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Phytochemistry 64 (2003) 153–161
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Phenylpropanoid compounds and disease resistance in transgenic tobacco with altered expression of L-phenylalanine ammonia-lyase

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Received 22 November 2002; received in revised form 18 January 2003

Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

Tobacco plants over-expressing L-phenylalanine ammonia-lyase (PAL^+) produce high levels of chlorogenic acid (CGA) and exhibit markedly reduced susceptibility to infection with the fungal pathogen *Cercospora nicotianae*, although their resistance to tobacco mosaic virus (TMV) is unchanged. Levels of the signal molecule salicylic acid (SA) were similar in uninfected PAL^+ and control plants and also following TMV infection. In crosses of PAL^+ tobacco with tobacco harboring the bacterial *NahG* salicylate hydroxylase gene, progeny harboring both transgenes lost resistance to TMV, indicating that SA is critical for resistance to TMV and that increased production of phenylpropanoid compounds such as CGA cannot substitute for the reduction in SA levels. In contrast, PAL^+/NahG plants showed strongly reduced susceptibility to *Cercospora nicotianae* compared to the NahG parent line. These results are consistent with a recent report questioning the role of PAL in SA biosynthesis in *Arabidopsis*, and highlight the importance of phenylpropanoid compounds such as CGA in plant disease resistance.

Keywords: *Nicotiana tabacum*; Solanceae; *Cercospora nicotianae*; Chlorogenic acid; L-phenylalanine ammonia-lyase; *Pseudomonas syringae*; Salicylic acid; Tobacco mosaic virus

1. Introduction

Phenylpropanoid compounds are widely used by plants as part of their antimicrobial defense arsenal. For example, flavonoids, isoflavonoids, stilbenes, monolignols and lignans serve as inducible phytoalexins or pre-formed phytoanticipins in many plant species (Dixon, 2001), and the phenylpropanoid polymer lignin can act as an inducible physical barrier against pathogen ingress (Mitchell et al., 1999). However, most studies linking specific phenylpropanoid compounds with disease resistance have been essentially correlative. Only a few examples provide more direct genetic evidence for a role of a particular phenylpropanoid compound in defense. Thus, increased production of isoflavonoid phytoalexins enhances disease resistance in alfalfa (He and Dixon, 2000), and reduced fungal detoxification of isoflavonoids decreases fungal pathogenicity on pea (Mackintosh et al., 1998). The stilbene resveratrol can confer resistance to fungal infection when expressed in transgenic tobacco and alfalfa, species in which it is not naturally produced (Essenberg, 2001; Hain et al., 1993; Hipskind and Paiva, 2000).

L-Phenylalanine ammonia-lyase (PAL), the entry-point enzyme into the phenylpropanoid pathway (Fig. 1), and downstream enzymes such as caffeic acid *O*-methyltransferase (COMT), are strongly induced following infection of tobacco plants with viral pathogens or exposure to fungal elicitor (Jaeck et al., 1992; Pellegrini et al., 1993, 1994). Reduction of phenylpropanoid biosynthesis in tobacco by down-regulation of PAL seriously compromises local disease resistance to fungal

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doi:10.1016/S0031-9422(03)00151-1

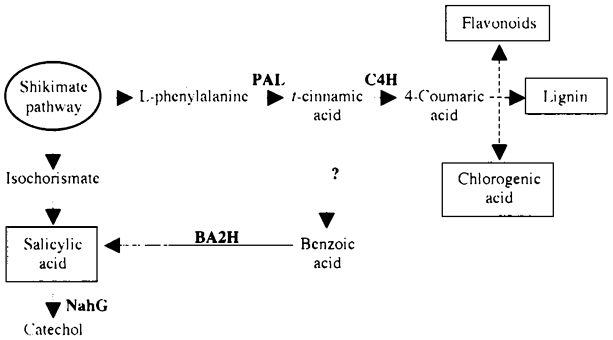

Fig. 1. Schematic pathway for the biosynthesis of phenylpropanoid compounds associated with defense in tobacco. Enzymes are PAL, L-phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; BA2H, benzoic acid 2-hydroxylase; NahG, bacterial salicylate hydroxylase (transgene). Dotted arrows represent pathways with multiple enzymes.

infection (Maher et al., 1994), and both local resistance and systemic acquired resistance (SAR) to viral infection (Pallas et al., 1996). However, it is not clear which phenylpropanoid derivatives confer resistance in wild-type tobacco. Salicylic acid (SA) acts as a signal molecule for local and systemic resistance of tobacco and other plants to viral, fungal and bacterial pathogens (Dempsey et al., 1999; Klessig and Malamy, 1994; Shah and Klessig, 1999; Silverman et al., 1993). Based upon the similarity in disease phenotypes between PAL-reduced tobacco (Pallas et al., 1996) and plants expressing the *NahG* salicylate hydroxylase gene (Gaffney et al., 1993), the product of which removes SA by converting it to catechol, it has been assumed that reduction in SA levels is an important component of the PAL$^-$ phenotype.

Until recently, it was believed that SA was primarily synthesized from L-phenylalanine via cinnamic and benzoic acids (Lee et al., 1995; Verberne et al., 1999) (Fig. 1). However, genetic evidence has recently been presented indicating that a significant proportion of the SA produced during disease responses in *Arabidopsis* originates from isochorismate by-passing the phenylpropanoid pathway (Wildermuth et al., 2001) (Fig. 1). These findings necessitate a re-evaluation of the role of PAL in SA biosynthesis and the role of SA in the defense responses of plants with modified PAL expression.

Transgenic tobacco plants over-expressing a bean *PAL* transgene (PAL$^+$) produce elevated levels of the hydroxycinnamic acid ester chlorogenic acid (CGA) in their leaves (Howles et al., 1996), and exhibit increased systemic resistance to tobacco mosaic virus (TMV) infection (Felton et al., 1999). PAL$^+$ plants are therefore useful tools with which to examine the relationships between PAL activity, SA production, and the role of phenylpropanoid compounds in disease resistance. We here examine the responses of PAL-modified tobacco plants to a viral and a fungal pathogen in relation to the levels of CGA and SA produced and, using PAL$^+$ plants harboring a bacterial salicylate hydroxylase gene, assess the importance of SA to the resistance of PAL$^+$ plants.

2. Results

2.1. Infection phenotypes of transgenic tobacco in response to tobacco mosaic virus

When compared with wild-type (WT) control plants (C1), lesions on PAL$^+$ plants caused by infection with the U1 strain of TMV were more uniformly black, whereas lesions on PAL$^-$ plants were much lighter in color (Fig. 2A and insets). Lesions on WT and PAL$^-$ plants always had a darker area surrounding a light center, whereas, in lesions on PAL$^+$ plants, the light center was either smaller or completely absent, and the dark outer ring was significantly darker (Fig. 2A). TMV lesions on NahG plants differed from those on WT plants by their increased size (Gaffney et al., 1993; Murphy et al., 1999; Vernooij et al., 1994), but their overall coloration was similar (Fig. 2A).

The sizes of the primary TMV lesions on PAL$^+$ and control lines were similar at 7 days post-inoculation (dpi) (Fig. 2B). Note the two different control tobacco lines included in these and following experiments. Both lines were of cultivar Xanthi; one was of the exact genotype used for transformation with the bean *PAL2* transgene (C1) and the other was of the exact genotype used for transformation with the bacterial *NahG* transgene (C2). Although lesions on NahG plants were significantly larger than on control lines, over-expression of PAL in the NahG background did not lead to reduced lesion size (Fig. 2B). However, lesions on PAL$^+$/NahG plants exhibited the darker coloration characteristic of PAL over-expression (Fig. 2A). The increased size of TMV lesions on PAL$^-$ as compared with control plants has been described previously (Pallas et al., 1996).

Fig. 2C compares lesion sizes 7 days after a secondary inoculation on plants that had either been mock inoculated or pre-inoculated with U1 TMV on another leaf seven days prior to the secondary inoculation. These treatments should result in SAR following pre-inoculation with TMV but no SAR following the mock inoculation. Again, lesions on NahG and PAL$^+$/NahG plants were larger than on PAL$^+$ or control lines. A comparison of lesion sizes between mock and pre-inoculated lines demonstrated a similarly reduced lesion size (i.e. manifestation of systemic resistance) on PAL$^+$ and the two control lines. However, there was no difference between mock- and pre-inoculated lines in relation to secondary lesion size for the NahG or PAL$^+$/NahG lines (Fig. 2C), indicating loss of SAR following

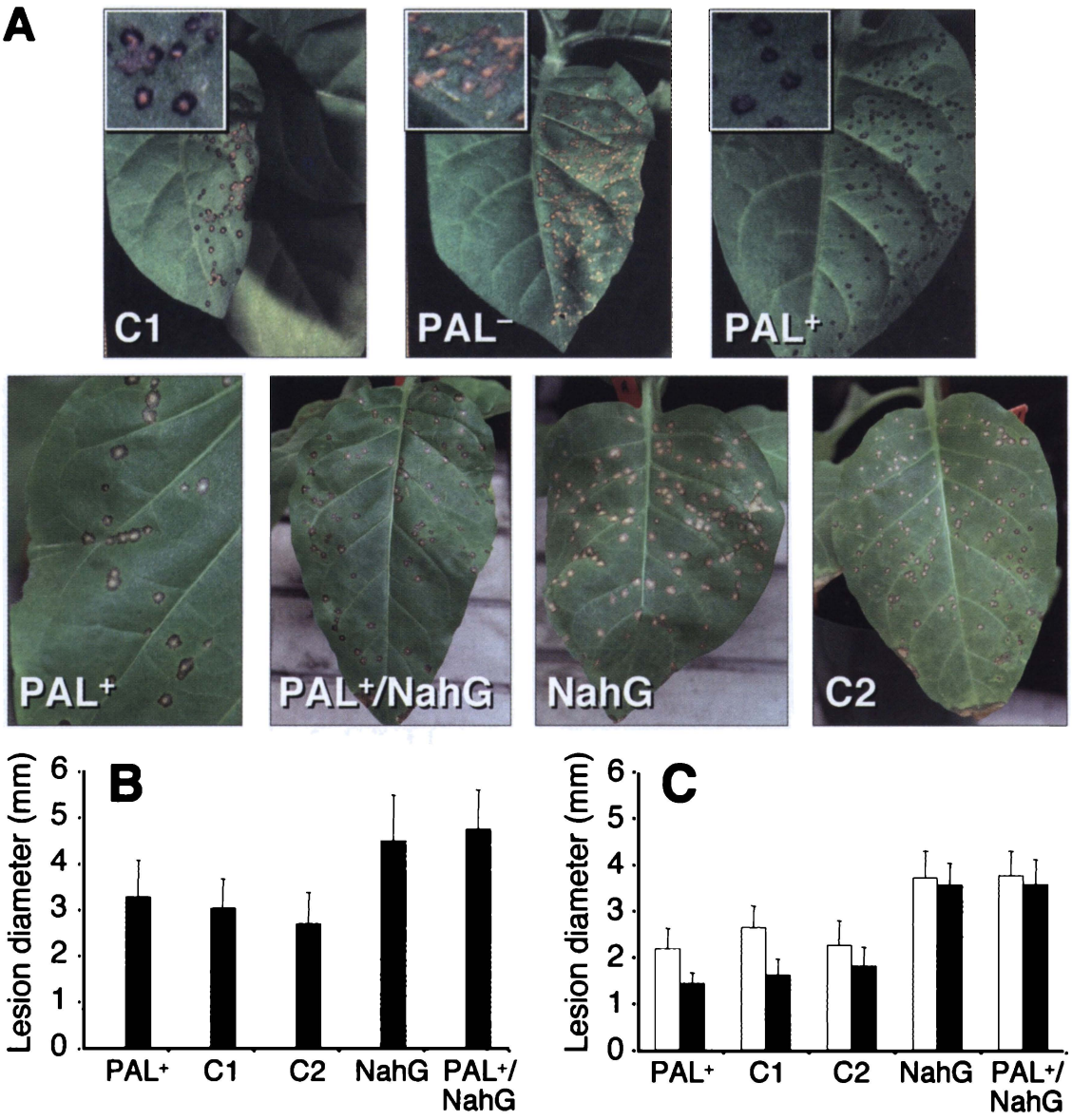

Fig. 2. Tobacco mosaic virus lesions on leaves of PAL-modified, NahG and control tobacco plants. (A) visible appearance of lesions (see Experimental for details of the transgenic and control lines). Insets show a magnification of selected lesions from each panel. The photographs in the upper panel are of plants grown at a different time from those in the lower panel; within each panel, leaves were from plants at the same physiological stage and inoculations and incubations were carried out in parallel under identical conditions. The level of pigmentation in the lesions can differ between experiments, perhaps depending on the exact environmental conditions in the greenhouse. (B) Primary lesion sizes on leaves of the various lines inoculated with TMV and analyzed 7 days post-inoculation. (C) Secondary lesion sizes on leaves of the various lines 7 days after a secondary inoculation with TMV on plants that had either been mock inoculated (open bars) or pre-inoculated with U1 TMV (filled bars) on another leaf 7 days prior to the secondary inoculation. Bars represent means and standard deviations from four determinations.

reduction of SA levels in NahG plants, and the failure of PAL over-expression to compensate for this effect.

2.2. Levels of CGA and SA in response to TMV infection

There is a direct relationship between PAL activity and levels of CGA, the major leaf phenylpropanoid compound in tobacco (Howles et al., 1996). Thus, high CGA levels are diagnostic of PAL over-expression in the transgenic lines. CGA levels in the various tobacco lines following TMV infection were determined by HPLC analysis as described previously (Howles et al., 1996). Levels of CGA in non-inoculated WT control plants were 0.71 mmol/gfw, compared with 3.48 mmol/gfw in non-inoculated PAL^+ plants. Comparison of the PAL^+ line and its corresponding control seven days after primary inoculation with TMV revealed an approximately six-fold increase in CGA levels as a result of *PAL* transgene expression, and similar elevated

CGA levels were seen in the PAL$^+$/NahG progeny (Fig. 3A). Thus, expression of NahG does not affect CGA levels in TMV-infected PAL over-expressing plants. The slightly higher CGA levels in the NahG control line C2 than in the WT control C1 are most likely due to the two control lines not possessing identical genetic backgrounds. Similar relative CGA levels among the different lines were observed in naïve systemic leaves (uninoculated leaves from a pre-inoculated plant) seven days after a primary inoculation on a separate leaf (Fig. 3B), although CGA levels in the NahG background were somewhat higher than in Fig. 3A. There was no systemic induction of CGA accumulation following primary inoculation. However, CGA levels in secondarily inoculated leaves on PAL$^+$ and PAL$^+$/NahG plants that had received an earlier primary inoculation on separate leaves were approximately twice as high as in plants that had received a single inoculation (Fig. 3C).

SA levels were monitored by GC/MS analysis as described in Experimental procedures. Total (free plus esterified) SA levels in non-inoculated NahG plants were 0.08 μg/gfw, compared with 0.28 μg/gfw in non-inoculated PAL$^+$ plants. SA levels in leaves of the PAL$^+$ and control lines were quite similar seven days after a primary inoculation with TMV, whereas levels in plants expressing NahG, either alone or with the bean *PAL* transgene, were significantly lower (Fig. 3D), as would be predicted. SA levels in naïve systemic leaves of plants previously inoculated on separate leaves were very low for all the lines (similar to levels in inoculated NahG plants; Fig. 3E). Thus, increases in total SA levels were only seen in leaves directly inoculated with TMV, and were not further increased as a result of PAL over-expression, in contrast to the increases in CGA that arose as a result of constitutive *PAL* transgene expression.

Fig. 3F compares total SA levels in secondarily inoculated leaves following a primary TMV- or mock-inoculation. Importantly, PAL$^+$ plants had SA levels similar to those of the control lines and, in PAL$^+$/NahG plants, SA levels were close to those of the NahG parent. Taken together, the SA analyses indicate that SA levels are induced by TMV infection, are strongly reduced as a result of *NahG* transgene expression, but are not significantly affected by PAL over-expression in either wild-type or NahG backgrounds.

2.3. Infection phenotypes of transgenic tobacco in response to Cercospora nicotianae

In contrast to the lack of effect of PAL over-expression on the size of TMV lesions, PAL$^+$ tobacco plants developed significantly fewer and smaller lesions than corresponding WT control plants following infection with the virulent fungal pathogen *C. nicotianae* (Fig. 4 A). Although the coloration of the lesions was similar in WT (C1), NahG, PAL$^+$, PAL$^-$ and PAL$^+$/NahG lines, the rates of lesion development differed greatly, with much greater spread and coalescence of lesions in NahG and PAL$^-$ plants compared to controls. In contrast, lesions developed very little, if at all, on PAL$^+$ plants, as seen from a comparison of lesion development rates (Fig. 4B) and the infected leaf area data from two independent experiments (Fig. 4C, D). Note that the data in Fig. 4B are for plants that had fewer than average lesions, such that the lesions developed independently and did not coalesce. Individual lesions with a more or

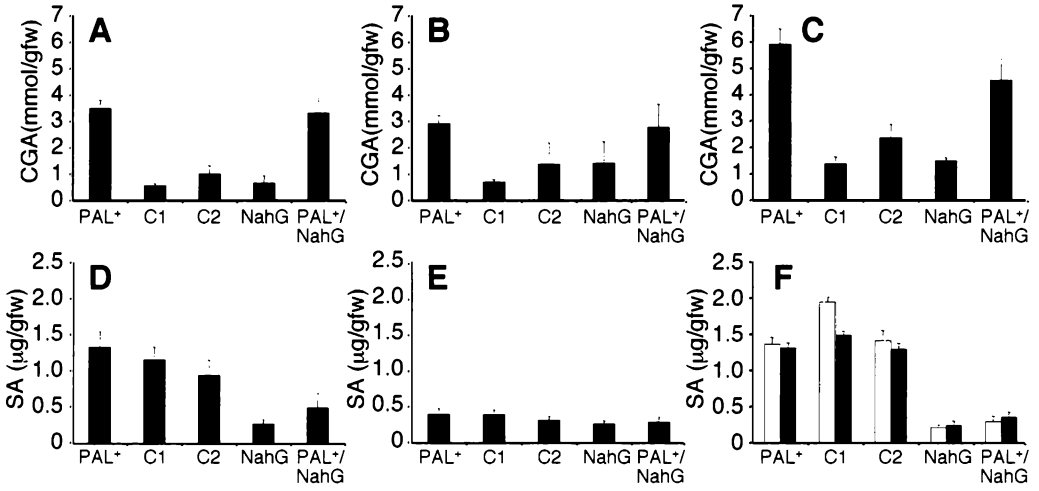

Fig. 3. Levels of CGA and SA in leaves of PAL-modified, NahG and control tobacco plants following inoculation with TMV. (A) CGA levels 7 days after a primary inoculation with TMV. (B) CGA levels in uninoculated leaves 7 days after primary inoculation of a separate leaf with TMV. (C) CGA levels in secondarily inoculated leaves (7 dpi) that had received an earlier primary TMV inoculation on a separate leaf. (D) SA levels 7 days after a primary inoculation with TMV. (E) SA levels in uninoculated leaves 7 days after primary inoculation of a separate leaf with TMV. (F) SA levels in leaves 7 days after a secondary inoculation with TMV on plants that had either been mock inoculated (open bars) or pre-inoculated with U1 TMV (filled bars) on another leaf 7 days prior to the secondary inoculation. Bars represent means and standard deviations from four determinations.

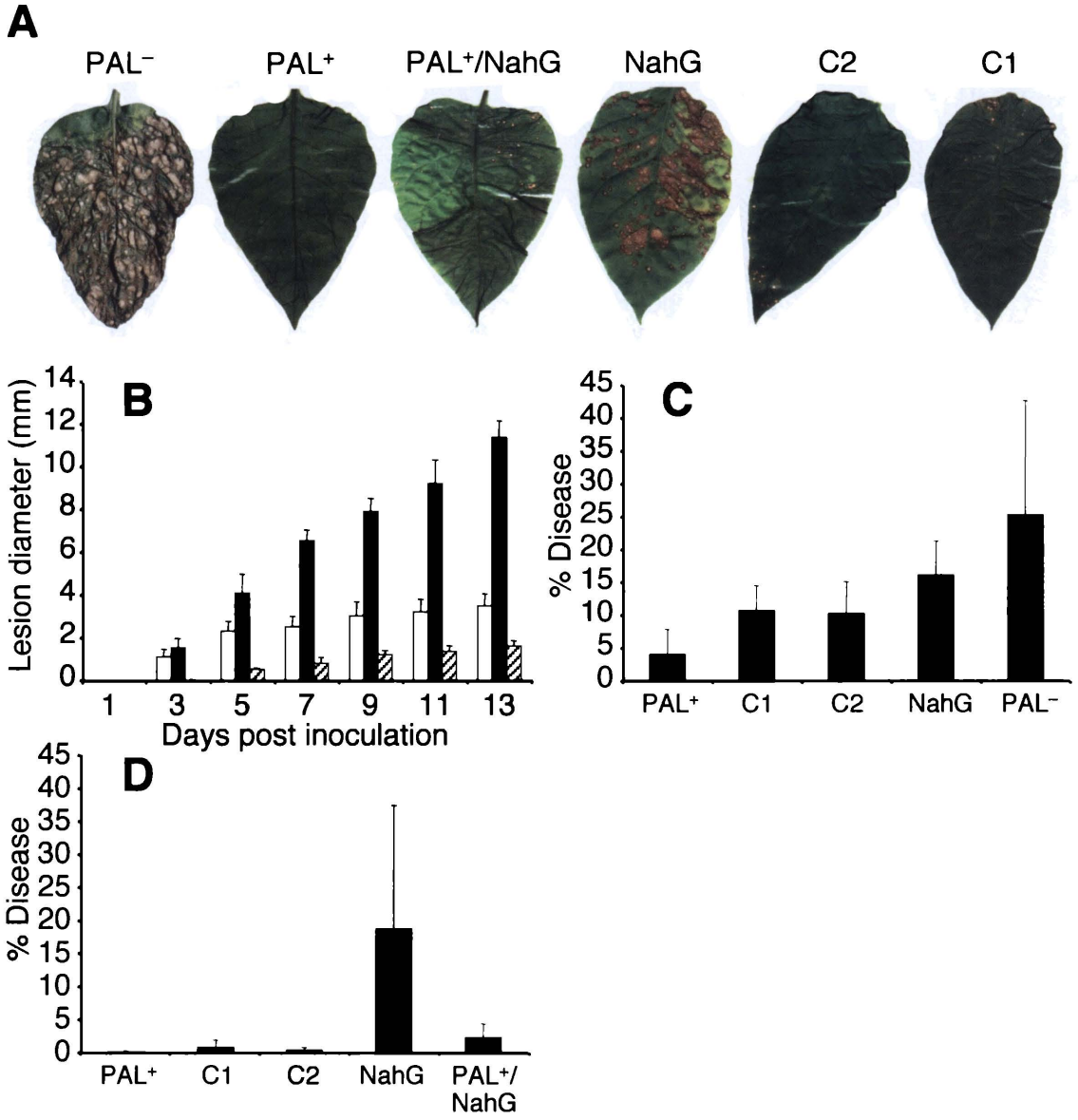

Fig. 4. *Cercospora nicotianae* lesions on leaves of PAL-modified, NahG and control tobacco plants. (A) Visible appearance of lesions at seven dpi. (B) Lesion development as a function of time in wild-type plants (C1, open bars), PAL^- plants (filled bars) and PAL^+ plants (hatched bars). Up to 30 lesions of uniform size were selected on each plant genotype, and their diameters monitored independently during the time course. (C, D) Two independent experiments in which disease was measured as total infected leaf area determined by image analysis. Lesions were measured at 7 dpi.

less circular morphology were selected and their diameter followed as a function of time. In the data in Figs 4C and D, no selection for specific lesion phenotypes was made, and the total infected area was measured. Whereas NahG plants exhibited a severe, coalescing lesion phenotype, similar to that observed on PAL^- plants, lesion size was, by comparison, much reduced in PAL^+/NahG plants (Fig. 4D).

2.4. Levels of CGA and SA in response to Cercospora

The high levels of CGA in PAL over-expressing plants did not significantly increase following fungal inoculation (Fig. 5 A), as shown previously for CGA levels in wild-type plants (Maher et al., 1994), and were not significantly reduced in the PAL^+/NahG progeny. The apparent increase in CGA levels following fungal inoculation of the NahG line was unexpected but statistically significant.

SA levels were low and similar in all lines and treatments following analysis of the same leaf material as analyzed for CGA earlier (Fig. 5B), with the exception of elevated SA following inoculation of the PAL^+ plants. This level was approximately 60% of that observed following primary inoculation of PAL^+ plants with TMV (Fig. 3D, F). Importantly, PAL over-expression in the NahG background did not lead to increased SA levels above those of the NahG control C2.

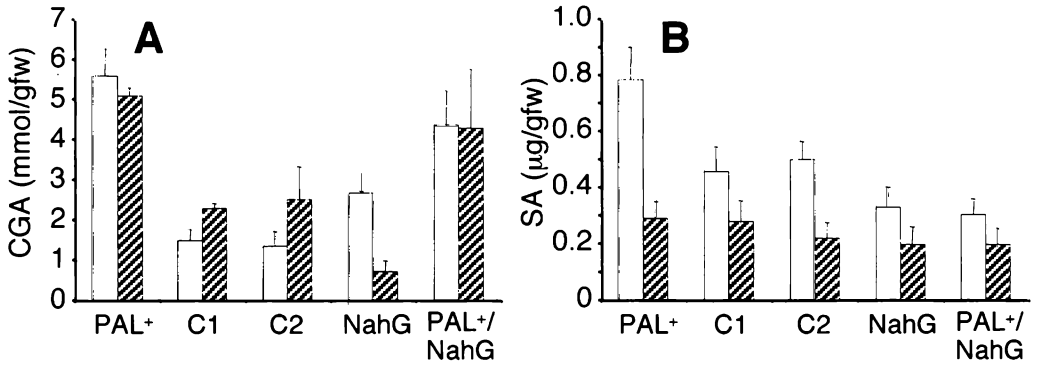

Fig. 5. Levels of CGA and SA in leaves of PAL-modified, NahG and control tobacco plants following inoculation with *Cercospora nicotianae*. (A) CGA levels in uninfected leaves (hatched bars) or in leaves 7 days after infection with *C. nicotianae* (open bars). (B) SA levels in uninfected leaves (hatched bars) or in leaves 7 days after infection with *C. nicotianae* (open bars). Bars represent means and standard deviations from four determinations.

3. Discussion

PAL over-expression changes the lesion phenotype on tobacco plants inoculated with TMV. As the tobacco lines used in the present work contained the *N* gene (Whitham et al., 1994), they are resistant to U1 TMV and the lesions become limited. The lesions on PAL over-expressing plants were dark in color, presumably as a result of increased deposition of phenolic compounds. Conversely, as described previously (Pallas et al., 1996) lesions on PAL$^-$ plants were lighter than those on WT plants. Whereas lesions on PAL$^-$ plants were larger than those on WT plants (and more similar to those on NahG plants), the intensely dark primary lesions on PAL over-expressing plants were not of reduced size. This places in question the function in viral resistance of the increased phenylpropanoid and lignin biosynthesis associated with induction of PAL and COMT that occurs in and around the developing TMV lesion (Fritig et al., 1973; Jaeck et al., 1992; Legrand et al., 1978). Perhaps a major function of PAL induction here is the production of the coumarin scopoletin and its glucoside scopolin which have been shown in other studies to reduce TMV symptoms, either through anti-viral activity or ability to buffer active oxygen intermediates (Ahl-Goy et al., 1993; Chong et al., 2002)

Over-expression of PAL effectively reduced disease severity in tobacco plants infected with a virulent fungal pathogen. This presumably results from the production of one or more phenylpropanoid compounds, including CGA (as measured here) and coumarins, consistent with the converse observation that down-regulation of PAL increases disease severity in the same plant-pathogen combination. A similar reduction in lesion size, and reduced lesion number, is observed on PAL$^+$ plants following infection with the virulent bacterial pathogen *Pseudomonas syringae* (V. Wesley, unpublished results), suggesting that PAL over-expression might be a general strategy for engineering disease resistance. It has recently been shown that constitutive over-expression in tobacco of a *PAL* transgene from the tropical pasture legume *Stylosanthes humilis* also leads to increased resistance to *Cercospora nicotianae*, as well as to the Oomycete pathogen *Phytophthora parasitica* pv. *nicotianae* (Way et al., 2002). However, the PAL$^+$ plants exhibited significantly reduced growth rate and delayed flowering, phenotypes that were not observed in the present study with tobacco. The reason for this discrepancy is currently not clear. It might reflect quantitative differences in the level of PAL over-expression between the two studies (five-fold in the present work, up to eight-fold for transgenics expressing *Stylosanthes* PAL), or qualitative effects such as perturbation of metabolic channeling between PAL and C4H (Fig. 1; Rasmussen and Dixon, 1999) resulting from the use of different PAL species in the two studies.

We had previously suggested that loss of resistance following PAL down-regulation resulted from a reduction in SA levels, and that, in this respect, PAL$^-$ plants could essentially be viewed as NahG phenocopies (Pallas et al., 1996). This idea was supported by studies indicating reduced SA levels in PAL compromised plants (Felton et al., 1999; Pallas et al., 1996). However, results from the present work, in which PAL over-expression has been studied, do not support a strong correlation between PAL activity and total SA levels. Thus, in contrast to the clear relationship between increased PAL activity and CGA levels in all the experiments here reported, the only conditions under which we observed an increase (albeit small) in total SA levels attributable to PAL over-expression were following *Cercospora* infection of PAL$^+$ compared with WT control plants. No difference in total SA levels was observed when comparing highly susceptible NahG plants and PAL$^+$/NahG plants with little susceptibility to infection by *Cercospora*. Thus, total SA production is unlikely to be a factor in the reduced susceptibility of PAL over-expressing tobacco to *Cercospora*.

PAL over-expression in tobacco results in strongly increased levels of the major leaf phenylpropanoid compound CGA in both WT and NahG backgrounds, but CGA levels do not significantly increase following infection with virulent *C. nicotianae*, as previously observed in WT plants (Maher et al., 1994). Thus, increased constitutive CGA production is a potential cause of the increased fungal resistance in PAL over-expressing plants. A similar role for CGA in fungal resistance in potato has been proposed based on results showing increased susceptibility of plants with reduced CGA levels as a result of the creation of a new metabolic sink that reduced phenylalanine levels (Yao et al., 1995). At the same time, tobacco cultivars with increased scopoletin levels show improved resistance to *Cercospora* (Ahl-Goy et al., 1993); future studies on disease resistance in PAL over-expressing tobacco should therefore also address scopoletin levels.

Recent studies have questioned the role of the phenylpropanoid pathway in SA biosynthesis in *A. thaliana*, where a significant proportion of bacterially induced SA production occurs via the isochorismate synthase pathway (Wildermuth et al., 2001). However, it was suggested that constitutive SA levels in *Arabidopsis* leaves might originate via the PAL reaction, and that the inducible SA involved in triggering host cell death may also arise by this route (Wildermuth et al., 2001). Large increases in free SA following insect herbivory have been recorded in PAL over-expressing tobacco compared to wild-type controls (Felton et al., 1999). It is possible that the origin of SA depends upon the nature of the trigger and can vary both temporally and spatially. The present data are inconsistent with the phenylpropanoid pathway as being the only, or major, source of SA during microbial challenge. Discrepancies between the present data and previous studies on SA levels in PAL-modified plants (Felton et al., 1999; Pallas et al., 1996) may be explained by differences in the pools being measured (i.e. total vs. free SA) and the direction of PAL modulation. For example, it is possible that there is cross-talk between the shikimate and phenylpropanoid pathways such that PAL down-regulation might reduce flux through the isochorismate synthase reaction whereas PAL up-regulation may not affect this pathway. With the existence of at least two potential biosynthetic pathways for SA, and multiple pools, perhaps distinct both structurally (free and conjugated; Enyedi et al., 1992) and functionally/spatially (e.g. for activation of the hypersensitive response, PR protein production, the oxidative burst and cell death; Dorey et al., 1997; Mur et al., 1996; Rate et al., 1999; Shirasu et al., 1997), the relationship between SA biosynthesis and disease resistance is clearly complex.

Several of the disease susceptibilities of highly bred cultivated crops may have resulted from the successive loss of natural products during years of selection for yield or palatability traits. Although some of these pathways can now be restored by transgenic approaches, it has been argued that the levels of natural products required may be impractically high (Stuiver and Custers, 2001), that pathogens may rapidly overcome effects of single antimicrobial compounds by the evolution of detoxification pathways (Covert et al., 1996; Wasmann and VanEtten, 1996) and that large numbers of genes may have to be transferred, and coordinately regulated, in order to introduce many of the most effective antimicrobial compounds. Expressing bacterial SA-producing enzymes in transgenic plants results in increased fungal and viral resistance (Verberne et al., 2000), but high-level constitutive SA production leads to constitutive defense gene expression, dwarf phenotypes and correspondingly reduced plant fitness (Heil and Baldwin, 2002). Increasing production of endogenous antimicrobial compounds such as CGA and related phenylpropanoids through over-expression of a single entry point enzyme is a simple strategy that does not appear to put a metabolic stress on the plant. This strategy may be of more general utility than many approaches for engineering natural products in view of the common occurrence of hydroxycinnamic acid derivatives in plants.

4. Experimental procedures

4.1. Plant material

Four different types of tobacco lines were examined. PAL over-expressing plants (PAL^+) transgenic for the bean *PAL2* transgene have been described previously (Howles et al., 1996); OX434 was used as the PAL^+ line and has PAL activities up to five-fold higher than wild-type. Corresponding controls for OX434 were plants of the same cultivar (Xanthi) accession (C1). A transgenic tobacco line expressing the bacterial NahG SA hydroxylase transgene (NahG) was provided by Dr. John Ryals and has been described elsewhere (Delaney et al., 1994); a control line in the identical genetic background (C2) was also included in the present studies. Four progeny lines from a cross of OX434 with the NahG line (PAL^+/NahG A-2A, A-2B, A-9A, A-9B) were shown by PCR to contain both the bean *PAL* and *NahG* transgenes (data not shown). In some studies, plants in which PAL expression had been silenced following introduction of the bean *PAL2* transgene (PAL^-) were also included. This line (YE-10-6, with 75-fold reduced PAL activity in leaves) has been described previously (Elkind et al., 1990). Plants of each of the earlier lines were vegetatively propagated. In each experiment, plants of each line examined were grown together under identical environmental conditions in the greenhouse.

4.2. TMV inoculation

Inoculations with TMV strain U1 were done as described previously (Felton et al., 1999; Pallas et al., 1996). The adaxial surface of a newly expanded leaf was inoculated with 75 μl of virus (0.5 μg/ml). Lesion measurements were taken at seven days after initial inoculation using digital calipers. Challenge inoculations were performed 7 days after initial inoculation and lesion measurements taken 7 days after secondary inoculation.

4.3. Cercospora inoculation

Cercospora nicotianae (ATCC 18366) mycelia were suspended in sterile water, spread-plated on V8 agar plates (200 ml V8 juice, 3.0 g $CaCO_3$, and 15 g agar/l) and incubated at 20 °C under low relative humidity. Six to seven days after inoculation, spores were dislodged from the culture by flooding with 4–5 ml sterile water per plate followed by gentle brushing. The spore suspension was then filtered through cheesecloth, diluted to 50,000 spores/ml, and aerosolized on the tobacco leaves. Plants were kept covered with transparent bags for 2–3 days, then monitored for disease symptoms. Lesion measurements were taken over a period of 13 dpi using digital calipers (for measurements of selected, uniform lesions). Images of the infected leaves were also scanned into a computer, and the percent of diseased tissue versus healthy tissue was determined using the Sigma Scan Pro program (Jandel Scientific).

4.4. Metabolite analyses

Leaves from infected and control plants were harvested, frozen in liquid nitrogen, and ground into a fine powder. Soluble phenolic compounds were extracted from ground leaf tissue and CGA levels were measured by HPLC as described previously (Howles et al., 1996). SA was extracted from ground leaf tissue as described by Raskin et al. (1989) with the following modifications. 3-Hydroxybenzoic acid was added to ground tissue samples as internal standard. After the residue was resuspended in trichloroacetic acid, SA was extracted in diethyl ether and the extract dried under N_2, silylated by MSTFA and subjected to GC/MS analysis. This was performed on a Hewlett-Packard 5890 series II gas chromatograph with a 5971 series mass selective detector (column: HP-1, 60 m × 0.25 mm × 0.25 μm film thickness) using electron ionization (70 eV). The SA amount was determined using selected ion monitoring at $m/z = 267$.

Acknowledgements

We thank Dr. Margot Daub (North Carolina State University) for *Cercospora* cultures and advice as to their use, Jack Blount for assistance with HPLC, Gregory May and Lahoucine Achnine for critical reading of the manuscript, and Cuc Ly for assistance with artwork. This work was supported by the Samuel Roberts Noble Foundation.

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