insect resistance is that agrichemical modulation or transgenic manipulation of components of the signal pathway at, or upstream of, the

SA–JA interaction might have unwanted side effects in the field. Such cross-talk may not, however, occur in all cases. For example, JA is a necessary component of the resistance response of *Arabidopsis* roots to *Pythium* root rot [20] and, in rice, wounding results in elevated JA levels and systemic resistance to microbial infection [21].

Materials and methods

The PAL-modified tobacco plant lines have been described elsewhere [12,13]. The *NahG* transgene in the NahG-10 line is in a different line of cv Xanthi N from that harboring the bean *PAL* transgene, which explains the different absolute values for resistance level. Plants were grown in a greenhouse at an average temperature of approximately 24°C under a 16 h:8 h light:dark regimen. For TMV experiments, 6–8-week-old plants at the same physiological stage of growth were paired by approximating their plastochron indices. Systemic leaves were inoculated with a challenge inoculation of U1 TMV 7 days after mock inoculation with buffer (0.01 M phosphate pH 7.0) or pre-inoculation of a single leaf with U1 TMV (0.5 μg/ml), and lesion sizes determined after 3 days. Three independent lines of each phenotype were analyzed, and the overall experiment repeated twice with identical results. Four replicated insect feeding experiments were performed, each with five cuttings per treatment. Plants were pre-exposed (induced) by 'caging' two third-instar *H. virescens* on a single lower leaf for 3 days, and then exposed to a challenge with 20 larvae on an upper terminal leaf. Control plants were not exposed to the pre-challenge. The extent of herbivory on the challenged lower leaves was generally greater than 50% of the leaf area, and was independent of the genotype. Larval weight and mortality were scored five days after the test challenge. Data were analyzed for statistically significant differences at $P < 0.05$, using Fisher's LSD test. Systemic leaf tissues from naïve and induced plants were harvested from five replicate plants for each treatment following the 3 day induction period for analysis of metabolite levels. Nicotine [22], SA [23] and JA [22] levels were analyzed by high performance liquid chromatography or gas chromatography, as described.

Acknowledgements

This work was supported by the Samuel Roberts Noble Foundation and by the USDA NRICGP and the NSF. We thank John Ryals (Novartis Inc) for providing NahG-10 and corresponding control tobacco plants.

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