Over-expression of phenol-oxidising peroxidases alters the UV-susceptibility of transgenic *Nicotiana tabacum*

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Received: *17 February 2004*
Accepted: *22 April 2004*

Summary

- Class III peroxidases catalyse the oxidative crosslinking of UV-absorbing phenolics. The effect of changes in the activity of phenol oxidising peroxidases (EC 1.11.1.7) on UV-tolerance in *Nicotiana tabacum* plants has been determined.
- The UV-sensitivity of transgenic *N. tabacum* lines, altered in their peroxidase expression pattern, was studied by measuring radiation effects on photosynthetic efficiency.
- Analysis of the effect of UV-radiation on the relative variable chlorophyll fluorescence showed that the SPI-2 line, which over-expresses a defence-related cationic peroxidase, is markedly UV-tolerant. By contrast, the ROPN3-line, which overexpresses a synthetic horseradish peroxidase-C gene, was found to be UV-sensitive. The increased activity of indole-3-acetic acid (IAA) inducible peroxidases in homozygous IAA-overproducing transgenic plants was also found to correlate with UV-sensitivity.
- It is concluded that only specific peroxidase isozymes, through their effects on phenolic metabolism, contribute to the UV protection response. Thus, the analysis of the role of isozymes in UV-protection addresses fundamental questions of isozyme diversity and/or redundancy in relation to phenolic substrates.

Key words: UV-radiation, auxin, stress, peroxidase, chlorophyll a fluorescence, *Nicotiana tabacum* (tobacco).

New Phytologist (2004) **163**: 585–594

© *New Phytologist* (2004) **doi**: 10.1111/j.1469-8137.2004.01132.x

Introduction

Ultraviolet-B (UV-B; 280–315 nm) radiation is a ubiquitous component of solar radiation. Although the UV-wavelengths constitute only a minor component of sunlight, they are biologically very effective in driving a range of photo-modification and photo-sensitisation reactions (Greenberg *et al.*, 1997). In plants, photo-excitation of nucleotides, amino acids, lipids, and pigments (Jordan, 1996) affects gene expression, growth, development, photosynthesis, flowering, pollination and transpiration (Jansen *et al.*, 1998; Mackerness & Jordan, 1999; Frohnmeyer & Staiger, 2003). However, many of these detrimental UV-B effects are alleviated by naturally occurring UV-protection mechanisms (Jansen *et al.*, 1998; Frohnmeyer & Staiger, 2003). The effectiveness of these mechanisms is shown by the difficulty in detecting adverse UV effects on the photosynthetic productivity of plants grown under environmentally relevant levels of UV-B (Allen *et al.*, 1998). However, not all plants are equally effective in alleviating the effects of UV-radiation (Hofmann *et al.*, 2001); levels of UV-tolerance vary between genera, species, ecotypes and cultivars (Jansen *et al.*, 1999).

UV-protection responses include increased oxygen radical scavenging activity (Rao *et al.*, 1996), the light-dependent restoration of UV-damaged DNA by photolyases (Britt, 1999) and the accumulation of UV-screening pigments (Jin *et al.*, 2000; Mazza *et al.*, 2000). Flavonoids play a major role in UV-screening. However, work on flavonoid deficient mutants has established that other phenolics (e.g. sinapate esters) also contribute significantly to UV protection (Landry *et al.*, 1995; Booij-James *et al.*, 2000). Indeed, a wide range of phenolics,

including flavonoids, cinnamate esters, lignin and tannin accumulates during UV acclimation (Rozema *et al.*, 1997).

We have hypothesised that phenol oxidising class III peroxidases contribute to the alleviation of UV-radiation stress (Jansen *et al.*, 2001). Peroxidases (EC 1.11.1.7) are monomeric hemeproteins that catalyse the oxidation of a range of substrates by hydrogen peroxide. These enzymes are implicated in many physiological processes that include phenol-oxidation and the crosslinking of phenolic compounds to proteins and polysaccharides, deposition of polyphenols and lignin, suberisation, pathogen resistance and the oxidative degradation of indole-3-acetic acid (Hiraga *et al.*, 2001). We have proposed that peroxidase catalysed changes in phenolic content, composition and/or distribution are a determinant of the level of UV-tolerance (Jansen *et al.*, 2001). Increased UV-tolerance correlates with increased peroxidase activity and accumulation of lignin-like polymers in the cell walls of natural duckweed ecotypes and transgenic *Nicotiana sylvestris* with altered peroxidase expression profiles (Jansen *et al.*, 2001). Lignin accumulation was also observed in field studies where plants were exposed to supplemental UV-B (Rozema *et al.*, 1997). Ecologically and agronomically, UV-induced phenolic polymerisation is important since it has been shown to directly effect the decomposition of plant litter in the soil (Rozema *et al.*, 1997).

Peroxidase mediated UV-protection is also linked to changes in IAA (Indole-3-acetic acid) metabolism in UV-tolerant duckweed ecotypes and in transgenic *Nicotiana* over-expressing an anionic peroxidase (Jansen *et al.*, 2001). It has been proposed that the decrease in IAA levels in transgenic *Nicotiana* is associated with the increase in peroxidase activity (i.e. increased oxidative decarboxylation of IAA). In turn, lowered IAA levels affect leaf and plant architecture (Lagrimini *et al.*, 1997a, 1997b). Morphological changes resembling those caused by changes in phytohormone metabolism have been observed in many UV-acclimation studies (Barnes *et al.*, 1996; Jansen, 2002).

Plants contain multiple peroxidase isozymes. For example, in *Arabidopsis thaliana* > 73 genes, encoding putative peroxidases, have been identified (Tognolli *et al.*, 2002; Welinder *et al.*, 2002). A challenging question concerns the functional role of this (and other) large gene families in plant biology. At present, it is not clear whether the correlation between increased peroxidase activity and UV-protection is isozyme specific. In this study we studied transgenic *Nicotiana tabacum* lines, in which peroxidase expression patterns were altered. We investigated alterations in peroxidase expression levels that were either a direct (SPI-2 and ROPN3 lines) or an indirect effect (C and X-lines) of the transformation event. Physiological responses of *N. tabacum* plants over-expressing class III peroxidases (SPI-2 and ROPN3 lines), and of plants over-producing IAA (C and X-lines) or an IAA-lysine synthetase (IAAL-lines) were studied with respect to their sensitivity to various abiotic stresses. Data presented in this paper demonstrate a clear, but complex, link between peroxidase activity and UV-tolerance.

Materials and Methods

Biological system

The generation of homozygous *Nicotiana tabacum* (L.) cv. 'samsun NN' transgenics overexpressing a synthetic horseradish peroxidase C (HRP-C) gene containing the natural N-terminal extension, but not the C-terminal extension, and under control of the CaMV 35S promotor, has been detailed previously (ROPN3-line) (Pellegrineschi *et al.*, 1995). High levels of transcription of the horseradish gene, as determined by Northern and rt-PCR analysis, were demonstrated, and the large (up to 10-fold) increase in total peroxidase activity correlated with an abundance of horseradish protein as shown by immunoblot analysis (Kis *et al.*, 2004). Histochemical analysis shows that the majority of both recombinant enzyme and elevated peroxidase activity was in the cytoplasm, with a smaller activity increase in the apoplast (Kis *et al.*, 2004). Transgenic seedlings were selected using kanamycin, and plants were routinely checked for increased levels of peroxidase activity.

The generation of homozygous *N. tabacum* cv. 'Wisconsin 38' transgenics overexpressing a Norway spruce defence related cationic peroxidase (*spi 2*), under control of the CaMV 35S promotor, has been detailed (Elfstrand *et al.*, 2002). The *spi 2* transcript was identified in the transgenic lines (SPI 2–6 and SPI 2–15), and total peroxidase activity was found to be several fold increased. Seedlings of the transgenic plants were selected for kanamycin resistance.

The generation of *N. tabacum* cv. 'Wisconsin 38' and 'Petit Havana SR1' IAA-overproducing transgenics overexpressing the *Agrobacterium tumefaciens* T-DNA *iaaM* and *iaaH* genes (C- and X-lines) has been detailed (Sitbon *et al.*, 1992; Eklöf *et al.*, 2000). In the 'SR1' background the *iaaM* and *iaaH* genes were under the control of the CaMV 35S and the endogenous iaaH promotor, respectively (35S-*iaaM*/*iaaH* C-line; Sitbon *et al.*, 1992), whereas in the cv. 'W38' line 35S-*iaaM*x35S-*iaaH* both genes were expressed from the CaMV 35S promotor and brought together by crossing (X-line). Homozygous C-, and X-line transformants contained 3–5-fold more free IAA in leaves and internodes and were characterised by phenotypic aberrations, that is, stunted growth, adventitious root formation, and decreased axillary bud outgrowth (Sitbon *et al.*, 1992; Eklöf *et al.*, 2000). Heterozygous X-line transformants (cross between *iaaM* and *iaaH* expressing lines) were characterised by more moderate phenotypic aberrations. Both heterozygotes and homozygotes were selected by screening for their distinct, stunted phenotype. (Sitbon *et al.*, 1999).

In addition, we studied *N. tabacum* 'W 38' transgenics overexpressing a bacterial IAA-lysine synthetase gene (*IAAL*) under the control of the CaMV 35S. Transgenic plants (IAAL-line) were selected by screening for their distinct phenotype (wrinkled leaves, reduced apical dominance, reduced root growth). This phenotype was very close to that of the similarly transformed tobacco plants described by Romano *et al.* (1991),

in which free IAA levels were found to be up to 19-fold decreased. Moreover, the phenotype of our transgenic plants (IAAL-line) was associated with a significantly decreased expression of tobacco auxin-regulated *IAA/Aux* genes (F. Sitbon, unpublished), indicative of a decrease of free IAA levels.

All *N. tabacum* plants were grown from seed under a long day regime (daylength > 14 h), in glasshouses. The minimal light intensity was 120 μmol m^{-2} s^{-1} (High Pressure Sodium bulbs). Intensities increased up to 500 μmol m^{-2} s^{-1} on sunny days. Such growth in the absence of UV-B radiation resulted in UV-sensitive plants that were ideally suited for our studies. Plants were grown in 2.5 l pots that were watered daily. Young, but fully expanded leaves from bolting plants were used for experiments.

Transgenic plants were routinely identified by measuring the relatively high level of peroxidase activity in leaf tissue (ROPN3 and SPI 2 lines). Tissue was homogenised using phosphate-buffered saline (PBS) buffer containing polyvinyl-polypyrrolidone (PVPP), centrifuged (10 000 ***g*** for 3 min) and the peroxidase activity of the supernatant was determined by following the oxidation of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) at 415 nm in potassium acetate buffer (pH 4.5) and in the presence of 2 mM H_2O_2. ABTS is a general peroxidase substrate and its oxidation rate (pH optimum between 4.5 and 6.0) will give an estimate of total peroxidase activity. The use of such a general substrate is justified because we do not know the substrate preference of isozymes relevant for UV-protection. Protein content was measured using Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA).

Experimental treatments

UV treatments consisted of exposure of tobacco leaf discs to UV radiation, generated by Philips TL12 fluorescent tubes (λ_{max} 315 nm) (Philips, Eindhoven, The Netherlands). The output of the bulb was filtered through either a UV-blocking 233-filter [50% Transmission (= T) at 378 nm; 10% T at 367 nm; 5% T at 364 nm] or a UV-transmitting 2458 filter covered with a single layer of cellulose acetate (50% T at 299 nm; 10% T at 293 nm, 5% T at 292 nm) (Wientjes, Nieuw Vennep, The Netherlands). Under our experimental conditions, *N. tabacum* cv.'s 'Samsun' and 'Petit Havana SR1' are slightly more UV sensitive than *N. tabacum* cv. 'W38'. Therefore, exposure times and irradiance conditions were adjusted accordingly. Leaf discs were exposed to either 6 h of 4.4 W m^{-2} (cv.'s Samsun and Petit Havana SR1) or 14 h of 3.3 W m^{-2} (cv. W-38), which is equivalent to 22 and 38.5 kJ m^{-2} biologically active radiation, respectively (Jansen & van den Noort, 2000). The irradiance level represents radiation in the spectral range between 295 nm and 345 nm, which was measured under the 2458 filter. Discs were floated on distilled water, with their adaxial side facing the UV-sources. The decrease in photosynthetic activity was attributed to the UV-B wavelengths since the low level of UV-A radiation is ineffective in decreasing PSII activity (Jansen *et al.*, 1999). No additional PAR (Photosynthetically active radiation) was applied during any of the UV-treatments. UV-levels were measured using an optometer (United Detector Technology Inc., Hawthorn, CA, USA) equipped with a probe specific for UV-wavelengths and/or a PMA2200 UV-meter (Solar Light Co., Philadelphia, PA, USA).

Photo-inhibitory treatment consisted of a 1 h exposure of tobacco leaf discs to a fluence rate of 1400 μmol m^{-2} s^{-1} PAR. Radiation was generated by a projector containing a 1000 W tungsten-halogen bulb (Hanimax, Tokyo, Japan), the output of which was filtered through 1 cm of saturated $CuSO_4$ and a KG-4 heat absorbing filter. Leaf material was dark adapted for at least 40 min before the measurement of photoinhibitory damage. Discs were floated on distilled water, with their adaxial side facing the radiation source.

Heat shock treatment consisted of floating tobacco leaf discs on preheated water (5 min 46°C for cv.'s Samsun and Petit Havana SR1; 9 min 46°C for cv. W-38). Plant material was kept in the dark during the treatment. Damage was assessed after 30 min of recovery in the dark.

Chilling treatment consisted of a 16 h exposure of tobacco leaf disks to temperatures of −1°C (cv.'s Samsun and Petit Havana SR1) or −4°C (cv. W-38). The cold treatment was followed by a slow thawing process (4 h) and damage was measured at room temperature. During the cold treatment, plant material was kept in the dark on half-strength PBS.

Assays

The photosynthetic efficiency of PSII was determined by the saturating pulse fluorescence technique, using a plant efficiency analyser (Hansatech, King's Lynn, UK). The minimal fluorescence (F_o), maximal fluorescence (F_m), and the variable fluorescence ($F_v = F_m - F_o$) were all measured according to van Kooten & Snel (1990). The photochemical yield of open PSII reaction centres, commonly known as the relative variable fluorescence, was calculated as F_v/F_m. It reflects the maximal efficiency of PSII which was measured in dark adapted tissue.

The analysis of soluble, UV-absorbing pigments was based on extractions with acidic methanol. Leaf discs of equal size were incubated in a mixture of methanol, water and concentrated 37.5% HCl (70%: 29%: 1%) for at least 3 d at 4°C, in the dark. Absorbance of the extracts was measured spectrophotometrically.

Results

Differences in UV sensitivity among *N. tabacum* transgenics with altered peroxidase activities

N. tabacum transgenics were raised under glasshouse conditions in the absence of UV-B radiation. The UV-sensitivity of these plants was determined by exposing leaf discs to UV radiation and measuring deleterious effects on the photosynthetic

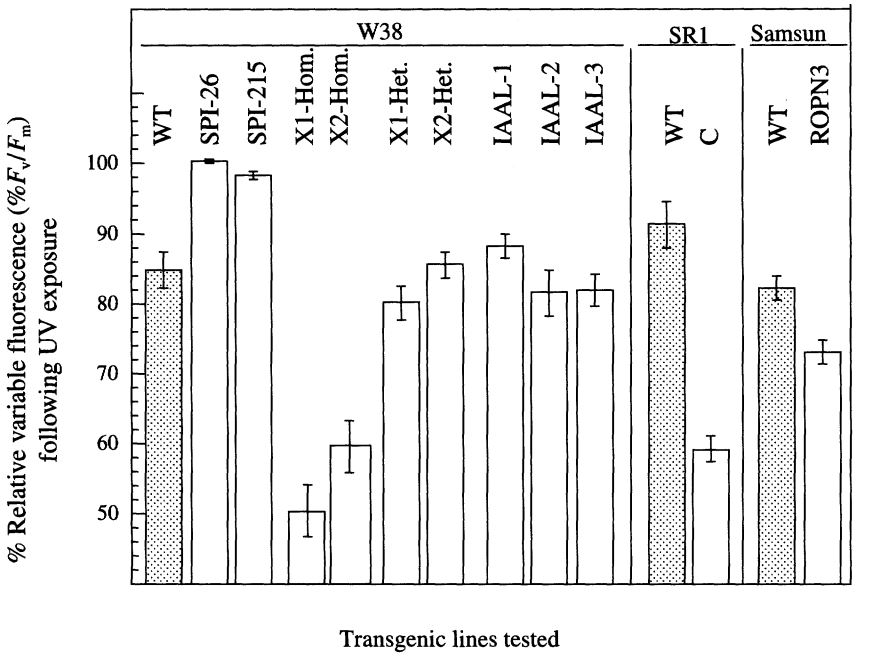

Fig. 1 Effects of UV-radiation on the relative variable fluorescence of *Nicotiana tabacum* transgenics with altered peroxidase expression patterns. The minimal fluorescence F_o and the maximal fluorescence F_m were measured on dark-adapted samples. The variable fluorescence was normalised to that of the non UV treated control (Table 1 gives the 100% values). Data represent average values of 30 (SPI 2–6 and SPI 2–15), 40 ('W38' homozygous X-line (X-Hom.)), 50 ('W38' heterozygous X-line (X-Het.)), 50 (IAAL), 38 ('Petit Havana SR1' C-line) and 35 (ROPN3) measurements. Standard errors of the mean are given. Statistical analysis (Student's *t*-test) showed that, following UV exposure, the SPI-2 6 and SPI-2 15 lines had a higher efficiency of PSII compared with their control ($P < 0.01$). Following UV-exposure, both the ROPN3-line and the homozygous C and X-lines-lines had a lower efficiency of PSII compared with their respective controls ($P < 0.01$).

efficiency of photosystem II (F_v/F_m). Prior to UV-treatment, the values for F_v/F_m were very similar for all the *N. tabacum* cultivars and the derived transgenic lines. However after UV-exposure (F_v/F_m) values differed significantly (Fig. 1).

A 14-h exposure of *N. tabacum* cv. 'W38' control leaf discs to 3.3 W m^{-2} UV-B resulted in a 15% decrease in F_v/F_m (Fig. 1). In comparison, leaf discs of the corresponding SPI 2 lines, overexpressing a cationic peroxidase, were not significantly affected by UV-radiation. The average decrease of the variable chlorophyll fluorescence was 0 and 2% for SPI 2–6 and SPI 2–15, respectively. At present, it is not clear whether these two UV-tolerant lines can be distinguished in terms of UV susceptibility under extreme radiation conditions.

A 6-h exposure of *N. tabacum* cv. 'samsun NN' leaf discs to 4.4 W m^{-2} UV-B resulted in a 18% decrease in F_v/F_m (Fig. 1). We found that the ROPN3 line, which over-expresses a synthetic horseradish peroxidase-C gene, was relatively UV-sensitive. The decrease in F_v/F_m, following UV-exposure of the ROPN3 line, was 27% (Fig. 1).

Homozygous, IAA-overproducing *N. tabacum* 'W38' 35S-*iaaM*x35S-*iaaH* X-line plants were found to be extremely UV-sensitive compared with their corresponding control. A 14-h exposure to 3.3 W m^{-2} UV-B resulted in a complete disruption of PSII activity in the X-line. Under these radiation conditions the relative variable fluorescence decreased by > 50% in the X-line leaves (i.e. out of the linear response range of the variable fluorescence), but just 15% in wild type leaves (Fig. 1). Similarly, the variable chlorophyll fluorescence of the 'Petit Havana SR1' 35S-*iaaM*/*iaaH* C-line decreased by > 40%, compared with 8% in corresponding wild type leaves, following UV-exposure (Fig. 1). Thus, the homozygous C and X lines are equivalent in terms of UV-sensitivity, despite a difference in their genetic background. In comparison, hetero-zygous *N. tabacum* 'W38' X-line plants were considerably less sensitive to UV radiation (Fig. 1). Their UV sensitivity was equal to that of the control line. Similarly, the *N. tabacum* 'W38' IAAL lines, which exhibit an IAA-underproducing phenotype, were just as UV-sensitive as the corresponding control lines (Fig. 1).

Similar effects of heat, chilling and excess visible radiation on *Nicotiana* transgenic plants

Alterations in the level of UV-tolerance could be the result of the up- or down-regulation of general stress tolerance pathway(s), induced in response to the nonphysiological level of peroxidase activity in the transgenic plants. An increase in the activity of scavengers of active oxygen species commonly results in cotolerance to a range of abiotic stresses (Gressel & Galun, 1994). Therefore, it was important to investigate whether observed changes in stress tolerance are specific for UV-radiation stress (Table 1). We exposed tobacco leaf discs to excessive intensities of visible light (photoinhibition), heat and cold and measured the effects on the photochemical efficiency of PSII. The cultivar W38 was found to be slightly more tolerant to extreme temperatures than Samsun and Petit Havana SR1. Therefore the W38 lines were exposed to somewhat more severe stress conditions.

Compared with their wildtypes, the UV-sensitive ROPN3-, and C-lines (Fig. 1), were not sensitive to heat, chilling or excessive PAR (Table 1). On the contrary, we did note a small increase in the ability of the UV-susceptible C-line leaves to withstand chilling, possibly as a result of increased leaf thickness. This effect is also observed in the 'W38' X-line heterozygotes. The observed increase in UV-tolerance of the SPI 2 lines was not paralleled by any significant change in sensitivity

Table 1 Effects of heat, chilling and excessive PAR on the variable chlorophyll a fluorescence of transgenic *Nicotiana* plants

Genetic background	Variable fluorescence (F_v/F_m)				
		Plants exposed to:			
	Control	UV-B	Heat	Chilling	Excessive PAR
N. tabacum 'W38'					
WT	0.77 ± 0.01	0.65 ± 0.02	0.69 ± 0.02	0.62 ± 0.02	0.62 ± 0.02
SPI-2 6	0.81 ± 0.01	0.81 ± 0.01*	0.66 ± 0.03	0.61 ± 0.02	0.64 ± 0.04
SPI-2 15	0.81 ± 0.01	0.80 ± 0.01*	0.70 ± 0.03	0.62 ± 0.02	0.65 ± 0.04
X1-Homozygote	0.81 ± 0.01	< 0.4*	ND	ND	ND
X2-Homozygote	0.80 ± 0.01	< 0.4*	ND	ND	ND
X1-Heterozygote	0.79 ± 0.01	0.64 ± 0.02	0.71 ± 0.02	0.69 ± 0.02	0.62 ± 0.01
X2-Heterozygote	0.79 ± 0.01	0.68 ± 0.02	0.71 ± 0.02	0.70 ± 0.02*	0.65 ± 0.02
IAAL-1	0.78 ± 0.01	0.69 ± 0.01	ND	ND	ND
IAAL-2	0.78 ± 0.01	0.64 ± 0.03	0.66 ± 0.02	0.64 ± 0.02	0.69 ± 0.02
IAAL-3	0.78 ± 0.01	0.65 ± 0.02	0.70 ± 0.02	0.58 ± 0.02	0.66 ± 0.02
N. tabacum 'Samsun'					
WT	0.82 ± 0.01	0.68 = 0.01	0.66 ± 0.01	0.72 ± 0.01	0.72 ± 0.01
ROPN3-line	0.82 ± 0.01	0.60 = 0.01*	0.65 ± 0.01	0.72 ± 0.02	0.71 ± 0.01
N. tabacum 'Petit Havana SR1'					
WT	0.75 ± 0.01	0.69 = 0.03	0.57 ± 0.03	0.69 ± 0.02	0.56 ± 0.04
C-line	0.77 ± 0.01	0.46 = 0.01*	0.62 ± 0.02	0.75 ± 0.02*	0.51 ± 0.02

All data are mean ± SE of the mean. n = 8–40. *Difference with WT is significant (t-test; $P < 0.05$). ND, not determined.

to heat, chilling or excessive PAR (Table 1). Thus, the changes in the UV-sensitivity of the ROPN3-, SPI 2, homozygous C-, and heterozygous X-lines are unlikely to be caused by the up- or down-regulation of general stress tolerance pathway(s).

Changes in peroxidase activity and levels of soluble UV-screening pigments

Peroxidases catalyse the crosslinking of UV-absorbing phenolics in to polymers and to other cell wall components. Peroxidases can also catalyse the oxidative degradation of IAA. Therefore, we studied whether the IAA overproducing lines contain increased peroxidase activity. Extracts from the leaves of X-line plants were found to contain significantly increased peroxidase activity. The increase in peroxidase activity was found to be stronger in the homozygotes compared with the heterozygotes (Fig. 2). A several-fold increase in total peroxidase activity, comprising the up-regulation of several isozymes, has previously been observed in the C-line (Sitbon *et al.*, 1999) (Table 2). By contrast, extracts from IAAL-line leaves were

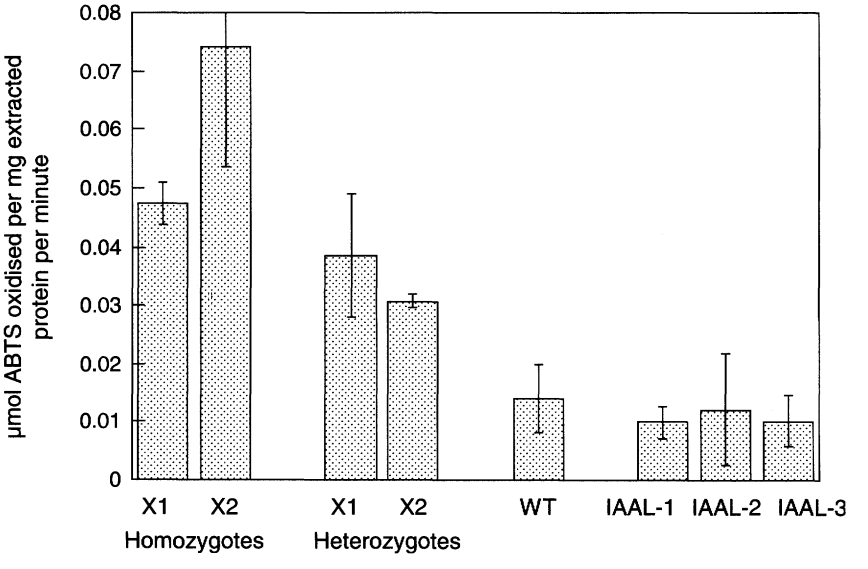

Fig. 2 Quantitative analysis of peroxidase activity in leaf extracts of *Nicotiana tabacum* 'W38'-WT, IAAL, and X-lines. Plants were homogenised in extraction buffer containing PVPP, centrifuged and peroxidase activity in the supernatant was quantified by measuring the absorption change of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) at 415 nm in potassium acetate buffer (pH 4.5) in the presence of 2 mM H_2O_2. n = 4.

Table 2 Relative activity of soluble peroxidases in transgenic *Nicotiana tabacum* lines

Genetic background	Peroxidase activity relative to WT
N. tabacum 'W38'	
WT	1.0
SPI-2 6[(1)]	2.6
SPI-2 15[(1)]	4.0
X1-Homozygote	3.4
X2-Homozygote	5.3
X1-Heterozygote	2.8
X2-Heterozygote	2.2
IAAL-1	0.7
IAAL-2	0.9
IAAL-3	0.7
N. tabacum 'Samsun'	
WT	1.0
ROPN3-line[(2)]	3.4
N. tabacum 'Petit Havana SR1'	
WT	1.0
C-line[(3)]	5.4

[(1)]Elfstrand *et al.* (2002); [(2)]Kis *et al.* (2004); [(3)]Sitbon *et al.* (1999). All other data Fig. 2.

not altered with respect to their peroxidase activity. Leaves of the ROPN3 and SPI 2 transgenes contain elevated levels of peroxidase activity, as has previously been reported (Pellegrineschi *et al.*, 1995; Elfstrand *et al.*, 2002) (Table 2), and this was routinely reconfirmed as part of our transgenic line selection programme.

The role of soluble, UV-screening phenolics in protecting plants from UV-damage, has been established (Jin *et al.*, 2000; Booij-James *et al.*, 2000; Mazza *et al.*, 2000). Peroxidases catalyse the one electron oxidation of phenolics to generate free-radical intermediates that polymerise and/or cross-link to other cell wall components. We have determined the peroxidase mediated changes in soluble phenolics for plants raised in the absence of UV-B radiation. Soluble UV-screening pigments, extracted with methanol or acidified methanol, exhibited strong absorbance in the UV spectral region, with the main absorbance band broadly centred at 327 nm (Fig. 3). UV-absorbance varied among the *N. tabacum* transgenics. A comparison of the spectra of the peroxidase over-expressing SPI 2-line with that of the control, showed a near doubling of the absorbance at 327 nm in the transgenic line (Fig. 3). By contrast, the ROPN3-line, which over-expresses horseradish peroxidase-C, was found to contain significantly less (20%) UV-absorbing pigments than its corresponding control. Overproduction of IAA also impacts on phenolic content. Homozygous C-, and X-line transgenics were found to contain 60 and 90% less UV-absorbing capacity, respectively (Fig. 3), compared with their corresponding controls. Heterozygous X-line transgenics contained 45% less UV-absorbing capacity. The expression of an IAA-lysine synthetase gene in the IAAL lines did not affect absorbance at 327 nm.

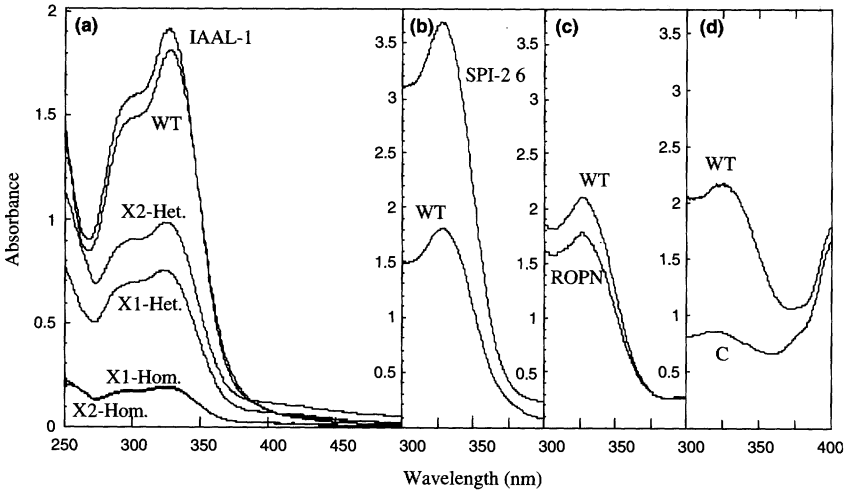

Fig. 3 Absorption spectra of pigments extracted from *Nicotiana* transgenics with altered peroxidase expression patterns. Absorbances were measured spectrophotometrically. Values were normalised for equal leaf area. One typical measurement is shown. (a) and (b) W38 background. (c) Samsun background. (d) Petit Havana SR1 background. The average absorbance at 327 nm (a.u.; corrected for equal leaf area) was in the W38 background (n = 6) 2.01 ± 0.44 (WT), 0.24 ± 0.08 (X1-homozygous (X1-Hom.)), 0.27 ± 0.06 (X2-homozygous (X2-Hom.)), 0.78 ± 0.27 (X1-heterozygous (X1-Het.)), 1.08 ± 0.10 (X2-heterozygous (X2-Het.)), 2.01 ± 0.62 (IAAL-1) 2.39 ± 0.64 (IAAL-3), 4.19 ± 0.95 (SPI-2 6) and 5.82 ± 1.53 (SPI-2 15); in the Samsun background (n = 12) 2.13 ± 0.08 (WT) and 1.76 ± 0.08 (ROPN3); in the Petit Havana SR1 background (n = 23) 2.56 ± 0.21 (WT) and 1.12 ± 0.10 (C-line). Statistical analysis (t-test) shows that both the ROPN3-line and the C- and X-lines contain significantly less UV-screening pigments (327 nm point taken as point of comparison) than their respective controls ($P < 0.01$). The SPI-2-lines contain more UV-screening pigments compared with their control wildtype ($P < 0.01$).

Discussion

We have used measurements of UV-induced alterations in the chlorophyll fluorescence of PSII as a sensitive, quantitative, *in situ* indicator of the efficiency of UV-protection mechanisms (Jansen *et al.*, 2001). We have shown that genetically induced changes in peroxidase activity result in changes in UV-susceptibility in two separate sets of different transgenic *N. tabacum* lines (SPI 2 and ROPN3) (Fig. 1). Up-regulation of peroxidase activity can result in either an increase or a decrease in UV-tolerance. The increase in UV-tolerance was found to be quantitatively similar for two independently created *spi 2* transformants. The decrease in UV-tolerance was found to be qualitatively similar for the ROPN3 line and a related transgenic line in which HRP-C expression was under control of the SSU promotor (Heggie *et al.*, in preparation). These data confirm that changes in UV-tolerance are caused by peroxidase expression, rather than a genomic insertion artefact. Our data are consistent with our previous observation that overexpression of the tobacco anionic peroxidase results in increased tolerance to UV-B radiation in *Nicotiana sylvestris* (Jansen *et al.*, 2001). However, our data also reveal a new level of complexity; seemingly contradictory results were obtained with the SPI 2 and ROPN3 peroxidase overexpressing lines.

The apparent paradox that enhanced peroxidase activity can be associated with both increased and decreased levels of UV protection appears not to be related to overall peroxidase expression levels. Relative peroxidase activity levels were found to be similar for the UV-tolerant SPI 2 transformants and the UV-sensitive ROPN3 line (Table 2). Rather, diverging effects on UV-susceptibility can be explained if different peroxidase isozymes have distinct physiological roles *in planta*. Peroxidases determine the fate of the phenolic products by catalysing their oxidation and/or crosslinking (Hiraga *et al.*, 2001). Peroxidases can also catalyse the degradation of phenolic compounds, as has been shown for anthocyanins located in the vacuole (Lopez-Serrano & Barcelo, 1999). The UV-sensitive ROPN3-line constitutively (CaMV 35S-promotor) over-expresses synthetic HRP-C. Previously, we showed that the constitutive overexpression (CaMV 35S-promotor) of the tobacco anionic peroxidase resulted in increased UV-tolerance (Jansen *et al.*, 2001). The biochemical properties of the tobacco anionic peroxidase have been extensively compared with those of HRP-C and were found to be distinct (Gazaryan & Lagrimini, 1996). The presence of a negatively charged glutamate near the entrance of the heme binding pocket of the tobacco anionic peroxidase (but not HRP-C) is thought not only to affect the stability of compounds I, II and III (intermediate forms of peroxidases that are formed during the catalytic cycle), but also to affect substrate binding. Compared with HRP-C, the substrate specificity of the tobacco anionic peroxidase is decreased for iodide and guaiacol, but increased for ferrocyanide (Gazaryan & Lagrimini, 1996). It remains to be proven whether such subtle differences in catalytic properties can affect the physiological role of isozymes *in planta* and hence levels of UV-protection. Indeed, differences in tissue and cellular localisation should also be considered. Secretory class III peroxidases are normally targeted towards the apoplast. However, in the ROPN3 line over-expressed HRP-C is expressed both in cytoplasm, and cell wall (Kis *et al.*, 2004). Clearly, such a difference in the cellular distribution should be considered when assessing the physiological roles of peroxidases and the effects on UV-tolerance. Conversely, the isozyme specificity of the UV protection response might potentially shed light on the physiological role of large gene families in biology and the importance of substrate specificity and/or cellular localisation.

The degree of UV-protection in relation to UV screening phenolics

Alterations in peroxidase activity in the SPI 2 and ROPN3 lines significantly affect the susceptibility of transgenic tobacco plants to UV-radiation stress, but not to other stresses (Table 1). These data are consistent with a UV-screening based mechanism. Phenolics absorb strongly in the UV region of the spectrum, making them excellent screening compounds (Landry *et al.*, 1995; Booij-James *et al.*, 2000). Oxidation and crosslinking of phenolics by peroxidases will affect the composition of the phenolic pool and will lead to alterations in the accumulation and/or distribution of phenolics. Crosslinking of phenolics to cell wall components such as proteins and polysaccharides will complement the screening by soluble phenolics that are located in the vacuoles. Our data clearly show that the increase in UV-tolerance in the SPI 2 line parallels the increased levels of soluble phenolics in the leaves (Fig. 3). Similarly, the UV-susceptibility of the ROPN3-line and of the homozygous C- and X-lines, is matched by low levels of soluble phenolics in the leaves of these lines (Fig. 3). Transgenic lines with altered auxin levels are characterised by alterations in leaf morphology that include leaf thickening (Sitbon *et al.*, 1992; Sitbon *et al.*, 1999). Therefore, if the levels of soluble phenolics are normalised per unit weight, rather than per unit leaf area, the differences in UV-absorbance between wildtype and auxin overexpressing lines will be even greater. The data on the SPI 2, ROPN3, IAAL and homozygous X and C lines clearly show a positive correlation between the total levels of soluble UV-absorbing phenolics and UV-protection, and this is consistent with a role for peroxidases in phenolic metabolism (Fig. 4) (Gazaryan & Lagrimini, 1996; Hiraga *et al.*, 2001). At present we do not know to what extent levels of bound phenolics are also affected in the transgenic tobacco lines. However, we note that in the case of the 'W38' X-line heterozygotes a near normal level of UV-tolerance (Fig. 1) corresponds with a significant (45%) decrease in soluble UV-absorbing pigments (Fig. 3). These data are interpreted as evidence for a complementary UV-protection mechanism which compensates for the lack of UV screening by soluble phenolics. Covalently bound phenolics, crosslinked by peroxidases to cell wall components, are a possible

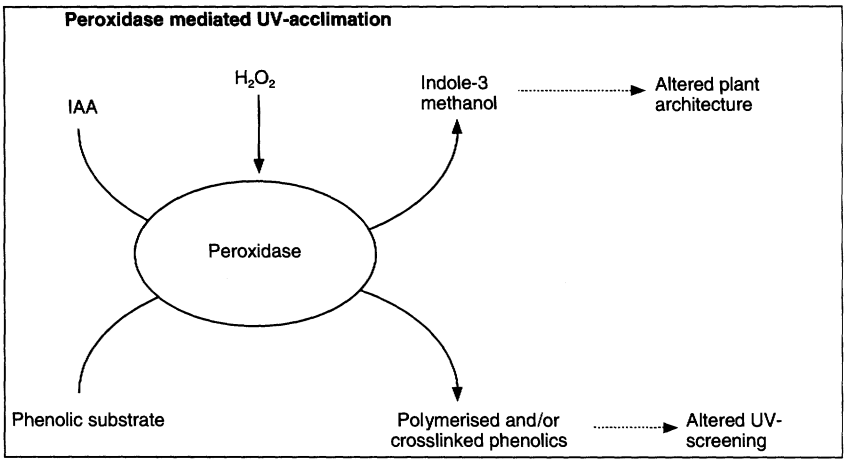

Fig. 4 Schematic representation of the central role of class III secretory peroxidases in linking indole-3-acetic acid (IAA) catabolism and phenolic metabolism.

candidate to complement UV-screening by soluble phenolics. Indeed, we have found that the heterozygote 'W38' X-line contains significantly increased peroxidase activity (Fig. 2). Lignin composition and levels are reportedly changed in several high peroxidase lines, including the UV-tolerant SPI 2 line (Elfstrand *et al.*, 2002), the UV-tolerant *N. sylvestris* line that overexpresses the tobacco anionic peroxidase (Lagrimini *et al.*, 1997b) and the UV-sensitive homozygous C-line (Sitbon *et al.*, 1999). Thus, bound phenolics can potentially contribute to the overall UV-protection of transgenic tobacco. However, the relative contribution of bound phenolics to UV-protection is difficult to ascertain as this will largely depend on the localisation of cells and tissues to which the phenolics are bound.

Peroxidases and auxin catabolism

We have previously found that increased activity of the tobacco anionic peroxidase is linked to an increase in UV-tolerance (Jansen *et al.*, 2001), as well as a decrease in auxin levels (Lagrimini, 1999) in *N. sylvestris*. We concluded that IAA catabolism is linked to UV-tolerance, through peroxidase activity (Fig. 4). This hypothesis was consistent with the established role of peroxidases in IAA catabolism (Normanly, 1997; Gazaryan *et al.*, 1998), and also with the colocalisation of peroxidases, phenols and IAA in the cell wall. Local decreases in IAA levels in *N. sylvestris* transgenics overexpressing the tobacco anionic peroxidase were reflected in a decrease in root development and hypocotyl length (Lagrimini *et al.*, 1997a; Lagrimini, 1999). Similar architectural alterations were observed in the SPI 2 and ROPN3 lines. In the ROPN3-line root development was decreased while hypocotyl length and axillary branching increased (Heggie *et al.*, in preparation). In the SPI 2 lines root development decreased and shoot/root ratio increased (Jansen, unpublished). However, it was not clear whether changes in IAA levels are an essential component of the link between peroxidase activity and UV-protection (i.e. see Fig. 4). We have now shown that UV-tolerance (as well as PAR, heat or chilling tolerance) is not affected in an IAA-underproducing phenotype (IAAL) in which expression of auxin responsive genes is decreased. By analogy, we are led to conclude that any potential decreases in free IAA-levels in peroxidase overexpressing lines (SPI 2, ROPN3) are not essential for the observed changes in levels of UV-absorbing phenolics, and hence UV tolerance. Nevertheless, an intriguing possibility is that peroxidase mediated changes in IAA levels, through their effect on plant architecture, could still contribute to UV-protection at the level of the whole plant. Architectural changes like leaf thickening, axillary branching and internode shortening, are commonly observed in UV-acclimated plants, and have been proposed to play a role in UV-avoidance (Barnes *et al.*, 1996; Jansen, 2002).

Unlike IAA-underproduction, the up-regulation of IAA levels (C and X-lines) dramatically altered the UV-screening response, although not general stress susceptibility (Fig. 1). It is not clear whether the decrease in the soluble phenolics in the C-, and X-lines is directly related to increased peroxidase activity (like in the ROPN3 line), or rather an indirect consequence of the dramatic changes in auxin homeostasis. Peroxidase activities were found to be strongly increased in the C-, and X-lines (Fig. 2) (Sitbon *et al.*, 1999), and this might be related to an increased rate of auxin catabolism. Consistently, it has been found that *in vivo*, the metabolic rate of IAA turnover is some three-fold increased in the C-line (Sitbon *et al.*, 2000). Remarkably, we have recently found that a rapidly IAA catabolising duckweed mutant (Tam *et al.*, 1995), containing increased levels of one particular peroxidase isozyme, is UV-tolerant (Jansen *et al.*, 2001). Based on the comparison of the UV-sensitive ROPN3 and the UV-tolerant SPI 2 lines, we speculate that the paradox of the opposite responses in duckweed and tobacco can be resolved if the isozyme specificity of the UV-protection response is considered.

UV-induced changes in peroxidase activity have been reported for a number of plant species (Rao *et al.*, 1996; Ambasht & Agrawal, 1997; Huang *et al.*, 1997; Tekchandani & Guruprasad, 1998; Egert & Tevini, 2003) and an UV-responsive *cis* element has been identified in the promotor region of a rice

peroxidase (Ito *et al.*, 2000). Unfortunately, none of these reports identified which isozymes contributed to the measured overall increase in peroxidase activity. Our results emphasise the specificity of the peroxidase mediated UV-protection response with only particular peroxidase isozymes being able to increase the protection of plants from UV-radiation.

Acknowledgements

R.N.F.T. and M.A.K.J. were supported by CSG funding from the UK Biotechnology and Biological Sciences Research Council to the John Innes Centre. L.H. and P.J.D. acknowledge support from the HEA-programme for research in Third Level Institutions, Ireland. M.A.K.J. and L.H. acknowledge support from the E.C. (TMR Network 'Peroxidases in Agriculture, the Environment and Industry, contract no ERB FMRXCT 980200).

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