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Role of polyisoprenoids in tobacco resistance against biotic stresses

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Infection with avirulent pathogens, tobacco mosaic virus (TMV) or *Pseudomonas syringae* pv. tabaci induced accumulation of polyisoprenoid alcohols, solanesol and a family of polyprenols [from polyprenol composed of 14 isoprene units (Pren-14) to -18, with Pren-16 dominating] in the leaves of resistant tobacco plants *Nicotiana tabacum* cv. Samsun NN. Upon TMV infection, solanesol content was increased seven- and eight-fold in the inoculated and upper leaves, respectively, while polyprenol content was increased 2.5- and 2-fold in the inoculated and upper leaves, respectively, on the seventh day post-infection. Accumulation of polyisoprenoid alcohols was also stimulated by exogenously applied hydrogen peroxide but not by exogenous salicylic acid (SA). On the contrary, neither inoculation of the leaves of susceptible tobacco plants nor wounding of tobacco leaves caused an increase in polyisoprenoid content. Taken together, these results indicate that polyisoprenoid alcohols might be involved in plant resistance against pathogens. A putative role of accumulated polyisoprenoids in plant response to pathogen attack is discussed. Similarly, the content of plastoquinone (PQ) was increased two-fold in TMV-inoculated and upper leaves of resistant plants. Accumulation of PQ was also stimulated by hydrogen peroxide, bacteria (*P. syringae*) and SA. The role of PQ in antioxidant defense in cellular membranous compartments is discussed in the context of the enzymatic antioxidant machinery activated in tobacco leaves subjected to viral infection. Elevated activity of several antioxidant enzymes (ascorbate peroxidase, guaiacol peroxidase, glutathione reductase and superoxide dismutase, especially the CuZn superoxide dismutase isoform) and high, but transient elevation of catalase was found in inoculated leaves of resistant tobacco plants but not in susceptible plants.

Abbreviations – APX, ascorbate peroxidase; CAT, catalase; CPT, *cis*-prenyltransferase; DPI, days post-infection; GR, glutathione reductase; HAE, hydroxyalkenals; HSP, heat-shock proteins; IPP, isopentenyl diphosphate; LOX, lipoxygenase; MDA, malondialdehyde; MEP, methylerythritol phosphate; MVA, mevalonate; NBT, nitroblue tetrazolium; POX, guaiacol peroxidase; PQ, plastoquinone; ROS, reactive oxygen species; SA, salicylic acid; SOD, superoxide dismutase; TMV, tobacco mosaic virus; TPT, *trans*-prenyltransferase; UQ-6, ubiquinone 6.

Introduction

Plants have evolved a wide range of mechanisms to cope with biotic and abiotic stresses. Upon adverse conditions, complex metabolic changes occur involving the primary metabolism and mobilization of so-called 'secondary metabolites', e.g. terpenoids that are the most numerous and diverse group of natural products. Numerous terpenoids shown to be involved in plant defense against pathogens include volatile mono-, sesqui- and diterpenes, which are thought to regulate the susceptibility to biotic and abiotic stress (Gershenzon 1994).

Polyisoprenoid alcohols, representatives of high-molecular terpenoids, are synthesized in all living cells, and their accumulation is significantly increased with the age of the tissue (Hemming 1985). The structure of polyprenols typically found in leaves of plants is shown in Fig. 1. Polyprenols isolated from the leaves and needles of various plants differ considerably in chain length (from 5 to more than 130 isoprene units). In photosynthetic tissues, these mainly-*cis*-polyisoprenoid alcohols have always been detected as mixtures of homologues, and the composition of the polyprenol mixture has been considered a species-specific chemotaxonomic criterion (Swiezewska and Danikiewicz 2005). Interestingly, up to now, there have been no data on the accumulation of typical, mainly-*cis*, polyprenols in tobacco leaves. Instead, tobacco and other *Solanaceae* plants have been known as sources of solanesol (Fig. 1), an atypical prenol consisting of nine isoprene units in the *trans* configuration [all-*trans*-polyprenol composed of 9 isoprene units (Pren-9)] (Rowland et al. 1956). More recently, traces of solanesol have also been discovered in leaves of other plant species (Hemming 1985). The solanesyl group also serves as a side chain of plastoquinone (PQ) (Fig. 1), a component of all photosynthetic tissues. Solanesol always occurs in the leaves as a single prenol in contrast to the 'family' of mainly-*cis* polyprenols.

Biosynthesis of polyprenols and solanesol is accomplished by two enzymes (Fig. 1), either *cis*- or *trans*-prenyltransferase (CPT or TPT, respectively), depending on the product to be formed. Both prenyltransferases elongate the starter, farnesyl diphosphate, by sequential condensations with isopentenyl diphosphate (IPP) until polyprenyl diphosphate(s) of the desired chain length is achieved. Data on prokaryotic and eukaryotic CPT and TPT have been recently summarized (Liang et al. 2002, Takahashi and Koyama 2006). Based on in silico analysis of the *Arabidopsis* genome, families of six CPT and two TPT encoding genes have been predicted (Lange and Ghassemian 2003); however, only one AtCPT- (Cunillera et al. 2000, Oh et al. 2000,) and two AtTPT-encoding genes (Hirooka et al. 2003, Jun et al. 2004) have been functionally characterized.

The biological role of polyisoprenoid phosphates is well established. Dolichyl phosphates serve as cofactors of protein glycosylation and biosynthesis of glucosyl phosphatidyl inositol anchor in animal and most probably also in plant cells (Burda and Aebi 1999, Samuelson et al 2005). Solanesyl diphosphate in plant cells is used as a donor of the isoprenoid side chain for PQ (Swiezewska

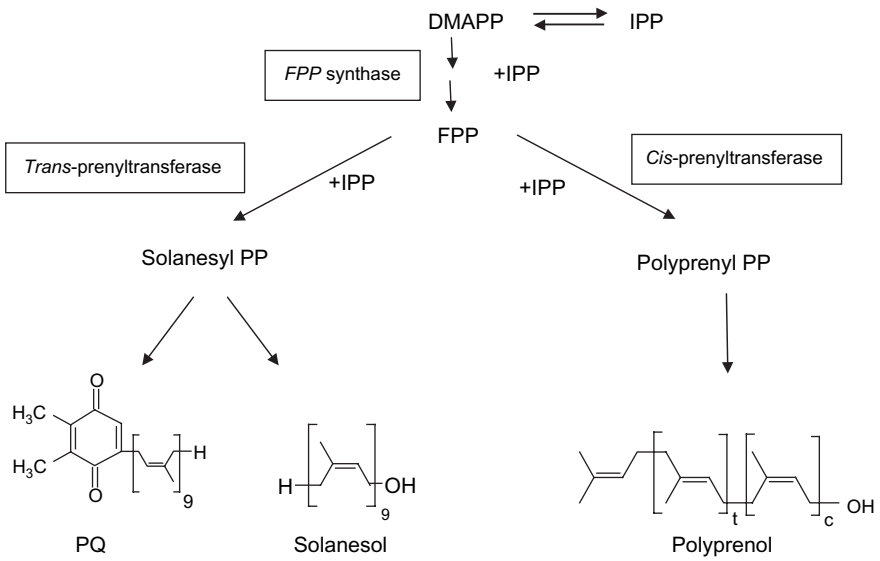

Fig. 1. Structures of analyzed lipids. Biosynthetic origin of solanesyl and polyprenyl diphosphates from IPP is indicated. t and c stand for *trans* and *cis* isoprenoid residue, respectively, t = 2 and c = 11 to 15; FPP, farnesyl diphosphate.

2004), while polyprenyl/dolichyl diphosphates are postulated to act as substrates for the biosynthesis of prenylated proteins (Gutkowska et al. 2004). No biological function has been ascribed to free polyisoprenoid alcohols or their carboxylic esters, the main accumulated forms. On the basis of polyisoprenoid-dependent fluidization of model membranes, their role as modulators of the physical properties of biological membranes has been postulated (Chojnacki and Dallner 1988 and references therein). Genetic studies in yeast have revealed that yeast-specific polyisoprenoid alcohols (2,3-dihydropolyprenols, trivial name dolichols) are involved in the transport of ER and vacuolar proteins (Belgareh-Touze et al. 2003, Sato et al. 1999). Recently, a new function of dolichols has been postulated, namely protection of cellular membranes against peroxidation. Accordingly, the polyisoprenoid chain might constitute a shield against reactive oxygen species (ROS) generated either by UV light or by chemicals (Bergamini 2003).

Stresses cause oxidative burst and production of ROS resulting from several pathways, both constitutive and induced, such as photosynthesis, photorespiration and mitochondrial respiration or NAD(P)H oxidases, amine oxidases and cell-wall bound peroxidases. The enhanced production of ROS can pose a threat to cells, but it is also thought that ROS act as secondary messengers involved in the stress-response signal transduction pathway and inducers of defense strategy, eventually leading to local cell death within the hypersensitive response (Mittler 2002). A special role of hydrogen peroxide as a 'master hormone' regulating a variety of stress responses is well documented (Slesak et al. 2007 and references therein). Nevertheless, the concentration of ROS has to be maintained within a certain range to avoid oxidative damage. This is ensured by both enzymatic and non-enzymatic antioxidants. Enzymatic antioxidant defense mechanisms include several well-characterized systems involved directly in ROS scavenging, e.g. catalase (CAT), superoxide dismutase (SOD), guaiacol peroxidase (POX), ascorbate peroxidase (APX) or regenerating antioxidant molecules, e.g. glutathione reductase (GR) responsible for regeneration of glutathione. Non-enzymatic antioxidants include hydrophilic (e.g. ascorbate) and hydrophobic molecules, with carotenoids and tocopherols being the two most abundant groups of lipid-soluble antioxidants in plant cells (Munne-Bosch 2007). Moreover, reduced ubiquinone (Popov et al. 2001) and PQ (Hundal et al. 1995, Kruk et al. 1997) have also been suggested to exert such function.

In the present report, the role of polyisoprenoid alcohols in plant resistance to biotic stress is analyzed. In tobacco leaves, accumulation of a family of polyprenols (from Pren-14 to -18, with Pren-16 dominating), besides solanesol, was found. The concentration of polyprenols and solanesol in resistant tobacco was increased considerably upon biotic stress; an increased polyisoprenoid level was observed both in inoculated and in upper leaves. In contrast, there was no significant change in the content of these lipids after wounding or in the leaves of susceptible plants after viral infection. The PQ concentration was doubled upon pathogen inoculation of resistant tobacco plants, both in the inoculated and in the upper leaves. Hydrogen peroxide stimulated the increase of polyisoprenoids and PQ accumulation. In contrast to susceptible plants, elevated activity of antioxidant enzymes was found in the leaves of resistant plants. Implications of these observations, indicating the involvement of polyisoprenoid alcohols in plant resistance response and suggestions of the putative mechanism by which polyisoprenoids exert such a function in biotic stress defense, are discussed.

Materials and methods

Plant material

Tobacco *Nicotiana tabacum* (Solanaceae) cv. Samsun NN or nn [resistant or susceptible to tobacco mosaic virus (TMV) infection, respectively] plants were used in most of the experiments. Plants were grown for 4 weeks in soil with a photoperiod of 16/8 h at 22°/18°C with light intensity 5000–6000 lux (75 μmol m^{-2} s^{-1}) as described previously (Talarczyk et al. 2002). In the leaves of Samsun NN plants, formation of necrotic lesions (hypersensitive response) was observed on the third day post-TMV infection. This was because of the presence of two alleles of the resistance gene N, encoding a protein that interferes with the TMV replicase. Susceptible plants (N gene not present) did not display necrotic changes.

TMV infection

Three neighboring, fully expanded leaves of 4-week-old plants of *N. tabacum* cv. Samsun NN or nn were infected with TMV, one half of each leaf was treated. For inoculation, carborundum-dusted leaves were rubbed with TMV strain U1 solution (0.5 mg ml^{-1}) or water (mock plants) (Talarczyk et al. 2002). After inoculation, plants were kept under the same environmental conditions. Samples were collected at the indicated times [3, 7, 14 or 21 days post-infection (DPI)]. Untreated halves of inoculated leaves and upper leaves (three adjacent leaves located above the inoculated ones) were collected. Similarly, untreated halves of water-treated leaves from the mock plants, and upper

leaves from mock plants (three adjacent leaves located above the water-treated ones) were collected. Because the content of polyisoprenoid alcohols increases with age of the tissue (Chojnacki and Dallner 1988, Hemming 1985), age-matched mock plants were always used for comparison. For each experiment, leaves of the age-matched untreated plants were collected on the day of inoculation and considered as reference. Three leaves, collected from each plant, were pooled and considered as one sample. Untreated halves of the leaves (infected or mock) were weighed, frozen in liquid nitrogen and kept at −80°C until used.

Pseudomonas syringae pv. tabaci infection

Leaves of *N. tabacum* cv. Samsun NN were infiltrated with avirulent bacteria *P. syringae* pv. tabaci (injection of freshly prepared suspension of overnight bacterial culture 10^8 cfu ml^{-1} in 10 m*M* $MgCl_2$) or 10 m*M* $MgCl_2$ (mock plants), one half of the leaf was treated in each case. *Pseudomonas* inoculum was prepared as described earlier (Katagrii et al. 2002). Untreated halves of inoculated and mock leaves were collected on the third day after treatment as described above.

Hydrogen peroxide and salicylic acid treatments

Leaves of *N. tabacum* cv. Samsun NN were either infiltrated with 300 m*M* H_2O_2 or sprayed with 50 m*M* salicylic acid (SA) in 5 m*M* phosphate buffer, pH 6.8; mock plants were treated with phosphate buffer. Challenged (H_2O_2 or SA) and mock leaves were collected on the third day after treatment as described above.

Wounding of tobacco leaves

Three neighboring, fully expanded leaves of *N. tabacum* cv. Samsun NN plants were cut (seven parallel cuts per leaf) with a razor blade. Treated leaves and untreated controls were collected on the third day after treatment as described above.

Extraction and purification of lipids

Tobacco leaves (6 g) were homogenized with Ultra-Turrax (IKA Ltd., Staufen, Germany) in 40 ml of chloroform/methanol (1:1 v/v.), thus the final chloroform/methanol/water ratio was 1:1:0.3 v/v/v. Lipids were extracted for 1 h at 38°C in the dark. Crude extract was filtered, and chloroform and water were added to reach the final solvent ratio (C:M:W 3:2:1 v/v/v). The lower, chloroform phase was collected and split into two parts: A – two-third volume for estimation of polyprenols and solanesol and B – one-third volume for estimation of PQ.

Purification of polyprenols and solanesol

Lipids were supplemented with 10 μg of the internal standard (Pren-11), and alkaline hydrolysis and purification were done as described previously (Skorupinska-Tudek et al. 2003). Pure lipids were dissolved in chloroform:methanol, 2:1 v/v.

Purification of PQ

Lipids were supplemented with 5 μg of the internal standard [UQ-6 (ubiquinone 6)], purified as described earlier (Maciejewska et al. 2002) and dissolved in chloroform:methanol, 2:1 v/v.

HPLC quantification of lipids

Lipids were analyzed by HPLC (Waters, Milford, MA) as described earlier for polyprenols (Skorupinska-Tudek et al. 2003) and PQ (Maciejewska et al. 2002).

MS analysis of polyprenols

MS experiments were carried out using an API 365 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) according to Skorupinska-Tudek et al. (2003).

Photosynthetic activity and gas exchange

Gas exchange was estimated using the infrared gas exchange analyzer Li-6400 (LI–COR, Lincoln, NE). Water-use efficiency was calculated by dividing the intensity of photosynthesis by the intensity of water transpiration. Fluorescence of Chl α was measured with the aid of the Handy PEA portable fluorescence measurement system (Hansatech, Pentney, UK).

Determination of antioxidative enzyme activities

Frozen leaf tissue was powdered and resuspended in ice-cold extraction buffer (50 m*M* phosphate buffer, pH 7.0) according to Milosevic and Slusarenko (1996). After centrifugation at 15 000 *g* for 15 min at 4°C, aliquots of crude homogenate were used for the estimation of the activity of subsequent enzymes. Protein concentration was determined by the method of Bradford (1976) using BSA as standard.

Activities of APX (EC 1.11.1.11), CAT (EC 1.11.1.6), POX (EC 1.11.1.7), GR (EC 1.6.4.2) and SOD (EC 1.15.1.1)

were measured as described earlier (Skorzynska-Polit et al. 2003/4).

Native PAGE and activity staining

To separate SOD isozymes, leaf extracts containing equal amounts of protein (40 μg) were subjected to discontinuous PAGE as described by Laemmli (1970) under non-denaturating, non-reducing conditions using 4% stacking gel and 12.5% resolving gel. For identification of SOD isozymes, a procedure described by Beauchamp and Fridovich (1971) and Martinez et al. (2001) was used with some modifications. After electrophoresis, enzymograms were incubated at 25°C for 30 min in the dark, either in 5 m*M* H_2O_2 or in 3 m*M* NaCN. Then, the gels were stained for SOD isoforms by immersion in 50 m*M* sodium phosphate buffer pH 7.8, containing 1 m*M* nitroblue tetrazolium (NBT) for 20 min in the dark followed by incubation in 50 m*M* sodium phosphate buffer pH 7.8, containing 28 μ*M* riboflavin and 28 m*M* tetramethyl ethylene diamine for 20 min in the dark and exposed to light at room temperature. The activities of SOD isoforms were visualized as achromatic bands by staining with NBT.

Determination of malondialdehyde and hydroxyalkenals

Fresh leaves (1 g) were homogenized in 3 ml of 20 m*M* phosphate buffer, pH 7.4 supplemented with butylated hydroxytoluene (10 μl of 0.5 *M* solution in acetonitrile per 1 ml of homogenate). Malondialdehyde (MDA) and hydroxyalkenals (HAE) in leaf extract were determined using the *Oxis*Research LPO-586 test (Oxis International Inc., Foster City, CA). The hydrochloric acid procedure, commonly used for estimation of MDA, was inappropriate because of the interference of anthocyanins; therefore, the methanesulfonic acid procedure was used; samples were incubated with N-methyl-2-phenylindole at 45°C and absorbance at 586 nm was measured.

Determination of lipoxygenase activity

The activity of lipoxygenase (LOX) (EC 1.13.11.12) was measured spectrophotometrically at 234 nm as described earlier (Skorzynska-Polit and Krupa 2003).

Statistical analysis

Statistical analysis of the results was by one-way ANOVA 'Student–Newman–Keuls' test using SigmaStat 3.5 software.

Chemicals

If not indicated otherwise, all chemicals were obtained from Sigma (St. Louis, MO) (pa grade). Standards of polyisoprenoid alcohols and PQ were from the Collection of Polyprenols (Institute of Biochemistry and Biophysics, Polish Academy of Sciences). UQ-6 standard was purchased from Sigma. Chromatographic materials (TLC plates, gels for column chromatography) were from Merck (Darmstadt, Germany). Organic solvents used for HPLC were from J. T. Baker B.V. (Deventer, Holland) and from Merck.

Results

Polyisoprenoid composition of tobacco leaves

The possible involvement of polyisoprenoids in plant response to biotic stress was elucidated using a well-characterized model of plant–pathogen interaction, *N. tabacum* cv. Samsun NN – resistant and Samsun nn – susceptible to TMV. Detailed analysis of the isoprenoid lipid composition of *N. tabacum* leaves revealed that in addition to a well-known solanesol, this tissue contained a family of mainly-*cis* polyprenols. Upon HPLC/UV analysis, a mixture of five homologues (Pren-14 to -18) with Pren-16 being the dominant form (Fig. 2) was detected. MS and NMR spectroscopy confirmed the structure of the newly described mainly-*cis* polyprenols. Careful inspection of the HPLC spectra of tobacco lipids revealed the presence of traces of polyisoprenoids of unknown structure, most probably geometric isomers of mainly-*cis* polyprenol. These additional polyisoprenoids were detected as low intensity, not fully resolved signals accompanying signals of polyprenols in the HPLC/UV spectrum (Fig. 2). Further analysis is required for their structural characterization. No detectable amounts of dolichols were found in the analyzed plant material.

Polyisoprenoid alcohol and PQ content was increased after hydrogen peroxide treatment but not after SA

The signaling cascade initiated after recognition of an invading pathogen engages various messenger molecules, hydrogen peroxide and SA being among the best recognized. Hydrogen peroxide is considered to play a key role in the regulation of cellular responses to a broad range of stressors. To mimic the role of H_2O_2 in the regulation of polyisoprenoid metabolism, the effect of exogenous hydrogen peroxide was tested. A higher level of solanesol (2-fold) and a somewhat higher level of polyprenols (1.3-fold) were found in the H_2O_2-infiltrated

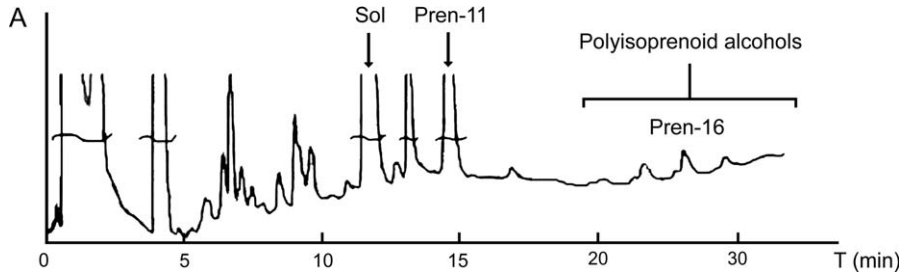

Fig. 2. HPLC/UV spectrum of polyisoprenoid alcohols isolated from the leaves of *Nicotiana tabacum* Samsun NN. Expanded spectrum is presented to facilitate the observation of polyisoprenoid alcohols because in the extract of the native tobacco leaves, solanesol:polyisoprenoid ratio is approximately 9:1. Arrows indicate the signals corresponding to solanesol (Sol) and Pren-11 (internal standard), clasp indicates the family of polyisoprenoid alcohols (Pren-16 dominating).

leaves than in the leaves of mock plants on the third day after treatment; however, the changes in polyprenol content were not statistically significant. The content of PQ was also doubled after the H_2O_2 treatment (Table 1).

SA has been shown to have a crucial function in mediating and orchestrating stress responses in plants. Thus, in parallel experiments, superfluous exogenous SA was applied to plant leaves. Neither polyprenol nor solanesol content was changed on the third day after SA treatment. A moderate increase (1.3-fold of mock) in PQ content was found in the leaves of SA-treated plants (Table 1).

Increased polyisoprenoid accumulation in the leaves of resistant tobacco after viral and bacterial infection

Different *N. tabacum* cvs, resistant (NN) and susceptible (nn), to TMV infection were used in the experiments. In susceptible plants, in contrast to resistant ones, TMV inoculation did not result in significantly increased polyprenol content (105–115% of mock) at any time point analyzed. In line with this observation, no significant change of solanesol or polyprenol content was observed in *N. tabacum* cv. Xanthi leaves infected with *Potato virus Y* (PVY) (110% of mock). These observations indicate that there was little or no change in the polyisoprenoid content in tobacco leaves during incompatible plant–pathogen interactions. For this reason, all subsequent experiments were done with *N. tabacum* NN exhibiting the hypersensitive response to TMV infection.

Table 1. Effect of bacterial infection, SA, hydrogen peroxide and wounding on accumulation of polyisoprenoids in resistant tobacco leaves. Content of solanesol and polyprenols was estimated in infected leaves 3 days after treatment. All values are % of mock.

Treatment	Solanesol	Polyprenols	PQ
Pseudomonas syringae	200	130	170
SA	95	100	130
H_2O_2	200	120	210
Wounding of leaves	85	102	140

Significant increases in the content of polyisoprenoid alcohols, polyprenols and especially solanesol were found in infected leaves of resistant tobacco plants (*N. tabacum* NN) after TMV infection. In comparison to mock leaves, solanesol content was increased two- and seven-fold on the third and seventh day post-infection (DPI), respectively (Fig. 3A). Polyprenol content was increased to a lower extent, reaching a three-fold higher level than the mock 7 DPI (Fig. 3B). As expected, during the initial 7 days of the experiment, there was no change in the content of polyprenols or of solanesol in mock leaves.

Analysis of the lipids in the upper leaves (three neighboring leaves above the inoculated ones) also showed increased content of solanesol (eight-fold of the mock 7 DPI) and polyprenols (two-fold of the mock 7 DPI) (Fig. 4). A further slight increase in polyprenol but not solanesol content was also found 14 DPI (data not shown). Values in this experiment were expressed as per cent of mock because of variability of the absolute values of lipid content found between two series of measurements. This discrepancy comes most probably from the experiments being done in different seasons of the year.

To verify whether the increased polyisoprenoid content might be considered a general component of resistance plant response, *N. tabacum* cv. Samsun NN leaves were infected with the avirulent bacterium *P. syringae* pv. tabaci. As also for TMV, bacterial infection increased the content of polyisoprenoids (2-fold for solanesol and 1.3-fold for polyprenols, Table 1); however, this increase was not statistically significant.

The PQ content was almost doubled in the TMV-infected leaves of resistant tobacco plants in comparison to mock (3 and 7 DPI, Fig. 3C). The same was observed for upper leaves (not shown). Similarly, the PQ content

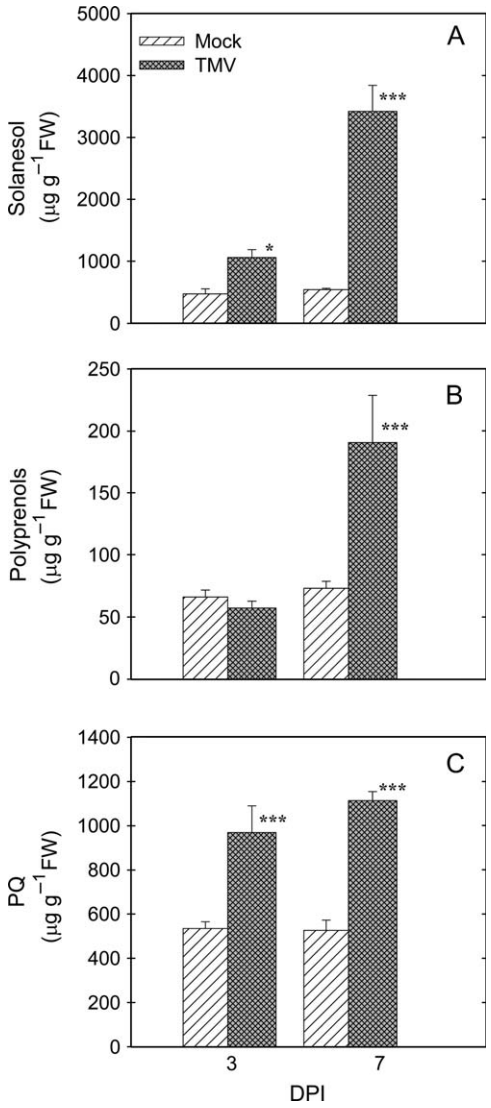

Fig. 3. Content of solanesol (A), polyprenols (B) and PQ (C) in inoculated leaves of *Nicotiana tabacum* cv. Samsun NN estimated 3 and 7 DPI with TMV. All values are means + SD. Asterisks denote significant difference of the lipid content over the age-matched mock leaves: $*P < 0.05$; $***P < 0.001$.

was increased 1.7-fold in the tobacco leaves inoculated with *P. syringae* (Table 1).

Wounding did not cause increased accumulation of polyisoprenoid alcohols

The increase in polyisoprenoid content observed in the leaves of resistant plants during biotic stress prompted us to test whether polyisoprenoid accumulation in leaves was induced by abiotic environmental factors. Therefore, the effect of wounding, as an example of an abiotic stress,

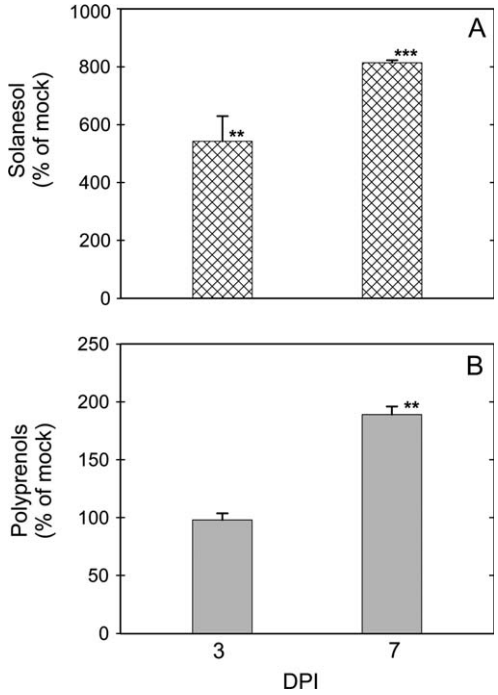

Fig. 4. Content of solanesol (A) and polyprenols (B) in the upper leaves (three neighboring leaves located above TMV-inoculated ones) expressed as the per cent of the mock. All values are means + SD. Asterisks denote significant difference of the lipid content over the age-matched mock leaves: $**P < 0.01$; $***P < 0.001$.

on the accumulation of analyzed lipids was tested in a parallel experiment (Table 1). There was no significant change in polyprenol content in wounded leaves in comparison to mock, while the solanesol content was slightly decreased (0.85-fold). The PQ content was increased moderately (approximately 1.4-fold). Changes in lipid content upon wounding were not statistically significant.

Minor changes in photosynthetic parameters in tobacco leaves after TMV infection

Because the PQ content was considerably increased in the leaves of inoculated resistant tobacco plants, it was estimated whether photosynthesis was enhanced simultaneously. In fact, no changes in the intensity of photosynthesis (approximately 95% mock) were observed in inoculated leaves 3 DPI. In contrast, stomatal conductance and water transpiration were decreased to approximately 50% of mock (Table 2). The calculated water-use coefficient for TMV-infected leaves was twice as high as for the mock leaves. No significant changes in the fluorescence of Chl were noted on the third day after TMV infection because the F_v:F_m ratio calculated for

Table 2. Photosynthetic parameters and gas exchange in mock and TMV-infected resistant tobacco measured on the third day after infection[a]. [a]Values are mean of three experiments ± SD.

Leaves	Photosynthesis (μmol CO_2 m^{-2} s^{-1})	Stomatal conductance (mol H_2O m^{-2} s^{-1})	Intracellular CO_2 concentration (μmol CO_2 mol^{-1} air)	Water transpiration (mol H_2O m^{-2} s^{-1})	Water-use efficiency coefficient
Mock	15.2 ± 0.6	0.26 ± 0.09	308 ± 3	3.3 ± 0.3	4.8 ± 0.1
Infected	14.5 ± 0.4	0.12 ± 0.03	154 ± 13	1.1 ± 0.1	10.7 ± 0.6

TMV-infected leaves was 0.84 ± 0.01 vs 0.85 ± 0.01 for the mock leaves.

Activity of antioxidant enzymes in tobacco leaves after TMV infection

TMV infection caused the increase in ROS production in resistant tobacco plants as shown by the finding that the level of peroxidation of lipids (content of MDA and HAE) increased by about 50% in infected leaves in comparison to mock leaves 3 and 7 DPI (Table 3). At the same time, accumulation of PQ in response to biotic stress might cause the change in the redox homeostasis. These reasons prompted us to measure the activity of a broad range of antioxidant enzymes from susceptible and resistant cultivars of *N. tabacum* after TMV infection (Fig. 5). Considerable differences in enzyme activities were noted for the resistant and susceptible plants on 3 DPI. The activities of APX, CAT, POX and GR were increased considerably (approximately 180, 360, 200 and 260% of mock, respectively) in the leaves of resistant plants (NN), while only POX activity was significantly enhanced for the susceptible (nn) plants, reaching 200% of mock. Elevated levels of APX, POX and GR activities (180, 190 and 120% of mock, respectively) were sustained on 7 DPI, while CAT activity was diminished to that of mock in the leaves of resistant plants. Complex fluctuations of the activities were observed 7 DPI in the leaves of susceptible plants because GR activity declined (55% mock), while POX activity increased (210% of mock), APX and CAT activities were similar to mock. In susceptible tobacco (nn) leaves, SOD activity was decreased after infection (42 and 21% of mock 3 and 7 DPI, respectively) in contrast to resistant (NN) plants (130 and 180% mock 3 and 7 DPI, respectively) (Fig. 6A).

Table 3. Content of peroxidation products in resistant tobacco leaves after TMV infection.

	Mock 3 DPI	TMV 3 DPI	Mock 7 DPI	TMV 7 DPI
MDA + HAE (nmol mg^{-1} protein)	2.8 ± 0.2	3.8 ± 0.2	3.2 ± 0.3	4.8 ± 0.1

In the latter case, CuZn SOD activity was particularly induced (Fig. 6B).

The activity of LOX was estimated over a broad pH range (from 5 to 10) to follow the activities of the putative LOX isoforms. Induction of LOX activity in TMV-inoculated leaves was observed only on 3 DPI in the susceptible plants when two optima were found at pH 7.5 and 8.5, while 7 DPI the activity of LOX was decreased and only one pH optimum (8.5) was found (Fig. 7A). In the resistant tobacco leaves, LOX activity was decreased (25 and 65% of mock 3 and 7 DPI, respectively) and only one optimum at pH 8.5 was observed (Fig. 7B).

Discussion

Polyisoprenoids are involved in tobacco resistance to pathogen

It is widely accepted that environmental factors affect the content and yield of secondary metabolites. Thus, modulation of the composition of secondary metabolites is considered as a mechanism of plants' adaptation to environmental changes. Experiments described here were aimed at elucidating the involvement of polyisoprenoids in plant response to stress. The increased content of polyisoprenoids found upon application of superfluous hydrogen peroxide confirmed this supposition. Because H_2O_2 is considered a common cellular signal used by signaling pathways of biotic and abiotic stresses (Hancock et al. 2001), several environmental factors were analyzed as potential inducers of polyisoprenoid accumulation. Four variants of plant–pathogen interactions were tested, two representing the resistance response (TMV or *P. syringae* inoculation of *N. tabacum* Samsun NN) and two representing the disease response (TMV inoculation of *N. tabacum* Samsun nn or PVY inoculation of *N. tabacum* cv. Xanthi). Accumulation of solanesol and polyprenols was increased exclusively in the leaves of tobacco plants challenged with avirulent pathogens. The highest increase in polyisoprenoid accumulation was observed during the first 7 DPI in inoculated and upper leaves. This time window seems typical for mobilization of secondary metabolism in

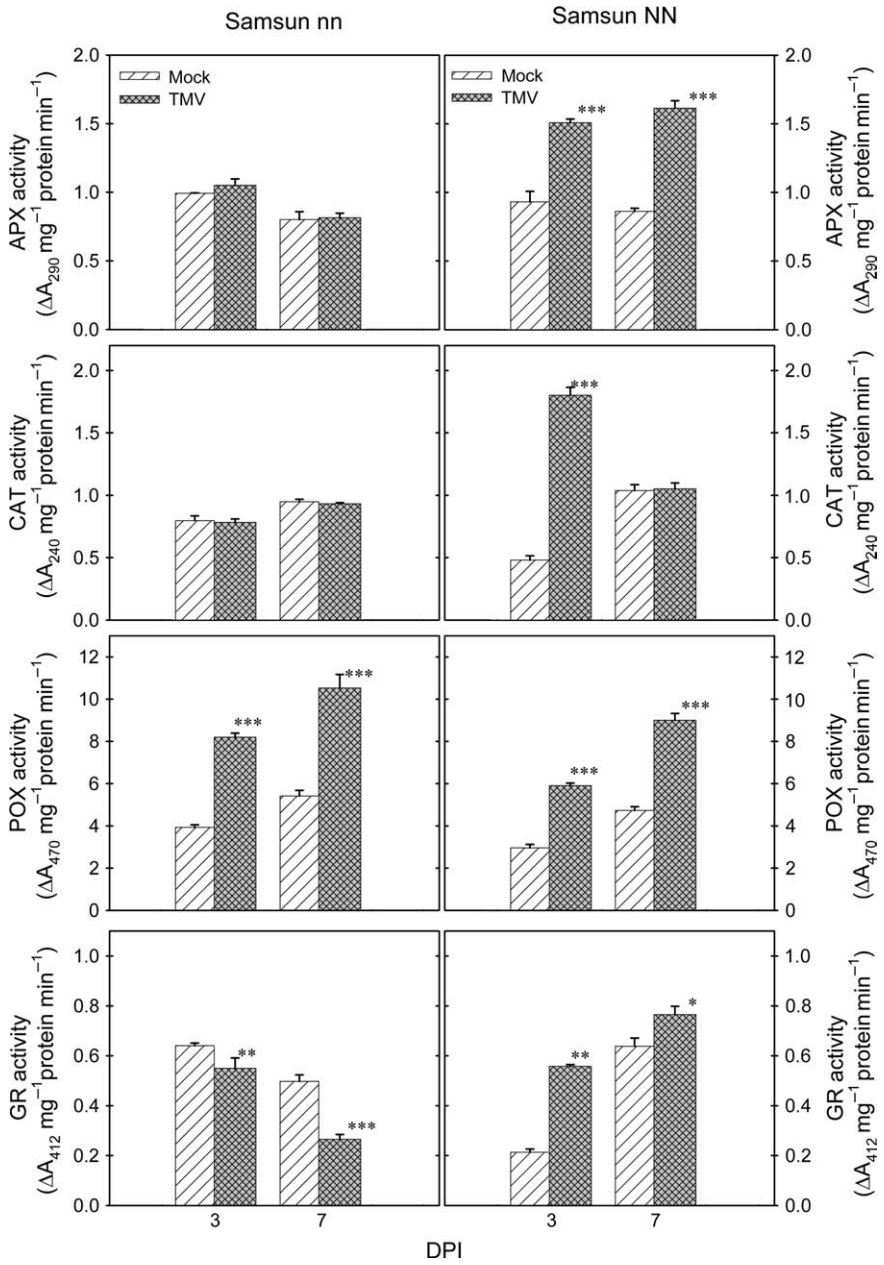

Fig. 5. Activity of APX, CAT, POX and GR in tobacco leaves inoculated with TMV; left panel – susceptible (Samsun nn) and right panel – resistant (Samsun NN) tobacco plants. All values are means + SD. Asterisks denote significant increase of activity of antioxidant enzymes: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

tobacco upon viral infection. Thus, in a recently published high-throughput study (Choi et al. 2006), a series of different metabolites were found to be upregulated in the leaves of resistant tobacco between 7 and 10 DPI, and the content of sesquiterpenoids and diterpenoids (e.g. capsidiol and cembranoids) was found to be especially increased in the upper leaves and thus linked to the systemic resistance. Nevertheless, in the quoted study, polyisoprenoids and other hydrophobic metabolites escaped analysis because of the extraction methods applied. Our experiments add hydrophobic polyisoprenoid alcohols and PQ to the list of secondary metabolites mobilized in plant leaves by avirulent pathogens.

In contrast to the resistance response to pathogen, polyisoprenoids do not seem to be involved in responses to mechanical wounding, at least not in our experimental

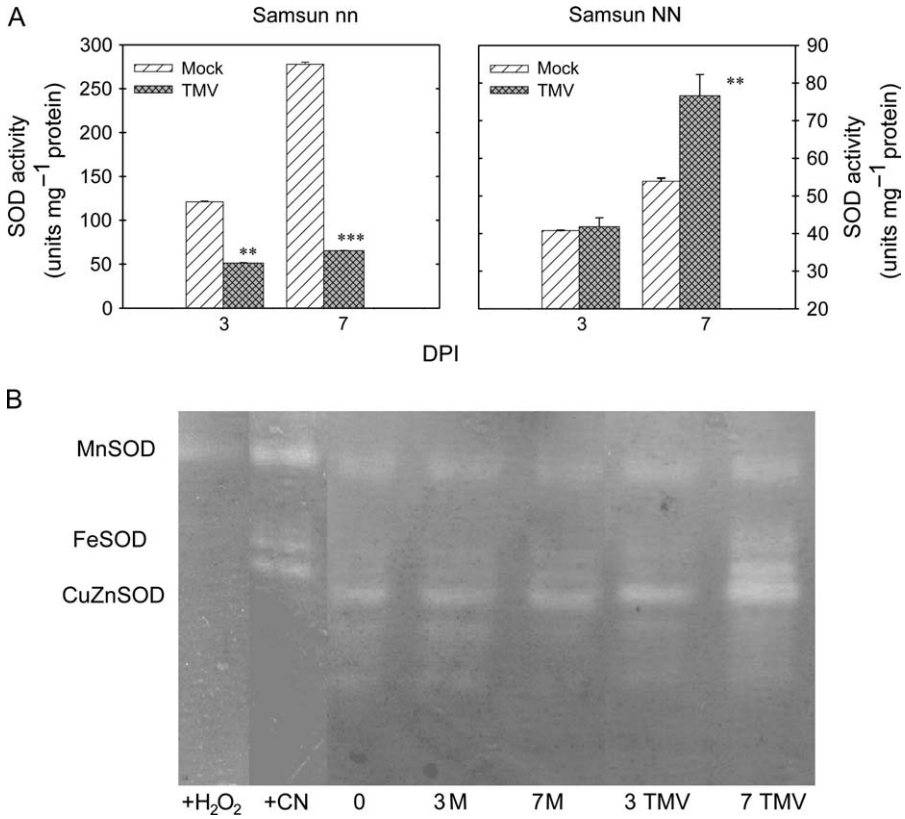

Fig. 6. Activity of SOD in leaves of *Nicotiana tabacum* infected with TMV. (A) Total SOD activity estimated for susceptible (left panel) and resistant (right panel) tobacco plants; (B) activity profile of SOD isoforms in *N. tabacum* Samsun NN. CN designates the supplementation of reaction with sodium cyanide, M and TMV stand for mock and TMV-inoculated leaves, respectively. All values are means + SD.

conditions. This requires further study because biotic stress (pathogen attack) as well as abiotic stress (in particular heavy metals) often induce synthesis and accumulation of the same defense-related secondary metabolites in higher plants (Mithöfer et al. 2004). Indeed, increased accumulation of polyisoprenoid alcohols has been found in plant roots treated with cadmium salts (Bajda and Skorupinska-Tudek, unpublished). Interestingly, several xenobiotics as well as pathogenic conditions (e.g. cancer) have been shown to induce accumulation of dolichol in mammalian liver (Chojnacki and Dallner 1988).

Regulation of polyisoprenoid accumulation

Accumulation of polyisoprenoids seems to result from induction of their de novo biosynthesis. Analysis of expression of *Arabidopsis* CPT and TPT (AtGenExpress project) revealed induction of transcripts of both leaf-specific AtCPT-encoding genes (1.5- to 3.5-fold) and one of the two genes encoding AtTPT (synonym solanesyl diphosphate synthase 1, SPS1) (1.5-fold) after infection with a virulent strain of *P. syringae* pv. tomato DC3000. Infection with a corresponding avirulent strain (*P. syringae* pv. tomato avrRpm1) induced the expression of only one of the AtCPT-encoding genes (two-fold) and none of AtTPTs. In contrast to CPT, expression of AtTPT-encoding genes was also induced by *Phytophthora infestans* (up to 3- and 3.7-fold for SPS1 and SPS2, respectively). Induction of CPT and TPT transcription in *Arabidopsis* upon infection with the avirulent strain of *P. syringae* agrees with the increase in polyisoprenoid biosynthesis observed here. Lack of enhanced accumulation of polyisoprenoid alcohols in the leaves of infected susceptible tobacco plants is not in line with the induction of *Arabidopsis* prenyltransferases caused by virulent *P. syringae*. This discrepancy might be possibly explained by differences between tobacco and *Arabidopsis* in mechanisms of response to pathogen.

In plant cells, there are two sources of IPP used by both prenyltransferases as building blocks for sequential elongation of the hydrocarbon skeleton. The two pathways are the classical mevalonate (MVA) pathway localized in cytoplasm and the newly recognized

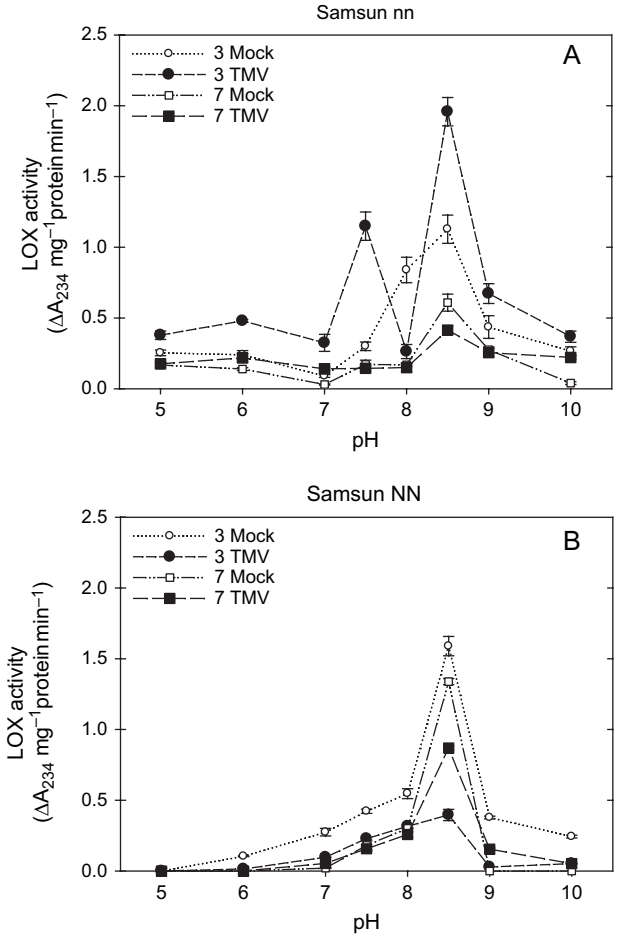

Fig. 7. LOX activity in tobacco leaves infected with TMV; left panel – susceptible and right panel – resistant tobacco plants.

methylerythritol phosphate (MEP) pathway localized in plastids (Eisenreich et al. 2004, Rohmer 1999). Similarly to dolichols accumulated in plant roots, polyisoprenoids in tobacco leaves are presumably 'mosaic compounds' derived from both pathways (Skorupinska-Tudek et al. 2008). A mixed biosynthetic origin might enable the plant cell to tightly regulate the polyisoprenoid level upon changing environmental conditions. Enzymes of both pathways have been shown to undergo upregulation as a result of pathogen attack. For example, transcription of 3-hydroxy-3-methylglutaryl CoA reductase, a key regulator of the MVA pathway, was induced in TMV-infected tobacco leaves (Kang et al. 1998), while transcripts of two key enzymes of the MEP pathway, 1-deoxyxylulose 5-phosphate synthase and 1-deoxy-xylulose 5-phosphate reductoisomerase, were amplified by a chitin elicitor in rice cells (Okada et al. 2007).

Because exogenous SA did not stimulate accumulation of polyisoprenoids, it seems that a different signal triggers the induction of polyisoprenoid biosynthesis in tobacco, e.g. phytohormones.

So far, significant increases in polyisoprenoid content have been found in all tissues upon aging. Increased content of polyisoprenoids upon aging and pathogen infection is in line with the observed relationship between senescence and disease development, suggesting the induction of a common set of genes by these two processes. This link could involve programmed cell death (Lim et al. 2007).

Putative mechanisms of polyisoprenoid involvement in plant defense strategy

One possibility to consider is a function of polyisoprenoids as modulators of the physical properties of biological membranes. A recent study has shown that incorporation of polyisoprenoids into model membranes (built of synthetic phosphatidylcholine analogues) is disruptive for the bilayer. Polyisoprenoids and their phosphates perturbed the orientation of the fatty acyl chains, which likely results in the polyisoprenoid-induced conformational change of the model membranes leading to the formation of a hexagonal II phase (Zhou and Troy 2005). Pivotal processes regulated by membrane fluidity include cellular expansion as well as uptake, secretion and compartmentalization of nutrients and metabolites. Furthermore, adjustment of membrane fluidity maintains an environment suitable for the function of critical integral membrane proteins during stress, e.g. photosynthetic machinery proteins in plants (Upchurch 2008) or heat-shock proteins (HSP) in mammals (Vigh et al. 2007). It has been suggested that physical properties of the membranes are important for regulation of the HSP response. Consequently, chemical modulation of membrane properties has been proposed as a 'membrane-lipid therapy' (Vigh et al. 2007).

Modification of membrane fluidity is believed to be mediated mainly by changes in the levels of unsaturated fatty acids. Increased fatty acid unsaturation in the membranes (Upchurch 2008, Wallis and Browse 2002) has been correlated with increased stress tolerance in plants. It seems plausible to assume that polyisoprenoids work in concert with polyunsaturated fatty acids and exert their bilayer-fluidizing effect also in vivo. Such a requirement is even better understood in the context of the increased content of α-tocopherol and carotenoids observed during biotic stress because these antioxidants increase the rigidity of the membranes (Gruszecki and Strzalka 2005).

Another possible mechanism by which polyisoprenoids protect biological membranes is by 'shielding' of other lipids and of integral membrane proteins. Several

reports suggest that volatile isoprenoids confer additional plant protection that can cooperate with carotene and tocopherols or serve as alternative defense system when the former mechanisms are not sufficient upon oxidative stress (Penuelas and Munne-Bosch 2005). The ROS scavenging function of membrane components seems of special importance for the reduction of ROS generated by integral membrane enzymes, e.g. plasma membrane NADPH oxidase. Additionally, it seems possible that membrane lipids are the first targets for the destructive ROS species because it is energetically cheaper to remove and replace a damaged lipid molecule than a protein molecule.

PQ involvement in plant defense against stress

The content of PQ was doubled in tobacco leaves in all types of stress tested. Because solanesyl diphosphate is a substrate for PQ biosynthesis, simultaneous increases in Sol and PQ content might be possibly explained by utilization of Sol for PQ biosynthesis (Fig. 1). Such a shunt would, however, require the activity of a solanesol kinase. So far, there are no data concerning this enzyme, although kinases of mainly-*cis* polyprenols and dolichols from plants and other organisms have been described (Rymerson et al. 1992 and references in Swiezewska and Danikiewicz 2005).

The doubled content of PQ together with the negligible changes in photosynthetic intensity indicate that the increased cellular pool of PQ is most probably used for defense against ROS rather than for the maintenance of the photosynthetic chain. Thus, the content of PQ was doubled in detached potato leaves treated with a *P. infestans*-derived elicitor (Maciejewska et al. 2002). A possible effect of the increased concentration of PQ and/or its redox state on the regulation of gene transcription might also be considered (Pfannschmidt et al. 2001).

Enzymatic antioxidants are involved in tobacco resistance to TMV

In the leaves of resistant tobacco plants, in contrast to susceptible ones, enzymatic activities of all the tested enzymatic antioxidants were increased after TMV infection. Upregulation of APX was reported after TMV infection of tobacco (Kang et al. 1998) leaves. However, in many reports, decreases in APX and CAT activities with simultaneous enhancement of the ROS level during biotic stress have been noted (Guo et al. 2000). Simultaneous suppression of ROS scavenging activity and enhanced production of ROS may be crucial for the activation of programmed cell death (Mittler 2002). Biotic stress often results in lipid peroxidation of polyunsaturated fatty acids and two mechanisms were found to contribute to this process, an enzymatic mechanism driven by LOX and a non-enzymatic mechanism driven by direct attack of ROS. Surprisingly, LOX activity was decreased in inoculated leaves of resistant tobacco plants. An increased level of peroxidation (measured as MDA and HAE level) together with decreased LOX activity indicate a major role of non-enzymatic lipid peroxidation in the leaves of resistant Samsun NN plants after TMV inoculation.

In conclusion, we suggest that polyisoprenoid alcohols (polyprenols and solanesol) might play a protective role in tobacco leaves upon TMV infection, both locally and systemically. Polyisoprenoids might be of particular relevance in adaptation of plant species to adverse climatic conditions and might have provided plants with a selective advantage for growing in unfavorable habitats. PQ together with a broad range of antioxidant enzymes are involved in regulation of the antioxidant potential required for tobacco resistance against viral infection. These two antioxidant systems are most probably dedicated to protection of different molecular species/cellular compartments – hydrophobic plastoquinol acts within the lipid bilayer rather than in the cytoplasm. Moreover, activation of both these systems is shifted in time – upregulation of enzymatic antioxidant machinery persists for a shorter period of time than PQ. Further experiments are required for detailed elucidation of the mechanism by which polyisoprenoid alcohols and PQ participate in plant resistance response to pathogens. Finally, resistant response of plants against pathogen results in global metabolic changes, which are supposed to overcome the consequences of the pathogen invasion. However, such changes are also aimed at protection of the plant against subsequent infections. Thus, it is also possible that polyisoprenoids serve as components of the defense machinery designed for such repeated attacks of pathogens.

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