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ORIGINAL ARTICLE

A comparison of individual and combined L-phenylalanine ammonia lyase and cationic peroxidase transgenes for engineering resistance in tobacco to necrotrophic pathogens

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Abstract This study tested the relative and combined efficacy of *ShPx2* and *ShPAL* transgenes by comparing *Nicotiana tabacum* hybrids with enhanced levels of L-phenylalanine ammonia lyase (PAL) activity and cationic peroxidase (Prx) activity with transgenic parental lines that overexpress either transgene. The PAL/Prx hybrids expressed both transgenes driven by the 35S CaMV promoter, and leaf PAL and Prx enzyme activities were similar to those of the relevant transgenic parent and seven- to tenfold higher than nontransgenic controls. Lignin levels in the PAL/Prx hybrids were higher than the PAL parent and nontransgenic controls, but not significantly higher than the Prx parent. All transgenic plants showed increased resistance to the necrotrophs *Phytophthora parasitica* pv. *nicotianae* and *Cercospora nicotianae* compared to nontransgenic controls, with a preponderance of smaller lesion categories produced in Prx-expressing lines. However, the PAL/Prx hybrids showed no significant increase in resistance to either pathogen relative to the Prx parental line. These data indicate that, in tobacco, the PAL and Prx transgenes do not act additively in disease resistance. Stacking with *Prx* did not prevent a visible growth inhibition from *PAL* overexpression. Practical use of *ShPAL* will likely require more sophisticated developmental control, and we conclude that *ShPx2* is a preferred candidate for development as a resistance transgene.

Keywords Plant defence · Genetic engineering · Genetic modification · Disease resistance · Lignin · Transgene stacking · Fungal pathogen · Phenylpropanoid pathway

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Introduction

The phenylpropanoid pathway has long been thought to play a critical role in disease resistance through the production of antimicrobial compounds and cell wall lignification (Dixon 2001). The enzyme L-phenylalanine ammonia lyase (PAL) (EC 4.3.1.5) catalyses the first committed step in the phenylpropanoid pathway (Vogt 2010) that leads to the synthesis of diverse phenolic compounds, including the phenolic alcohol monolignols which are the immediate precursors for lignin synthesis (Bonawitz and Chapple 2010). Peroxidases (Prx) (EC 1.11.1.7) are believed to catalyse the final step of crosslinking monolignols to synthesise the complex lignin polymer (Passardi et al. 2004b; Weng et al. 2008). There is a large body of correlative evidence supporting roles of the PAL and Prx enzymes in disease resistance, and direct evidence from several studies where the expression of these enzymes has been manipulated in transgenic plants, resulting in altered disease reactions. These studies not only demonstrated the importance of PAL and Prx genes in plant defence, but they also suggested that their manipulation may be useful for genetically engineering disease resistance.

Downregulation of PAL in tobacco plants achieved by sense suppression compromised resistance to both tobacco mosaic virus and the fungal necrotroph *Cercospora nicotianae* (Maher et al. 1994; Pallas et al. 1996).

Furthermore, tobacco plants that overexpressed heterologous PAL transgenes to confer elevated PAL enzyme activity showed enhanced resistance to *C. nicotianae*, the oomycete *Phytophthora parasitica* pv. *nicotianae* and TMV (Felton et al. 1999; Way et al. 2002; Shadle et al. 2003). A systematic metabolomic analysis has not been undertaken on plants overexpressing PAL. However, a significant effect on phenylpropanoid pathway metabolite levels has been reported, with enhanced levels of chlorogenic acid (Shadle et al. 2003) as well as 4-coumaric acid and its glycoside in uninoculated plants. Salicylic acid levels were also increased following fungal inoculation (Howles et al. 1996). Transgenic tobacco plants with reduced PAL levels also had reduced lignin levels (Bate et al. 1994; Sewalt et al. 1997). Based on comparisons of PAL-overexpressing plants and plants with compromised SA accumulation due to *Nah-G* expression, it has been proposed that the accumulation of phenylpropanoid intermediates such as chlorogenic acid is primarily responsible for the enhanced disease resistance of PAL-overexpressing plants; they are not effects of SA accumulation and defence signalling (Shadle et al. 2003).

Plants contain two classes of haem-containing Prx enzymes (Passardi et al. 2007). Class I Prx enzymes are located intracellularly and resemble bacterial peroxidases, with ascorbate peroxidase being a well-known example. They will not be discussed further here. Class III Prx enzymes are secreted, highly diverse, encoded by large gene families in most plants, and have been implicated in lignification and pathogen defence (Duroux and Welinder 2003; Passardi et al. 2004b). The expression of some *Prx* gene family members is developmentally regulated (Passardi et al. 2004a), and others are inducible by environmental stresses. Plant responses to pathogens usually involve the induction of a specific subset of Class III *Prx* genes (Harrison et al. 1995; Sasaki et al. 2004), but the multiplicity of Prx enzymes has complicated their functional analysis. The roles played by specific *Prx* genes in plant defence are still unclear. Potential defensive functions include the strengthening of the cell wall by increasing the amount and specificity of polymerisation of monolignols to lignin (Elfstrand et al. 2002), the production of potentially toxic oxidised phenolics (Kobayashi et al. 1994; Dowd and Lagrimini 1997) and altered levels of reactive oxygen species (ROS), which can signal defence reactions and trigger programmed cell death (Bindschedler et al. 2006; Schweizer 2008; Kazan et al. 1998a; Wally and Punja 2010).

Transgenic tobacco plants that overexpress heterologous class III *Prx* genes have shown enhanced resistance to fungal, oomycete and bacterial pathogens (Kazan et al. 1998b; Way et al. 2000; Elfstrand et al. 2002). Similar increases in resistance to diverse pathogens have been obtained using *Prx* overexpression in transgenic food crops, including canola, tomato, carrot and wheat (Kazan et al. 1998b; Sarowar et al. 2006; Schweizer 2008; Wally and Punja 2010). Overexpression of an anionic *Prx* from tobacco has also conferred resistance in tobacco against numerous insect pests under both laboratory and field conditions (Dowd and Lagrimini 2006). These studies clearly demonstrate a role for class III *Prx* genes in plant defence, and strongly indicate that there may be considerable biotechnological potential in the use of peroxidase transgenes in crop protection.

In general, the PAL and Prx transgenes discussed above have conferred partial resistance, and there is an interest in higher resistance levels for practical application. Transgenic technology for many crops has now progressed to the stage that stacking of transgenes is feasible, but this opportunity has barely been explored for pest or disease resistance. For example, a synergistic effect on insect resistance was reported when an anionic peroxidase transgene was combined with a transgene encoding a ribosome inactivating protein believed to act through an unrelated mechanism (Dowd et al. 2006).

Because PAL and Prx function at very different stages along the phenylpropanoid pathway, we were interested to establish whether they could confer additive or synergistic effects in disease resistance. This seems feasible (1) by increased flux through the pathway for lignin biosynthesis or (2) through interaction between resistance components involving phenylpropanoid metabolites and independent Prx functions. Previously, we described transgenic tobacco lines with disease resistance enhanced through the expression of heterologous PAL (*ShPAL*) or class III cationic Prx (*ShPx2*) genes (Way et al. 2000, 2002). In the work described here, we set out to determine the relative and combined efficacies of these genes to confer disease resistance. We generated hybrid tobacco plants that overexpressed both *ShPAL* and *ShPx2* and compared the resistances of these plants and their parental lines to necrotrophic oomycete and fungal pathogens. The results show that stacking these genes does not increase lignification or disease resistance over that conferred by the *ShPx2* gene alone. Because of a growth penalty from *ShPAL* overexpression, *Shpx2* is a stronger candidate for development as a resistance transgene, possibly in combination with others that affect pathogens through unrelated mechanisms.

Materials and methods

Plant material and plant growth conditions

The production of transgenic *Nicotiana tabacum* cv. Xanthi tobacco expressing either the *ShPx2* cationic

peroxidase cDNA (NCBI# L36112, Harrison et al. 1995) or the *ShPAL* cDNA (NCBI# L36822, Manners et al. 1995) each under the control of the CaMV 35S promoter has been previously described (Way et al. 2000, 2002). The transgenes were accessed from the laboratory of Dr. J. Manners at the Cooperative Research Centre for Tropical Plant Pathology. The transgenic plant lines with the highest PAL and Prx enzyme activities in these previous studies each contained single copy inserts, and were used as parents for the present study. The parental line expressing *ShPx2* is termed P1, and that expressing *ShPAL* is termed P2. Reciprocal hybridisations were undertaken, and hybrid lines are designated H1 and H2 according to the female parent. All plants were maintained in controlled environment rooms with a 16-h photoperiod, a light intensity of 500 µmol m^{-2} s^{-1}, and a temperature regime of 28°C during the day and 25°C during the night.

Production of *PAL*/*Prx* hybrids

Homozygous *ShPx2* (P1) and *ShPAL* (P2) transgenic tobacco lines (Way et al. 2000, 2002) were used as parents. Flowers were selected for emasculation and pollination at a stage where anthesis was expected within 24 h. An incision was made into the corolla and the anthers were removed, then mature pollen from a donor line was applied to the stigma of the emasculated recipient (Wernsman and Matzinger 1980). These cross-pollinated flowers were bagged until seeds were harvested. The resulting F1 hybrid plants were selfed. DNA isolated from leaf tissue (Dellaporta 1983) was analysed by PCR to identify F2 plants that contained both transgenes. Amplification of a 454 bp region of the *ShPAL* transgene used the forward ATCAAC ACACTCCTCCAAGGCTAC primer and the reverse AT GGTCAGTGAACTCTGGCTTCCC primer, while amplification of a 517 bp region of the *ShPx2* transgene used the forward GGCGTTGTTAACAGCGTA primer and the reverse GCCCACCAACAAGGGTA primer. The amplification conditions used were 1 cycle of 94°C for 7 min, 30 cycles of 94°C for 1 min, 52°C for 90 s, and 72°C for 2 min, with a final extension of 7 min at 72°C, and products were examined by electrophoresis on 2% agarose gels. Amplification under these conditions using template DNA of control nontransgenic tobacco plants did not reveal any amplification products. Homozygous hybrid lines containing both transgenes were used for further experiments.

Enzyme and lignin analyses

The third leaf from the apex was sampled from plants with eight leaves for PAL and Prx enzyme activities, as described previously (Way et al. 2000, 2002). Lignin was assessed in stem samples by first obtaining an acid digestible fibre (ADF) insoluble fraction by the method of Van Soest (1963) using an Ankom fibre analyzer (http://www.ankom.com/). To extract acid detergent lignin (ADL), the ADF insoluble residue was shaken in 72% sulfuric acid for 6 h at room temperature, washed in water, dried overnight, weighed, then ashed by incineration and reweighed to correct for ash content, giving a final ADL value. Measurements of ADL are known to strongly correlate with other analytical estimates of lignin such as Klason lignin determinations (Jung et al. 1997).

Northern blot analysis

Total RNA was extracted as described by Higgins et al. (1985). Replicate samples of 5 µg RNA each were dotted directly onto nylon membrane using a slot blot apparatus (He et al. 1996). Sequential hybridisations, using conditions described by Stephenson et al. (1997), were conducted using PCR-amplified *ShPAL*, *Shpx2* cDNA inserts as probes.

Pathogenicity tests and statistical analysis

The strains of *Phytophthora parasitica* pv. *nicotianae* and *Cercospora nicotianae*, inoculation conditions and assessments employed were the same as those described previously (Way et al. 2000, 2002). Disease assessments were specifically designed to measure effects on necrotic lesion development. For *P. nicotianae* pv. *nicotanae*, stem lesion length (in cm) was measured, while for the detached leaf assays, total lesion area per leaf in cm^2 was determined. For *C. nicotanae*, the lesion area was measured and expressed as a % of total leaf area. Planting times were staggered to allow plants to be tested at the same developmental stage in order to accommodate the slower early growth of *ShPAL* lines, as described by Way et al. (2002). For each assay, 20 replicate plants were used for each genotype. For all inoculation experiments, plants were arranged in a random design and statistical analysis was conducted as described by Way et al. (2002). Briefly, data were subjected to the Anderson–Darling test for normality and Levine's test for variance homogeneity. Where normal distributions were observed, data were analysed by a one-way analysis of variance. In some instances, data were grouped into appropriately sized lesion size categories to conduct statistical analysis and analysed using either Pearson's chi-square test or a maximum likelihood chi-squared statistical test with Monte Carlo randomisation.

Results and discussion

Hybrids with elevated PAL and Prx enzyme activities

Transgenic tobacco lines expressing *ShPx2* (P1) and *ShPAL* (P2) were used in reciprocal crosses to obtain homozygous hybrid lines designated H1 and H2, respectively, according to their female parent. Northern slot blot analysis indicated that the H1 and H2 hybrid homozygous lines expressed both of the *ShPx2* and *ShPAL* transgenes (Fig. 1). As expected, homologues of the heterologous *ShPAL* and *ShPx2* mRNAs were not detected in RNA from nontransgenic control tobacco plants under the high-stringency conditions used for this northern hybridisation analysis (Fig. 1).

The leaf PAL and Prx enzyme activities of the hybrids were then assayed and compared to those of the respective transgene donor parents and nontransgenic controls. Both hybrids had Prx activities equivalent to the P1 parent and approximately ten times that in a nontransgenic control or in P2 (Fig. 2). PAL activities in both hybrids were equivalent to the P2 parent and 7–10 times that in a nontransgenic control or in P1 (Fig. 2). These results at RNA and enzyme activity levels show that there was no interference between the *ShPx2* and *ShPAL* transgenes or enzymes during expression.

Overexpression of the *ShPAL* transgene causes a reduction in growth rate, particularly at early growth stages, as described by Way et al. (2002). A similar retardation of plant growth through the overexpression of a PAL transgene has recently been reported by Bauer et al. (2011). This phenotype of slower development was also observed in both the H1 and H2 hybrid lines, with no additional visible adverse effects from the overexpression of both *ShPx2* and *ShPAL*. Interestingly, enhanced Prx activity did not alleviate the growth retardation incurred by the expression of *ShPAL* in tobacco. This indicates that the growth penalty from increased PAL activity cannot be simply corrected by combining with Prx to increase the demand for metabolites from PAL. Therefore, if the *ShPAL* transgene is to be used in engineering resistance, it may have to be expressed in a tissue-specific or infection-regulated manner. Transgenic tobacco plants overexpressing a French bean PAL enzyme were not reported to grow more slowly than controls (Shadle et al. 2003), but the PAL enzyme levels (~fivefold those of the controls) were lower than those obtained here using *ShPAL*.

Effect of elevated PAL and Prx on total lignin content

Both the PAL and Prx enzymes have been implicated in lignin biosynthesis, and it is possible that the combined overexpression of both of these enzymes may lead to enhanced lignin content. To test this, the lignin content of the transgenic parents and the H1 hybrid were analysed using the acid detergent lignin (ADL) procedure, which is known to correlate with other estimates of lignin content such as Klason lignin (Jung et al. 1997). The mean ADL levels (as % of dry matter) were 4.0 ± 0.7 (SE) for the nontransgenic control, 5.9 ± 0.6 for H1, 5.3 ± 0.3 for P1 and 4.1 ± 0.5 for P2. The mean ADL values for the stems of P1 and P2 were not significantly different to that of the

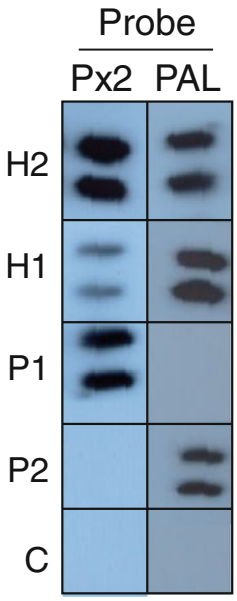

Fig. 1 Expression of *ShPx2* and *ShPAL* mRNAs in transgenic tobacco hybrids. Total RNA from leaves of the P1- and P2-transgenic parents expressing *ShPx2* and *ShPAL*, respectively, two homozygous hybrid lines (*H1* and *H2*), and a nontransgenic control (*C*), was examined in duplicate in slot blots hybridised to labelled probes from the *ShPx2* (*Px2*) and *ShPAL* (*PAL*) cDNAs

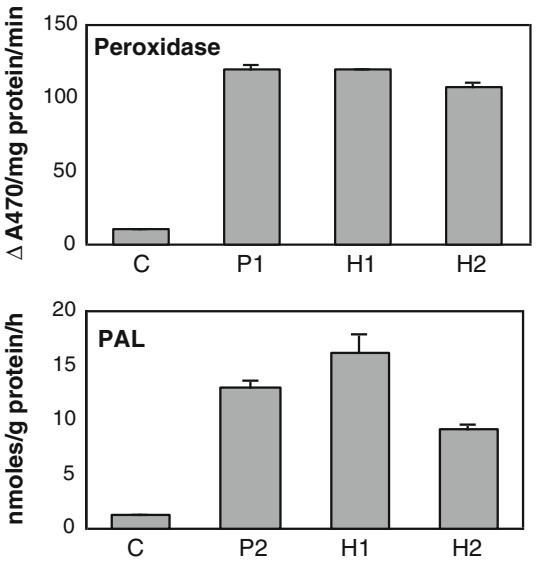

Fig. 2 Transgenic tobacco hybrids expressing *ShPx2* and *ShPAL* transgenes have elevated Prx and PAL enzyme activities. Leaf extracts from P1- and P2-transgenic parents expressing *ShPx2* and *ShPAL*, respectively, two hybrid lines (*H1* and *H2*), and a nontransgenic control (*C*) were assayed for enzyme activity. Means and standards errors from four replicates are shown

nontransgenic control (LSD > 0.05). These data demonstrate that overexpression of PAL does not have a discernible effect on lignin content, and this is consistent with the observations of others (Howles et al. 1996). The mean ADL for H1 was, however, 44% higher and significantly different to the control and P2 (LSD < 0.05), though not significantly different to the P1 parent. Therefore, combining PAL with Prx overexpression in H1 did significantly increase lignin content over nontransgenic controls and the P2 PAL overexpressing plants. However, the lack of a significant difference between the P1 and H1 lines indicates that it is the *ShPx2* gene that is the main contributor to the increased ADL content of H1. These comparisons represent a modulation of basal stem lignin levels, and as yet there have been no reports of lignin changes in plants overexpressing PAL or Prx transgenes following inoculation, and such experiments would be valuable in future studies aimed at understanding the mode of action of these transgenes in disease resistance.

Effect of individual and combined *ShPx2* and *ShPAL* transgenes on resistance to necrotrophic pathogens

Lesion development on inoculated leaves and stems was used to test the resistance of the transgenic lines and hybrids to necrosis caused by the oomycete pathogen *P. parasitica* pv. *nicotianae*. The stem lesion test has been shown to produce results that correlate with field responses (Robin and Guest 1994), and an image of the symptoms from this assay is shown in Way et al. (2000). No lesions developed on control stems and leaves that were mock inoculated with sterile agar plugs.

For the stem assay, an agar plug containing mycelium was applied to the cut surface of a freshly decapitated but otherwise intact pot-grown plant, and the spread of necrosis down the stem was measured over time for the 20 replicate plants used for each genotype (Robin and Guest 1994). Results are shown in Fig. 3 and Table 1. An ANOVA test revealed that the average stem lesion length for the transgenic parental and hybrid lines differed significantly for 14, 21 and 28 days post-inoculation when compared to the nontransgenic control plants ($P < 0.002$ for all time points). Mean lesion length after 1 week was reduced by approximately 66% in the P1 parent and the hybrids, and 50% in the P2 parent line, compared to controls. There was no significant difference in mean lesion length between the transgenic parental and hybrid lines (Fig. 3). These data are consistent with the observations previously reported by Way et al. (2000, 2002) on the parental lines.

To examine the distribution of *Phytophthora*-incited stem lesion length across control, parental and hybrid genotypes, the stem lesion lengths for the 20 replicates for each genotype were divided into eight size classes, and these are shown in

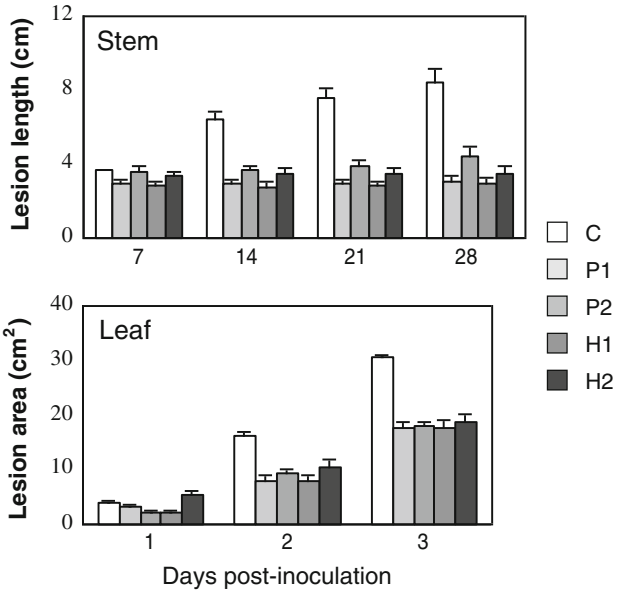

Fig. 3 Disease progression following inoculation of stems and leaves of P1 and P2 transgenic parents expressing *ShPx2* and *ShPAL*, respectively, their hybrids (*H1* and *H2*), and a nontransgenic control (*C*) with *P. parasitica* pv. *nicotianae*. Means and SE were derived from 20 replicate stems and leaves for each genotype

Table 1 Lesion length categories of the P1- and P2-transgenic parents expressing *ShPx2* and *ShPAL*, respectively, their hybrids (H1 and H2), and a nontransgenic control (cv. Xanthi)

Genotype	Lesion length		
	<4 cm	4–7 cm	>7 cm
Control	0	0	100
P1	100	0	0
P2	35	65	0
H1	100	0	0
H2	100	0	0

Lesions were measured 28 days after inoculation of decapitated stems with *P. parasitica* pv. *nicotianae*. Twenty replicate plants were used for each genotype, and values are % of plants in each category

Fig. 4 as an area plot. This clearly shows (1) that stem lesions in controls cover a distinctly larger lesion size distribution (7–10 cm) than all transgenic lines, (2) that the lesions on the P2 parent are mainly distributed across a range of longer lesion lengths (4–7 cm) than the P2 and hybrid lines, and (3) that there is little difference between the lesion distributions (2–5 cm) for P1 and hybrid lines. To enable a statistical analysis, lesion length was categorised into three size classes, as shown in Table 1, where significant differences in distribution ($P < 0.002$, Monte Carlo analysis) were observed between groups. For example, 100% of the lesions on the control were in the largest size class on day 28, and significantly different from the P1 parent and the hybrids, where 100% of the lesions were in the smallest size class. The

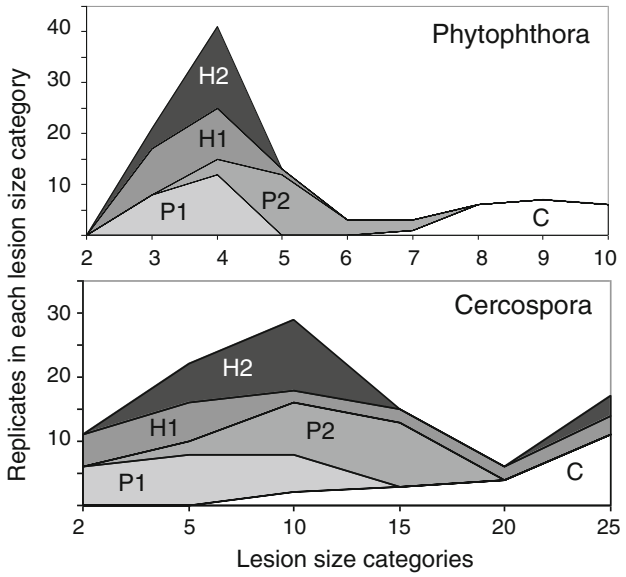

Fig. 4 Area plots of the distribution of lesions across control, transgenic parent and hybrid genotypes in stem inoculation assays with *P. nicotianae* pv. *nicotianae* and *C. nicotianae*. Twenty replicate measurements of stem lesion lengths (cm) for *Phytophthora* and lesion area (as % of total leaf area) for *Cercospora* were divided into eight and five size categories, respectively, and plotted against the number of replicates in each category for each genotype. Genotypes (*C*, *P1*, *P2*, *H1*, *H2*) are as in Fig. 3

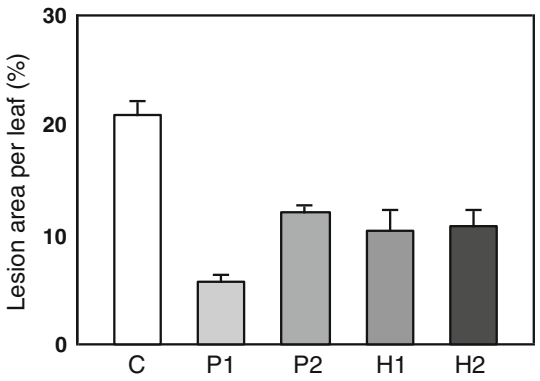

Fig. 5 Disease development on leaves of P1 and P2 transgenic parents expressing *ShPx2* and *ShPAL*, respectively, their hybrids (*H1* and *H2*), and a nontransgenic control (*C*) at 12 days after inoculation with *C. nicotianae*. Mean areas of infection as a % of total leaf area with SE from 20 plants per genotype are shown

P2 parent overexpressing PAL had a significant preponderance (65%) of lesions in the intermediate lesion class, distinguishing it from controls and P1 and hybrid lines (Table 1). Taken together, these analyses indicate that expression of the *ShPx2* transgene confers a higher level of resistance than the *ShPAL* transgene, with no evident additive effect observed against *Phytophthora* when these transgenes were combined in the hybrids.

The second assay involved placing a mycelial plug of *P. nicotianae* pv. *nicotianae* in the centre of each of 20 detached leaf replicates per genotype (Way et al. 2000) and measuring the lesion area in cm^2 using the ASSESS image analysis system (Tucker and Chakraborty 1997). Results are shown in Fig. 3. ANOVA tests showed that two days after inoculation there was a significant difference between H1 progeny and the nontransgenic control ($P < 0.02$). The following day, there was a significant difference between all the parental and hybrid lines compared to the nontransgenic control ($P < 0.04$). By day 4, leaves of the transgenic parental and hybrid lines were still intact, with lesion sizes being approximately 18 cm^2. In contrast, the lesion size on control leaves averaged 30 cm^2, and the leaves had collapsed into a soft, watery mass. There was no significant difference between the transgenic parental and hybrid lines, and there was insufficient variation in lesion size across the transgenic lines in this experiment to warrant classifying the lesions into size classes.

Leaves on intact plants were inoculated by spraying conidia of the necrotrophic ascomycete pathogen *C. cercospora* (Way et al. 2000). Twenty replicate plants were used for each genotype. An image of typical disease symptoms from this assay is shown in Shadle et al. (2003). A one-way ANOVA analysis indicated that disease progression, measured as the necrotic lesion area per leaf, was significantly ($P < 0.01$) reduced in all of the transgenic parent and hybrid lines compared to the nontransgenic control (Fig. 5). The lesion area in P1 was also significantly ($P < 0.01$) smaller than that of each of the other transgenic lines.

To examine the distribution of *Cercospora*-incited lesions across control, parental and hybrid genotypes, the lesion area as a % of leaf area for each of 20 replicate plants per genotype were divided into five size classes, and the lesion distribution patterns for each genotype are shown in Fig. 4 as an area plot. These data show that (1) lesion areas for controls are predominantly larger (15–25%) than for transgenic lines (2–15%), (2) the P2 parent predominated in intermediate lesions (10–20%) compared to P1 and hybrids (2–10%), and (3) the P1 parent predominated in the smaller lesion coverage category (2–5%), but this strongly overlapped with broader lesion areas for the hybrids. For statistical analysis, the percentage of the leaf area affected by necrosis was grouped into three size categories (Table 2). The majority of the lesions on P1 and hybrid lines fell into the smaller size categories, whereas the majority of the lesions on control plants were in the largest size category (Table 2). Monte Carlo analysis determined that the distribution over the three categories was significantly different for all of the lines ($\chi^2 = 74.22$, $df = 8$, $P < 0.00001$). Each of the transgenic lines was significantly different from the control ($P < 0.0001$). Among the transgenic plants, the P1 plant showed the

Table 2 Disease severity categories in leaves of the P1 and P2 transgenic parents expressing *ShPx2* and *ShPAL*, respectively, their hybrids (H1 and H2), and a nontransgenic control (cv. Xanthi)

Genotype	Lesion area as % of leaf area		
	<5%	10–15%	20–25%
Control	0	25	75
P1	70	30	0
P2	10	90	0
H1	55	20	25
H2	30	55	15

Lesion area as a % of total leaf area was measured on 20 detached replicate leaves per genotype at 12 days after inoculation with *Cercospora nicotianae*

Values are % of leaves in each category

lowest area of infection, and the P2 parent the greatest disease progression, with the hybrids showing an intermediate disease distribution.

Results from both the stem assay using *Phytophthora* and the leaf lesion assay using *Cercospora* indicate that expression of the *ShPx2* transgene confers a higher level of resistance than the *ShPAL* transgene, with no evident additive effect in hybrids. Indeed, there may have even been a slight reduction in resistance to *Cercospora* due to stacking relative to the *ShPx2* transgene alone.

Conclusions

In summary, the direct comparison of transgenic tobacco lines expressing individual and stacked *ShPx2* and the *ShPAL* transgenes (1) confirms previous reports that these transgenes confer enhanced resistance to two necrotrophic pathogens, (2) indicates that the *ShPx2* transgene is more promising for practical application due to a higher level of conferred resistance and an absence of visible adverse effects on growth, (3) indicates that stacking these resistance transgenes is unable to circumvent a growth penalty from *ShPAL* overexpression, and (4) shows no additive effect of stacking on either disease resistance levels or lignin biosynthesis. Given that the introduction of the *ShPAL* transgene led to reduced growth, future emphasis in resistance engineering should focus on the *Shpx2* transgene.

The reason for the lack of an additive effect on disease resistance when combining the *Shpx2* and *ShPAL* transgenes is not known, but it suggests that their modes of action do not operate independently and are either shared or antagonistic. In regard to the latter, one can speculate that enhanced Prx activity from the *Shpx2* transgene may neutralise effects on disease resistance from increased PAL by reducing the soluble pools of potentially antifungal phenylpropanoid metabolites. Future emphasis should be placed on understanding the mode of action of the *Shpx2* transgene for increased resistance. Once this us known, the *ShPx2* transgene could be rationally combined with other antifungal transgenes that have alternative modes of action.

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