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Overexpression of Plastidic Protoporphyrinogen IX Oxidase Leads to Resistance to the Diphenyl-Ether Herbicide Acifluorfen[1]

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The use of herbicides to control undesirable vegetation has become a universal practice. For the broad application of herbicides the risk of damage to crop plants has to be limited. We introduced a gene into the genome of tobacco (*Nicotiana tabacum*) plants encoding the plastid-located protoporphyrinogen oxidase of Arabidopsis, the last enzyme of the common tetrapyrrole biosynthetic pathway, under the control of the cauliflower mosaic virus 35S promoter. The transformants were screened for low protoporphyrin IX accumulation upon treatment with the diphenyl ether-type herbicide acifluorfen. Leaf disc incubation and foliar spraying with acifluorfen indicated the lower susceptibility of the transformants against the herbicide. The resistance to acifluorfen is conferred by overexpression of the plastidic isoform of protoporphyrinogen oxidase. The in vitro activity of this enzyme extracted from plastids of selected transgenic lines was at least five times higher than the control activity. Herbicide treatment that is normally inhibitory to protoporphyrinogen IX oxidase did not significantly impair the catalytic reaction in transgenic plants and, therefore, did not cause photodynamic damage in leaves. Therefore, overproduction of protoporphyrinogen oxidase neutralizes the herbicidal action, prevents the accumulation of the substrate protoporphyrinogen IX, and consequently abolishes the light-dependent phytotoxicity of acifluorfen.

Protoporphyrinogen oxidase (EC 1.3.3.4) (PPOX) is the last enzyme in the common tetrapyrrole biosynthesis pathway before the pathway branches toward chlorophyll and heme synthesis. PPOX catalyzes the oxygen-dependent oxidation of non-fluorescent protoporphyrinogen IX to fluorescent protoporphyrin IX and is associated with the chloroplast envelope and the thylakoid membrane (Matringe et al., 1992a). Parallel to the plastidic tetrapyrrolic pathway, activities of the last two enzymes of the heme synthesizing pathway, PPOX and ferrochelatase, were found in mitochondria (Jacobs et al., 1991; Smith et al., 1993) Thus, protoporphyrinogen IX is distributed between the plastidic pathway and the mitochondrial heme synthesis pathway. The control mechanism of the intercompartmental allocation is still not known. We recently identified two different cDNA sequences for PPOX in tobacco (*Nicotiana tabacum*). The deduced protein sequences designated as PPOX I and II have molecular masses of 60 and 55 kD, respectively, and share only 30% similarity. Translocation studies and immunological analysis proved that the proteins are imported either exclusively into plastids (PPOX I) or into mitochondria (PPOX II) (Lermontova et al., 1997).

PPOX I and PPOX II closely resemble known PPOX sequences, e.g. from Arabidopsis (Ward and Volrath, 1995; Narita et al., 1996), *Chlamydomonas reinhardtii* (Randolph-Anderson et al., 1998), human (Nishimura et al., 1995), or *Bacillus subtilis* (Hansson and Hederstedt, 1992). This eukaryotic-type PPOX is structurally different from the 21-kD HemG protein that is involved in protoporphyrinogen oxidation in *Escherichia coli* (Sasarman et al., 1993) and is assumed to be enzymatically active as a constituent of an electron transfer chain (Jacobs and Jacobs, 1977). The *hemG* mutation causes accumulation of protoporphyrin IX (Sasarman et al., 1993). HemG and PPOX share a common functional role and, consequently, the *E. coli hemG* mutant could be successfully complemented by eukaryotic PPOX cDNA sequences (e.g. Nishimura et al., 1995; Narita et al., 1996; Lermontova et al., 1997).

PPOX is the target site for photodynamically active herbicides of the diphenyl-ether type (Matringe et al., 1989; Duke et al., 1990). Their bicylic structure allows a competitive inhibition by filling the complementary space of the binding site for the natural substrate (Matringe et al., 1992b; Nandihalli et al., 1992). The toxicity of these herbicides is light dependent and involves intracellular peroxidation promoted by the accumulation of PPOX's substrate, protoporphyrinogen IX. It is assumed that excess protoporphyrinogen leaks out of the plastid and is oxidized to protoporphyrin IX by an unspecific plasma membrane-bound peroxidase, which is at least not sensitive to acifluorfen (Matringe and Scalla, 1988; Sandmann et al., 1990; Jacobs et al., 1991; Lee and Duke, 1994). The deleterious effects of protoporphyrin IX occur because it cannot be re-channeled into the plastid-located pathway (Jacobs et al., 1991; Jacobs and Jacobs, 1993; Lee et al., 1993).

Protoporphyrin(ogen) absorbs light energy that can only be used in detrimental reactions in which energy and or electrons are subsequently transferred onto oxygen, resulting in the formation of highly reactive oxygen species. The consecutive photooxidation leads to a rapid degradation of membranes, proteins, and DNA. Ultimately, this damage ends with cellular death. The necrotic phenotype of

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herbicide-treated plants displays leaf desiccation, veinal necrosis, and leaf deformation (Böger and Wakabayashi, 1999).

Deregulation of plant porphyrin biosynthesis results in injury symptoms similar to those after herbicide treatment. In transgenic plants, antisense RNA synthesis diminishes the expression of uroporphyrinogen decarboxylase and coproporphyrinogen oxidase, two preceeding enzymes in tetrapyrrole biosynthesis, and exhibits a light-dependent necrotic phenotype (Kruse et al., 1995; Mock and Grimm, 1997). This necrosis is due to accumulation of photosensitizing porphyrins derived from the enzyme's substrates, uroporphyrinogen and coproporphyrinogen. In a mutator-tagged maize mutant, deficiency in uroporphyrinogen decarboxylase activity leads to developmentally and light-dependent necrotic spots on leaves (Hu et al., 1998) and therefore mimics herbicide- and pathogen-dependent phenomena.

Natural tolerance against peroxidizing herbicides varies among plant species and is sometimes explained by variations in tetrapyrrole metabolism. Herbicide susceptibility corresponds to the accumulation of porphyrins, which depends on the rate of the metabolic flux through the pathway (Becerril and Duke, 1989; Sherman et al., 1991; Nandihalli et al., 1992). In general, younger leaves exhibit greater levels of protoporphyrinogen-associated destruction upon herbicide inhibition than older leaves, which is indicative of an active tetrapyrrole metabolism in developing leaves. Thus, older leaves appear to be more herbicide tolerant (Jacobs et al., 1996).

Several strategies have been evolved for obtaining plants resistant to the peroxidizing herbicides directed against PPOX. Screens for resistant spontaneous and induced mutants have been a useful tool. Resistance can be obtained by an alteration of the herbicide binding site of the catalytic cleft of the enzyme, preventing stable binding of specific herbicides. Mutant seedlings and cell cultures have been selected upon PPOX-inhibitor-containing medium with the aim of understanding the molecular mechanism of herbicide resistance and identifying the gene that confers this resistance. A photomixotrophic tobacco cell culture was selected after stepwise increasing of the concentration of the herbicide S23142 (Ichinose et al., 1995). A point mutation, Val389Met, of PPOX I of *C. reinhardtii* conferred herbicide resistance to the *RS-3* mutant (Randolf-Anderson et al., 1998). PPOX originating from microorganisms is poorly inhibited by the known tetrapyrrole-dependent photodynamic herbicides (Dailey et al., 1994). Expression of the less-herbicide-susceptible *B. subtilis* PPOX (*HemY*) in the cytoplasm of transgenic tobacco plants leads to a slight resistance against the herbicide oxyfluorfen (Choi et al., 1998). Jacobs and Jacobs (1993) described a protoporphyrinogen IX degradation mechanism that prevents protoporphyrin IX accumulation in plant cells.

We describe transgenic tobacco plants that overexpress Arabidopsis plastid PPOX I. The action of the herbicide acifluorfen was demonstrated in a comparative analysis of control and transgenic plants containing excessive levels of PPOX I. Laboratory and greenhouse studies were conducted to determine the tolerance of transgenic lines against acifluorfen applied to whole plants or in foliar incubation tests.

MATERIALS AND METHODS

Plant Growth and Harvest

Wild-type (*Nicotiana tabacum* var. Samsun NN) and transgenic tobacco plants were cultivated in growth chambers in a 12-h light (photon flux density 100 μmol m^{-2} s^{-1})/12-h dark cycle (light intensity) at 25°C. Leaves were harvested from 4- to 6-week-old plants, frozen in liquid nitrogen, and stored at −80°C until analysis. All experiments were done with primary transformants. Individuals of the primary transformants were obtained by vegetative propagation.

Construction of Protoporphyrinogen Oxidase Sense mRNA Expression Vector and Plant Transformation

A DNA fragment of approximately 1,600 bp encoding Arabidopsis PPOX I (accession no. D83139; Ward and Volrath, 1995; Narita et al., 1996) was amplified by PCR using the two primers 5′ AA GGA TCC ATG GAG TTA TCT CTT CTC C 3′ and 5′AA GTC GAC TTA CTT GTA AGC GTA CCG, cut with *Bam*HI and *Sal*I and inserted into the same restriction sites of the multiple cloning site of the Bin AR vector (Höfgen and Willmitzer, 1992), a pBIB derivative. This vector contains the selectable plant kanamycin resistance marker, the *Agrobacterium tumefaciens* T-DNA left and right border sequences and the PPOX I sequence in sense orientation between the 5′ cauliflower mosaic virus (CaMV) 35S promoter and the 3′ OCS transcription termination sequence. The resulting plasmid was transformed into the *A. tumefaciens* strain GV 2260 and transferred into tobacco plants by leaf disc transformation (Horsch et al., 1985). The insertion of copies of the transgene into the plant genome was confirmed by kanamycin resistance of regenerated explants and by genomic Southern-blot hybridization or PCR amplification using the Arabidopsis *PPOX I*-specific probe or oligonucleotide primers, respectively.

Porphyrin Analysis

Plant tissue (100 mg) was ground in 1 mL of methanol: acetone:0.1 N NaOH (9:10:1, v/v) and the homogenate was spun at 10,000*g* for 10 min to remove cell debris and proteins. Protoporphyrinogen IX was oxidized to protoporphyrin IX by adding 25 μL of 1 M acetic acid and 25 μL of 2 butanone peroxide per milliliter. Porphyrins were separated by HPLC on a RP 18 column (Novapak C18, 4-μm particle size, 4.6 × 250 mm; Waters Chromatography, Bedford, MA) at a flow rate of 1 mL/min. Porphyrins were eluted with a linear gradient of solvent B (90% [v/v] methanol, and 0.1 M ammonium acetate, pH 5.2) to solvent A (10% [v/v] methanol, and 0.1 M ammonium acetate, pH 5.2). The column eluate was monitored by a fluorescence detector (model 474, Waters, Milford, MA) at λ_{ex}405 nm and λ_{em}625 nm. Protoporphyrin IX was identified and quantified using authentic standards (Kruse et al., 1995).

Herbicide Treatment

Acifluorfen resistance of the transgenic tobacco plants was evaluated by using the leaf disc assay as described by Lee et al. (1993). Four 9-mm-diameter tobacco leaf discs were cut from leaves 5 and 6 (as counted from the top of the plants) and incubated at 25°C in darkness for 20 h in 5 mL of 1% (w/v) Suc and 1 mM 2-(*N*-morpholino) ethanesulfonic acid (pH 6.5) with various concentrations of acifluorfen. Porphyrins were extracted and their content measured as described above. In a parallel experiment, leaf discs were exposed to light for 6 h after 20 h of dark incubation. As an indication of cellular damage, electrolyte leakage was measured with a conductivity meter according to the method of Orr and Hess (1981).

Four- to 6-week-old plants were sprayed with a volume of 20 mL of a 10 μM acifluorfen solution (in 10 mM Tris/HCl, pH 7.7, and 0.05% [v/v] Tween 80) at the beginning of the dark period. For the analysis of protoporphyrin IX content, leaf material was harvested 18 h (12 h of dark incubation and 6 h of illumination) and 3, 5, and 7 d after foliar spraying.

Determination of Chlorophyll Content and 5-Aminolevulinic Acid (ALA)-Synthesizing Capacity

Chlorophyll concentration was determined in alkaline acetone extracts according to Porra et al. (1989). The ALA synthesizing capacity was measured as described by Papenbrock et al. (1999). Three leaf discs of the fourth leaf of the plant were harvested for each sample, incubated in 20 mM phosphate buffer containing 40 mM levulinic acid in the light for 6 h, and then frozen in liquid nitrogen. Samples were homogenized, resuspended in 1 mL of 20 mM K_2HPO_4/KH_2PO_4, pH 6.9, and centrifuged. The 500-μL supernatant was mixed with 100 μL of ethylacetoacetate, subsequently boiled for 10 min, and cooled down for 5 min. An equal volume of modified Ehrlichs reagent was added and the absorption of the chromophore was determined at λ 553 nm with the spectrophotometer (Mauzerall and Granick, 1956). Standard curves were used for calculating the amounts of accumulated ALA.

RNA and DNA Analysis

Total RNA was extracted as described by Chomczinsky and Sacchi (1987) and analyzed by northern blotting. DNA extraction and Southern analysis were carried out according to standard procedures (Sambrook et al., 1989). Aliquots of 15 μg of RNA and of 8 μg of DNA were analyzed. Filters containing RNA and DNA were probed with cDNA fragments radioactively labeled with [^{32}P]dCTP by random priming. Northern-blot analysis was performed with plant material from two harvests.

Extraction of Total Leaf Protein and Western-Blot Analysis

Plant material (100 mg) was ground under liquid nitrogen and suspended in 1 mL of solubilization buffer (56 mM Na_2CO_3, 56 mM dithiothreitol [DTT], 2% [w/v] SDS, 12% [w/v] Suc, and 2 mM EDTA). After 10 min of incubation at 70°C, cell debris were removed by centrifugation. Aliquots of the supernatant containing 10 μg of protein were subjected to western-blot analysis as previously described (Kruse et al., 1995). Antisera were raised against recombinant PPOX I and PPOX II proteins as described previously (Lermontova et al., 1997).

Protoporphyrinogen Oxidase Assay

Plastids were isolated from 8 g of leaf material of 4-week-old tobacco plants. Leaves were homogenized with a blender for 10 s in 40 mL of homogenization buffer (0.5 M sorbitol, 0.1 M Tris/HCl, pH 7.5, 1 mM dithiothreitol, and 0.1% [w/v] bovine serum albumin) and the homogenate was filtered through nylon gauze (100 μm). Plastids were collected by centrifugation at 5,000*g* for 10 min and resuspended in 2 mL of assay buffer containing 0.1 M Tris/HCl, pH 7.5, 5 mM DTT, 1 mM EDTA, and 0.03% (v/v) Tween 80. The incubation mixture contained 70 μL of chloroplast suspension, corresponding to 0.5 mg of protein and 130 μL of assay buffer. The reaction was started by adding 20 μL of approximately 130 μM protoporphyrinogen IX substrate. After 5 min, the reaction was stopped by adding 1 mL of ice-cold methanol:DMSO (8:2, v/v). The mixture was centrifuged, and the resulting supernatant was flushed with nitrogen and subsequently applied to HPLC. A boiled chloroplast suspension was assayed to determine the autoxidation to protoporphyrin IX (Guo et al., 1991). Inhibition of PPOX in the chloroplast suspension was tested by applying a final concentration of 1 μM acifluorfen to the assay. The plastid suspension was pre-incubated for 5 min before the reaction was started with protoporphyrinogen IX. Porphyrins were directly analyzed by HPLC as described above. The biochemical analysis of enzyme activities and the determination of tetrapyrrole endproducts and precursor were performed at least in triplicate on each independent sample.

RESULTS

Selective Germination and Growth of Transgenic Tobacco Seeds Expressing PPOX I in the Presence of Acifluorfen

The full-length cDNA sequence encoding the Arabidopsis PPOX I was inserted between the CaMV 35S promoter and the 3′ termination sequence of the octopine synthase gene of the binary plant vector BinAR. This gene construct was introduced into the tobacco genome by *A. tumefaciens*-mediated transformation. Approximately 125 individual transgenic lines grown on kanamycin-containing medium were generated, transplanted to soil, and grown to maturity in the greenhouse. Plants were analyzed for the accumulation of PPOX I by western-blot analysis. Thirty-eight lines showed significant increases in PPOX I levels compared with control plants. These lines were phenomenologically indistinguishable from control plants and displayed the same growth rate. None of the lines displayed bleached or necrotic leaf lesions as a result of transgene expression.

In pre-experiments the lethal concentration of acifluorfen in wild-type tobacco seeds was determined. Seeds were sterilized and germinated on sterile Murashige-Skoog agar medium containing 50, 100, 200, and 300 nM acifluorfen in the light. A the sublethal concentration of 100 nM acifluorfen caused delayed germination of the wild-type seeds and bleaching of leaves, while 200 nM acifluorfen completely suppressed germination. Seeds from selected primary transformants containing PPOX I transgenes germinated on medium containing 300 nM acifluorfen. Germination of the T_1 seeds was inhibited only at concentrations of more than 500 nM acifluorfen. Seedlings of a representative transgenic line germinating on 300 nM acifluorfen containing medium are shown in Figure 1. Wild-type seeds did not germinate in the presence of the herbicide.

Selection of Acifluorfen Tolerant Transgenic Plants by Determining Protoporphyrin IX Accumulation upon Herbicide Incubation

For the subsequent evaluation of the PPOX overexpressing plants, 38 selected lines were grown for 5 weeks in soil. Three discs of leaves 5 and 8 (counting from the top of the plant) were incubated in buffer containing 500 nM or 1 μM of acifluorfen for 20 h in the dark. Porphyrins were subsequently extracted and analyzed by HPLC. Less protoporphyrin IX accumulated in the cells of most transgenic lines compared with control plants. Protoporphyrin IX levels were slightly lower in leaf 8 of wild-type and transgenic plants, but the ratios between the wild-type and transgenic levels of accumulating protoporphyrin IX did not differ in young and old leaves.

For the detailed analysis of the effects of PPOX overexpression in transgenic lines, three lines designated S7, S16, and S19 were selected to determine the levels of accumulating protoporphyrin IX and cellular ion leakage as a result of membrane damage after herbicide treatment (Fig. 2). Peroxidative degradation of porphyrins in the cytoplasm affects membrane integrity. The degree of ion leakage is used to quantitatively define the phytotoxicity of the peroxidizing herbicide. Becerril and Duke (1989) showed that ion leakage of cell membranes is correlated with the content of accumulated protoporphyrin IX. Protoporphyrin IX contents are presented from discs of leaves 5 and 6 after a 20-h dark incubation in various concentrations of acifluorfen in the range of 0.5 to 100 μM are presented in Figure 2A. Control plants accumulated at least three times more protoporphyrin IX than the transgenic plants at all concentrations of acifluorfen. An additional exposure to light for 6 h resulted in an immediate increase of protoporphyrin IX content in wild-type leaves at low acifluorfen concentrations, while only incubation of 10 μM acifluorfen led to elevated protoporphyrin IX levels in the leaves of the transformants. The control leaves collapsed during incubation with acifluorfen concentration of more than 10 μM (Fig. 2B). The membrane permeability as indicated by conductivity measurements did not significantly change in leaf discs of the transgenic lines incubated with up to 10 μM acifluorfen, while control leaves showed a rapid increase of ion leakage in the presence of more than 1 μM acifluorfen (Fig. 2C).

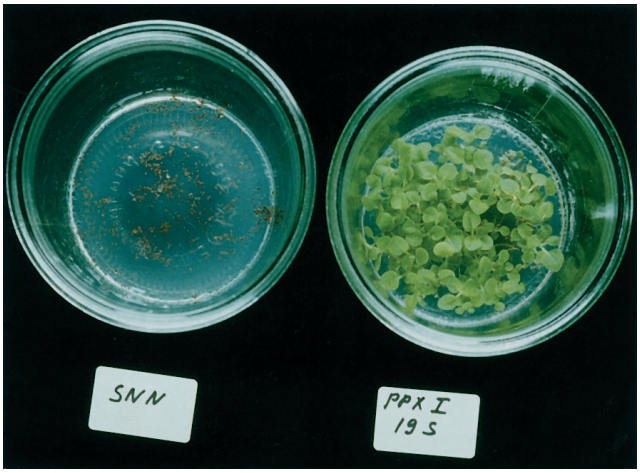

Figure 1. Germinating seeds from tobacco wild type (SNN) and the T_1 generation of the PPOX I-overexpressing line S19 on Murashige and Skoog medium containing 300 nM acifluorfen.

Physiological and Molecular Analysis of Plants Overexpressing PPOX I Protein

The differences in the chlorophyll content of wild-type and transgenic lines S7, S16, and S19 were low, indicating that overexpression did not have a significant effect on chlorophyll levels (Table I). Line S7 contained on average 13% less chlorophyll and line S19 contained approximately 10% more chlorophyll than control. The amount of accumulating protoporphyrin IX upon acifluorfen treatment depends on the rate of synthesized protoporphyrin IX. ALA formation is the limiting step in tetrapyrrole synthesis and determines the rate of precursors for the synthesis of end products. A lower metabolic rate would lead to less porphyrin accumulating in the presence of the inhibitor. The capacity of ALA synthesis was similar or slightly higher in the transgenic lines compared with wild type (Table I). Thus, the lesser accumulation of protoporphyrin IX and the resulting weaker phototoxic effects in the transformants are not due to a reduced ALA synthesis rate.

Total RNA was extracted from leaves 2, 4, and 6 of wild-type and transgenic tobacco plants. Northern-blot analysis was performed with radioactively labeled cDNA probes encoding the Arabidopsis *PPOX I* and tobacco *PPOX II* (Fig. 3). From our previous work it was known that the endogenous tobacco PPOX I transcript levels transiently increase during plant development, with a maximum in leaves with a high photosynthetic capacity and a subsequent decline toward older leaves (Lermontova et al., 1997). The tobacco PPOX I RNA did not hybridize with the Arabidopsis cDNA under the hybridization conditions used. The Arabidopsis *PPOX* I transcripts accumulated in the transgenic lines and the expression levels hardly varied during leaf development. PPOX II transcripts were most abundant in young leaves and did not differ between the corresponding leaves of wild-type and transgenic plants (Fig. 3, top).

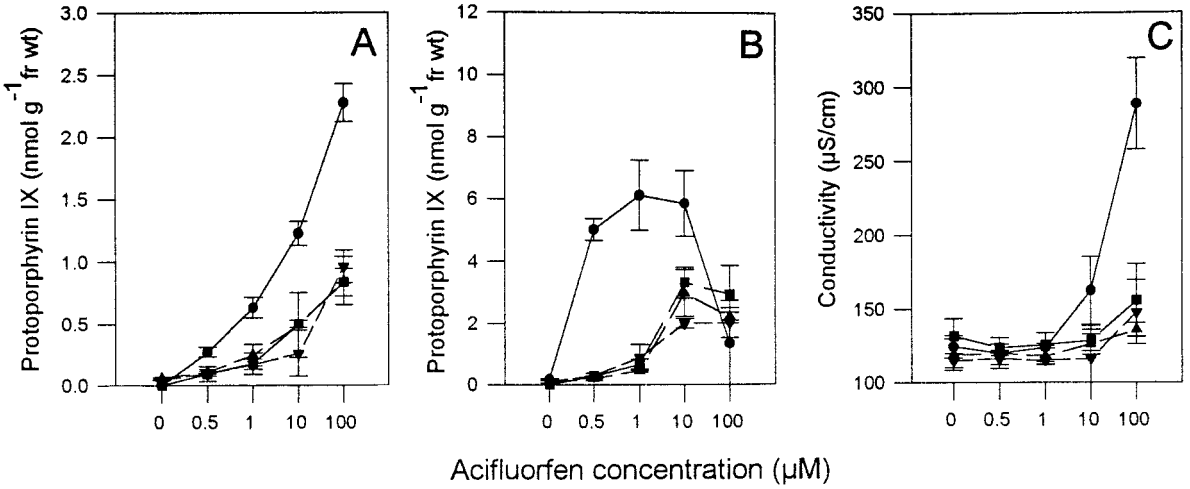

Figure 2. Effects of the peroxidizing herbicide acifluorfen on protoporphyrin IX content and membrane integrity. Discs of leaves 5 and 6 (counting from the top of the plant) of 4-week-old wild-type and PPOX I-over-expressing plants (lines S7, S16, and S19) were incubated at 25°C with various concentrations of acifluorfen either 20 h in darkness (A) or 20 h in darkness and 6 h in light (B and C). Protoporphyrin IX was extracted and quantified by HPLC as described in "Materials and Methods" (A and B). Electrolyte leakage was measured from leaf discs with a conductivity meter (C). Data are combined results from two independent experiments using three individual plants each. SDs are shown. ●, SNN; ■, S7; ▲, S16; ▼, S19.

The amounts of PPOX I and PPOX II protein in leaf extracts of transformants and control plants were determined using the antiserum against the respective recombinant tobacco PPOX isoform. The intensity of the immunodetectable protein band for PPOX I revealed enormous differences between wild-type and transgenic plants (Fig. 3, bottom): the PPOX I content was at least six times higher in the transgenic lines than in control plants. The steady-state levels of PPOX II protein were not altered in the PPOX I-overexpressing plants compared with the wild type.

Determination of PPOX Enzyme Activity

Activity of PPOX was determined from chloroplast suspensions prepared from 4-week-old transgenic and control plants. The activity assays with extracts from green plastids could be achieved by porphyrin extraction under strictly reducing conditions and a subsequent HPLC separation of protoporphyrin IX from the bulk of chlorophyll. This method distinguished between the amount of fluorescent protoporphyrin IX at the beginning and after different time points. Furthermore, the amount of protoporphyrin IX that is enzymatically formed can be determined by subtracting the amount of protoporphyrin IX formed in the heat-denatured sample. PPOX activity was increased in the three selected lines compared with the wild type. Extracts of lines S7 and S16 displayed a 6-fold-higher PPOX activity than the wild type, and line S19 a 5-fold-higher activity (Fig. 4).

The addition of 1 μM acifluorfen to the enzyme assays completely abolished the PPOX activity of control extracts.

Table I. *ALA-synthesizing capacity and chlorophyll content in leaves of wild-type (SNN) and transgenic tobacco plants overexpressing Arabidopsis PPX I*

ALA and chlorophyll were measured photometrically as described in "Materials and Methods." Values represent means ± SD of one extraction from identical leaves of three independent plants.

	ALA	Chlorophyll	
	Leaf no. 4	Leaf no. 4	Leaf no. 6
	nmol g^{-1} fresh wt	*$\mu g\ g^{-1}$ fresh wt*	
SNN	403.19 ± 63.12	947.22 ± 31.96	1,050.13 ± 78.76
S7	409.73 ± 25.29	824.53 ± 8.60	921.52 ± 11.72
S16	440.34 ± 51.79	925.13 ± 91.18	1,059.07 ± 183.98
S19	424.80 ± 44.74	1,049.94 ± 100.60	1,170.23 ± 5.38

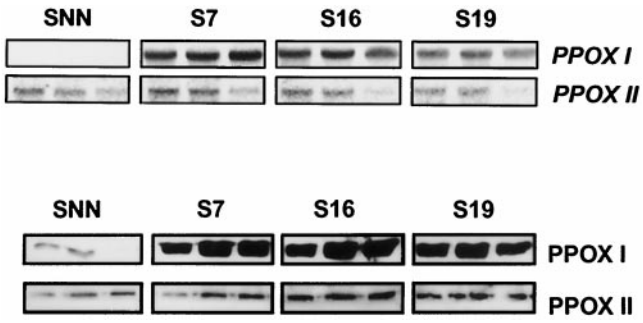

Figure 3. Expression studies in wild-type (SNN) and PPOX I-over-expressing plants (lines S7, S 16, and S19). Top, Northern-blot analysis of PPOX I and PPOX II mRNA levels in leaves 2, 4, and 6 of wild-type (SNN) and PPOX I sense plants. Equal amounts of RNA (10 μg) were separated on formaldehyde-containing agarose gels. Equal loading of RNA was proven by ethidium bromide staining. RNA was blotted onto nylon membranes. The filters were hybridized with fragments of Arabidopsis PPOX I and tobacco PPOX II cDNA probes. Bottom, Western-blot analysis of PPOX I sense and control plants. Total protein extracts were prepared from leaves 3, 5, and 7. Proteins were incubated with antibodies raised against PPOX I and PPOX II. The immune staining for PPOX I was brief in order to demonstrate the large difference between wild-type and transgenic levels of the enzyme.

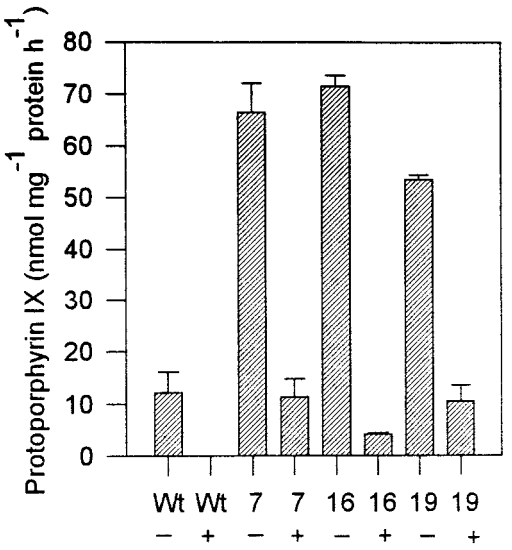

Figure 4. PPOX I enzyme activity in crude chloroplast extracts of PPOX I-overexpressing (lines S7, S16, and S19) and wild-type (Wt) tobacco plants. Crude chloroplast extracts were prepared as described in "Materials and Methods." Enzyme activity were measured without (−) and with (+) 1 μM of acifluorfen. Data are means of three replicates and SDs are shown.

In spite of a relatively high concentration of acifluorfen in the enzyme assays the protoporphyrinogen oxidation capacities of plastid extracts from the transformants were still in a similar range as the activities found in noninhibited wild-type extracts. PPOX I activity of line S16 was inhibited by acifluorfen to 6%, and that of line S7 and S19 to 17% and 13% remaining activity, respectively.

The catalytic oxidation of approximately 12 nmol protoporphyrinogen IX mg^{-1} protein h^{-1} in tobacco wild-type chloroplasts was similar to data obtained with other plant species. Spinach crude thylakoid fractions formed 1 to 4 nmol protoporphyrin IX mg^{-1} protein h^{-1} (Matringe et al., 1992a). Jacobs and Jacobs (1984) reported PPOX activities of spinach or barley chloroplasts of 8 nmol and 18 to 40 nmol protoporphyrin IX mg^{-1} protein h^{-1}, respectively. A PPOX activity of 29 nmol mg^{-1} h^{-1} was reported from a herbicide-resistant tobacco cell culture (Ichinose et al., 1995).

Figure 5. Comparison of the phenotype of transgenic PPOX I-overexpressing line S7 (right) and wild-type plant (left) 3 d after acifluorfen treatment. Each plant was sprayed with 20 mL of a 10 μM acifluorfen solution.

Acifluorfen Treatment of Tobacco PPOX I-Overexpressing and Control Plants

Five-week-old PPOX I-overexpressing and wild-type tobacco plants were sprayed with 20 mL of 10 μM acifluorfen at the beginning of the dark period. Leaf discs were harvested from leaves 3 and 5 for porphyrin analysis 18 h (12 h of dark and 6 h of light) and 3, 5, and 7 d after the treatment. Figure 5 shows a control and a transgenic plant (line S7) 3 d after the single acifluorfen treatment. The selected transgenic plants showed almost no necrotic lesions after the application of acifluorfen, while necrotic areas of entirely desiccated tissue became visible on wild-type leaves. The phenomenological differences between transgenic and control plants reflect the lower photosensitization in the transgenic leaf tissue. Protoporphyrin IX contents were compared from PPOX I-overexpressing and wild-type plants 18 h after herbicide application (Fig. 6). Porphyrin contents in transgenic plants were lower than in wild-type plants (corresponding to 20%–40% of the wild-type value) and were below a certain level that was not phytotoxic to tobacco plants. The amounts of accumulating protoporphyrin IX were continuously lowered in all plants from d 1 to 7 after application of the herbicide due to photooxidative degradation of protoporphyrinogen IX (data not shown).

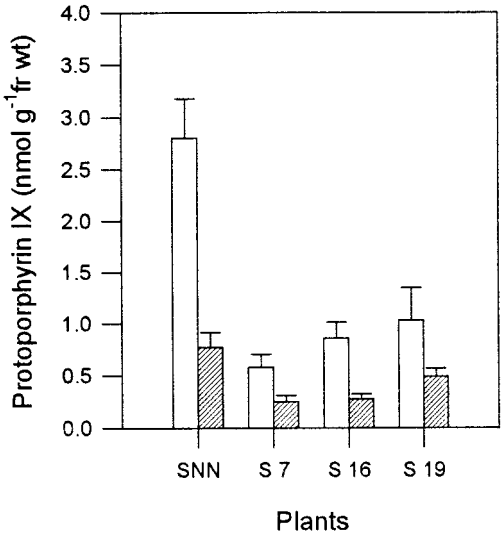

Figure 6. Accumulation of protoporphyrin IX in 5-week-old tobacco wild-type (SNN) and PPOX I sense transgenic plants (S7, S16, and S19) after an 18-h treatment with acifluorfen. Each plant was sprayed with 20 mL of a 10 μM acifluorfen solution. Porphyrins were analyzed from leaves 4 (white bars) and 6 (hatched bars). Data are means of two leaf extractions from two different harvests each.

DISCUSSION

We examined the molecular basis of PPOX I overexpression in tobacco plants leading to resistance against the peroxidizing PPOX inhibitor acifluorfen. A stable transformed gene construct consisting of the CaMV 35S promoter linked to the Arabidopsis cDNA sequence encoding the plastidic PPOX isoform resulted in increased expression (Fig. 3) and activity (Fig. 4) in tobacco transformants. The three transgenic lines analyzed had at least a five to six times higher plastidic PPOX activity than the wild type. PPOX would not be expected to control the metabolic flow through the pathway (Lermontova et al., 1997). It is assumed that regulation of PPOX I expression and activity prevents the accumulation of toxic amounts of protoporphyrin(ogen) IX. The experiments with the PPOX I-overexpressing lines demonstrated that excess PPOX I activity did not significantly enhance the synthesis of protoporphyrin IX and did not increase the chlorophyll pool (Table I). However, excess amounts of PPOX compensated for the herbicidal effects up to a certain inhibitor concentration at which residual PPOX activity still guarantees a normal metabolic flow in the pathway and prevents the leakage into the cytoplasm and the accumulation in the cytosolic membranes of photosensitizing protoporphyrin(ogen) IX as it is shown in leaf disc experiments (Fig. 2, A and B) and after acifluorfen spraying (Fig. 6). Therefore, the tolerance of transgenic plants to treatment with peroxidizing herbicides that is normally inhibitory to PPOX in control plants is explained by the reduced generation of reactive oxygen species as result of inhibited porphyrinogenesis.

Although the higher enzyme levels of PPOX I minimize the toxic effects of the inhibitors, it is likely that the mitochondrial isoform PPOX II is also inhibited by acifluorfen (Lermontova et al., 1997). We did not specifically test inhibitory effects of acifluorfen on PPOX II in the transgenic lines. However, it is conceivable that the inhibition of PPOX I in plastids is more critical because this isoform provides substrate for both chlorophyll and heme synthesis. It is assumed that the inhibition of mitochondrial PPOX II does not result in elevated levels of protoporphyrin IX causing the photodynamic damage. Additionally, a compensatory exchange of heme between plastids and mitochondria could substitute for the lack of heme in mitochondria. If this correct, then the plastidic heme-synthesizing branch could provide heme that is interchangeable among the different cellular compartments, as previously discussed (Watanabe et al., 1998).

Other resistance mechanisms against PPOX inhibition can be used as reference for our approach. Choi et al. (1998) demonstrated diphenyl ether herbicide resistance of transgenic tobacco plants expressing the *B. subtilis* HemY by means of an approximate 50% reduced electrolyte leakage of the transgenic compared with wild-type plants. However, it is not clear if accumulating bacterial protein contributes to the PPOX activity in the plants and if the herbicide tolerance can be explained by the reduced accumulation of protoporphyrin IX. In response to the application of the peroxidizing herbicide S23142, a mutant tobacco cell culture line revealed a 10-times higher transcript level encoding the mitochondrial PPOX II than the control culture; the PPOX I-RNA level was not changed (Watanabe et al., 1998).

The change in PPOX II-RNA content corresponded to a 2-fold increase of total PPOX activity of this cell culture line during photomixotrophic growth, which is sufficient to promote tolerance to the herbicide. The herbicide tolerance was demonstrated by low accumulation of protoporphyrin IX in the mutant culture after herbicide incubation compared with a five times or, transiently, a 20 times higher accumulation of protoporphyrin IX in wild-type culture during dark or light incubation, respectively (Ichinose et al., 1995). The *Chlamydomonas* cell line *RS-3* showed significant PPOX resistance against inhibition by peroxidizing herbicides, resulting in more than 10 times less reduced formation of Mg-porphyrins compared with the control strain (Sato et al., 1994). A point mutation in the PPOX I-encoding gene sequence was found in the RS 3 strain (Randolph-Anderson et al., 1998). Because of the other experimental systems that have been used to demonstrate resistance mechanisms against the peroxidizing effects of herbicides, direct comparison with the PPOX I-overexpressing plants is difficult. The attainment of herbicide tolerance of PPOX I-overexpressing tobacco lines was correlated with the expression of the PPOX I and PPOX II isoforms by means of corresponding antibodies and cDNA probes and the protoporphyrin IX levels accumulating in response to applying herbicide.

To compare with previous observations it is important to define precisely the conditions for the herbicidal inhibition experiments. Under the growth conditions described in "Materials and Methods," neutralization of the toxifying effects of acifluorfen was achieved in the PPOX I-overexpressing transgenic lines. Photodynamic symptoms caused by reactive oxygen species could not be observed in leaves of these lines compared with wild type (Fig. 5). The light dosage (light intensity $\times$ exposure time) and the time of application play a major role in the efficiency of the herbicide action and, inversely, in the protective response of the plant. Application of peroxidizing herbicides before dark causes an improved efficiency on the following day when plants are exposed to sunlight (Wakabayashi and Böger, 1999).

Apart from the environmental factors, the dosage effects of herbicide action depend on physiological conditions of the plants. Plants show a natural variation of the susceptibility to peroxidizing herbicides (Sherman et al., 1991). The resistance mechanisms are very complex and are not completely understood. They can include enzymatic resistance, increased degradation of the herbicide or the accumulating porphyrins, and an improved natural adaptive capacity of the antioxidative pathway for detoxification of reactive oxygen species generated during herbicide action (Böger and Wakabayashi, 1999). We are currently investigating in transgenic tobacco plants expressing Arabidopsis PPOX I whether other resistance mechanisms support the effects of PPOX overexpression. Future exploitations of these plant properties will be required to engineer higher resistance against peroxidizing herbicides.

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