Cloning, molecular biological and physiological characterization of two isoforms of tobacco protoporphyrinogen IX oxidase

Dissertation

zur Erlangung des akademischen Grades
doctor rerum naturalium (Dr. rer. nat.)

vorgelegt der
Mathematisch-Naturwissenschaftlich-Technischen Fakultät
(Fachbereich Pharmazie)
der Martin-Luther-Universität Halle-Wittenberg

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geb. am: 29.01. 1972 in: Weißrussland

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Halle (Saale), 08. 11. 2000

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List of abbreviations

ALA	5-aminolevulinic acid
ALAD	5-aminolevulinic acid dehydratase
APX	ascorbate peroxidase
AsA	ascorbate
A. tumefaciens	*Agrobacterium tumefaciens*
ATP	adenosine triphospate
BSA	bovine serum albumin
CaMV	Cauliflower Mosaic Virus
cDNA	complementary DNA
Chl	chorophyll
Chlide	chlorophyllide
Coprogen	coproporphyrinogen III
Copro	coproporphyrin III
CPO	coproporphyrinogen III oxidase
C. reinhardtii	*Chlamydomonas reinchardtii*
Da	Dalton
DasA	dehydroascorbate
DNA	deoxyribonucleic acid
DPEs herbicides	diphenyl ether-type herbicides
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
FAD	flavine adenine dinucleotide
FeCh	Ferro chelatase
FMN	flavine mononucleotide
GluTR	glutamyl-tRNA reductase
GR	glutathione reductase
GSA	glutamate-1-semialdehyde
GSAAT	GSA aminotransferase
GSH	glutathione (reduced)
GSSG	glutathione (oxidized)
GST	glutathione-S-transferase
HL	high light
HPLC	higher performance liquid chromatography
IPTG	Isopropyl-β-D-thiogalactopyranoside
k	kilo
L	litre
LL	low light
M	molarity
m	milli
µ	micro
MB	monobrombimane
MDAR	monodehydroascorbate reductase
MgCh	Magnesium chelatase
MOPS	N-morpholinopropanesulfonic acid
MS	Murashige and Skoog basal medium
mRNA	messenger RNA

List of abbreviations

n	nano
NADPH	nicotinamide adenine dinucleotide phosphate reduced
N. tabacum	*Nicotiana tabacum*
OD	optical density
PAGE	polyacrylamide gel electrophoresis
Pchlide	protochlorophyllide
PCR	polymerase chain reaction
POR	NADPH-protochlorophyllide oxidoreductase
PPOX	protoporphyrinogen IX oxidase
PPOX I	protoporphyrinogen IX oxidase plastidal isoform
PPOX II	protoporphyrinogen oxidase mitochondrial isoform
Proto IX	protoporphyrin IX
Protogen IX	protoporphyrinogen IX
RNA	ribonucleic acid
rRNA	ribosomal RNA
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
SOD	superoxide dismutase
TBA-RS	thiobarbituric acid-reactive substances
Tris	Tris-hydroxymethylaminomethane
tRNA	transfer RNA
UROD	uroporphyrinogen III decarboxylase
Urogen III	uroporphyrinogen III
URO III	uroporphyrin III

1 Introduction

Tetrapyrroles belong to a group of molecules with a common structure. They are synthesized in a branched pathway, in which various end products are formed to different amounts. The most abundant cyclic tetrapyrroles are chlorophyll (Chl) and heme, which are characterized by a chelated magnesium and ferrous ion, respectively. Chlorophyll is involved in light absorption and energy transduction during photosynthesis. Heme is a cofactor of hemoglobin, cytochromes, P450 mixed-function oxygenases, and catalases. Other members of the class of tetrapyrroles include siroheme (the prosthetic group of nitrite and sulphite reductases) and phytochromobilin, the chromophore of phytochrome, which is involved in light perception.

Tetrapyrrole biosynthesis has been the subject of numerous studies over several decades. But genetic and biochemical characterization of tetrapyrrole biosynthesis has progressed by using approaches to genetically dissect the tetrapyrrole biosynthetic pathway. Pigment-deficient mutants and antisense technology have proved to be useful for examining the mechanisms of metabolic control or for analyzing biochemically the enzymatic steps which are affected by the mutation or by the antisense RNA expression.

Tetrapyrrole intermediates are highly photoreactive. They can easily be excited and transfer the energy or electrons to O_2. Then reactive oxygen species (ROS) are produced upon exposure to light and oxygen. Under normal growth conditions the risk of photooxidative damage from intermediates in tetrapyrrole biosynthesis is low. Excessive accumulation of such intermediates is the result of deregulation of tetrapyrrole biosynthesis. Toxic effects of porphyrins are evident in human patients with deficiencies of one of the enzymes of heme biosynthesis. These patients are suffering from metabolic diseases, which are called porphyrias (Moore, 1993). The importance of avoiding accumulation of porphyrin intermediates causing photo-oxidative damage has been demonstrated by the analysis of transgenic tobacco plants with reduced uroporphyrinogen III decarboxylase (UROD) (Mock et al., 1995; Mock and Grimm, 1997; Mock et al., 1998) and reduced coproporphyrinogen oxidase III (CPO) activity (Kruse et al., 1995b; Mock et al., 1998). The deleterious effect of accumulated tetrapyrroles is also evident in plants treated with a variety of herbicides that act via inhibition of protoporphyrinogen oxidase (PPOX) (Duke et al., 1991; Matringe et al., 1989).

PPOX is the last common enzyme in chlorophyll and heme biosynthesis. There are two

isozymes in higher plants, one located in plastids and the other in mitochondria (Jacobs and Jacobs, 1977; Matringe et al., 1989; Lermontova et al., 1997). The effect of peroxidizing herbicides on PPOX is light dependent and involves intracellular peroxidation promoted by accumulation of protoporphyrin IX (Proto IX), the product of PPOX action (Matringe and Scalla, 1988; Sandmann et al., 1990; Jacobs et al., 1991; Lee and Duke, 1994). Application of herbicides becomes an universal practice in agriculture. Since diphenyl-ether type (DPEs) herbicides, one group of PPOX inhibiting herbicides, are effective at very low dosage, their mechanism of action is an attractive subject for detailed investigation. For a broad application of herbicides the risk of damage to crop plants has to be limited. Several strategies have been evolved for obtaining resistant plants towards the peroxidizing herbicides directed against PPOX.

1.1 Metabolic pathway of tetrapyrrole biosynthesis

A simplified flow diagram of the metabolic pathway for tetrapyrrole biosynthesis is given in Fig.1. The pathway can be subdivided into three sections: 5-aminolevulinate (ALA) synthesis, porphyrin formation from eight molecules of ALA, magnesium or iron ion insertion and modification of the metalloporphyrin to give the end products heme and Chl (Beale and Weinstein, 1990; Smith and Griffith, 1993). Chl synthesis exclusively takes place in chloroplasts, while the last two steps of plant heme synthesis are located in both mitochondria and plastids (Fig. 2). In contrast, in animals, heme synthesis starts in the mitochondria with ALA synthase, continues in the cytoplasm up to the formation of coproporphyrinogen and ends back in the mitochondria with the synthesis of protoheme.

The first committed precursor of tetrapyrrole pathway is ALA and there are two alternative pathways of ALA formation. In plants, algae (including the prokaryotic cyanobacteria), and most bacteria, ALA is formed from the C-5 skeleton of glutamic acid, in a pathway requiring three enzymatic reactions and $tRNA^{glu}$ (Smith and Griffith, 1993). In purple bacteria, in yeast and animal mitochondria ALA is formed by condensation of glycine and succinyl-CoA. This is mediated by the pyridoxal phosphate-requiring enzyme ALA synthase (EC 2.3.1.37) (Gibson et al., 1958). Some organisms, such as *Euglena gracilis* (Weinstein and Beale, 1983) and presumably *Scenedesmus* (Drechsler et al., 1993) possess both pathways. The C5 pathway in *Euglena* provides precursor of Chl synthesis, whereas ALA formed by ALA synthase is used for heme. All components of the C5 pathway are localized in the chloroplast stroma. In

the first step, glutamate is ligated to tRNA. This reaction step is catalyzed by glutamyl-tRNA synthetase (EC 6.1.1.17). The same enzyme functions simultaneously in protein and tetrapyrrole biosynthesis. Like aminoacyl-tRNA formation in general, this reaction requires ATP and Mg^{2+}. Next, the tRNA-bound glutamate is converted to a reduced form by glutamyl-tRNA reductase (GluTR) in a reaction that requires NADPH. The product of this reaction has been characterized as glutamate-1-semialdehyde (GSA) (Houen et al., 1983). The final step of the C5 pathway is catalyzed by the enzyme GSA aminotransferase (GSAAT, EC 5.4.3.8), that transfers an amino group of the C2 of GSA to C1 to form the product ALA. The active enzyme forms a homodimer and contains a vitamin B_6-derivative, either pyridoxamine phosphate or pyridoxal phosphate.

The next part of the pathway to form Proto IX, the last common intermediate between heme and chlorophyll, is probably mechanistically identical in all organisms. Condensation of two molecules of ALA to form the monopyrrole, porphobilinogen, is catalyzed by ALA dehydratase (ALAD, EC 4.2.1.24). Porphobilinogen deaminase (PBGD, EC 4.3.1.8) catalyzes the stepwise addition of four porphobilinogen molecules with the loss of a free amino group at each step, to form the very unstable linear tetrapyrrole 1-hydroxymethylbilan (HMB). The instability of the linear HMB suggests an association between PBGD and the next enzyme in the pathway, Uroporphyrinogen III synthase (UROS, EC 4.2.1.75), to allow a direct transfer of the metabolite. UROS catalyzes the inversion of ring D followed by ring closure of the linear tetrapyrrole to form Uroporphyrinogen III (Urogen III). Urogen III is the last common intermediate that leads to siroheme and Vitamin $B_{12.}$ Uroporphyrinogen decarboxylase (UROD, EC 4.1.1.37) catalyzes the formation of coproporphyrinogen III (Coprogen III) by the sequential removal of carboxyl groups from the four acetate side chains of Urogen III. The next enzyme Coprogen III oxidase (CPO, EC 1.3.3.3) catalyzes the oxidative decarboxylation of the two propionate groups to vinyl groups on rings A and B resulting in formation of protoporphyrinogen IX (Protogen IX). The last common step of the metabolic pathway to heme and chlorophyll is the removal of six electrons from Protogen IX to form protoporphyrin IX (Proto IX), which is catalyzed by Protogen IX oxidase (PPOX EC 1.3.3.4). In plants PPOX exists in two isoforms: plastidic-PPOX I and mitochondrial-PPOX II (Lermontova et al., 1997). Thus, Proto IX is distributed between the plastidic pathway and the mitochondrial heme synthesizing pathway. Insertion of Mg^{2+} into Proto IX initiates the chlorophyll synthesizing, while insertion of Fe^{2+} begins the heme/bilin synthesizing branch.

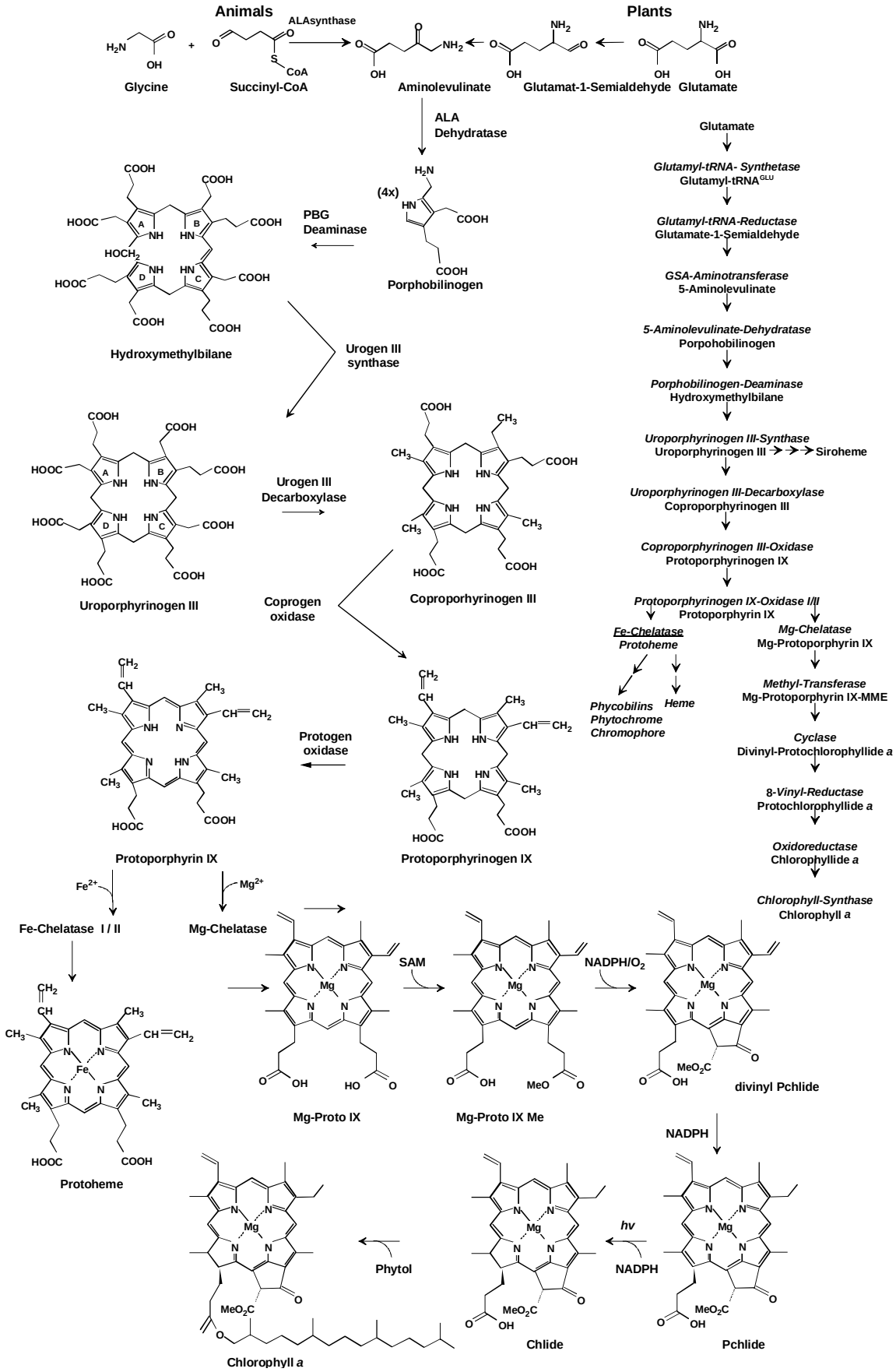


Figure 1: Scheme of the tetrapyrrole biosynthetic pathway (according to Smith and Griffiths, 1993, modified)

The first committed enzyme of the Chl synthesizing branch is the Mg chelatase (MgCh). The plant enzyme consists of three subunits designated CHL I, CHL H and CHL D. Ferro chelatase (FeCh, EC 4.99.1.1) channels the substrate Proto IX to the heme branch and, subsequently, to the phycobilins and phytochromobilins. Although the insertion of a ferrous ion into Proto IX seems to be a comparable reaction to Mg chelation, FeCh does not show any structural similarity to the competing enzyme. FeCh as well as PPOX exist in two isoforms. The enzyme S-adenosyl-L-methionine (SAM) Mg protoporphyrin IX methyl-transferase (MTF, EC 2.1.1.11) substitutes a methyl group from SAM for the hydrogen on the C_{13} propionate group of the Mg porphyrin to form Mg protoporphyrin IX monomethylester (Mg Proto IX MME). In the next step, the enzyme Mg protoporphyrin IX monomethylester cyclase forms a fifth isocyclic ring in the Mg porphyrin macrocycle. Mg Proto IX MME is converted to divinyl protochlorophyllide (Pchlide) using NADPH and oxygen. The 8-vinyl group on ring B of the divinyl Pchlide is reduced by vinyl reductase in the next reaction. Then the membrane associated enzyme NADPH-protochlorophyllide oxidoreductase (POR, EC 1.6.99.1) catalyzes the transreduction of the double bond in ring D to yield chlorophyllide (Chlide). This reaction has an absolute requirement for light. Esterification of the C_{17} propionate group of Chlide with a fatty alcohol, mainly phytol or geranylgeraniol, by Chlide a synthase leads to the formation of Chl a. The next step is an oxidation of the 7-methyl group of Chl a to the 7-formyl group of Chl b. The substrate specificity of this enzyme is broad and the oxygenase accepts, Chlide or Chl a.

1.2 Regulation of chlorophyll biosynthesis

Tetrapyrrole synthesis is regulated by environmental stimuli, such as light and temperature, and by tissue specific and cellular developmental programs. The most important exogenous stimulus for the chlorophyll biosynthetic pathway is light. In dicotyledons chlorophyll accumulation is controlled by light at least at two major sites in the pathway. ALA formation is stimulated by light (Kannangara and Gough, 1978b), and conversion of Pchlide to Chlide requires light (Mapleston and Griffiths, 1980). The control mechanism of tetrapyrrole biosynthesis has to guarantee a regular flow of substrate throughout the pathway to prevent accumulation of highly photoreactive tetrapyrrolic intermediates. It has many levels of regulation from transcriptional to posttranslational events. The earliest control point in the tetrapyrrole metabolic pathway is the synthesis of ALA, which is commonly accepted as the

rate limiting step. Feeding of ALA to etiolated seedlings drastically increases the level of Pchlide. Pchlide is the only intermediate that accumulates to detectable levels in angiosperm seedlings germinated in the dark and it is reduced to Chlide only in the light. On the base of ALA-feeding experiments it has been concluded that ALA synthesis is suppressed in the dark. An explanation for this phenomenon could be the inhibition of ALA formation by the end products of the different branches such as Pchlide and protoheme (Beale and Weinstein, 1990). It has been demonstrated that heme inhibits ALA synthesis in intact plastids (Chereskin and Castelfranco, 1982). As was mentioned before, synthesis of ALA in higher plants requires three enzymatic steps and $tRNA^{glu}$. As a possibility to explain the dark inhibition of ALA synthesis, it has been proposed that the level of $tRNA^{glu}$ may restrict chlorophyll synthesis in the dark. It was previously demonstrated that Pchlide and heme inhibit glutamyl-RNA synthetase from *Chlamydomonas reinhardtii* (*C. reinhardtii)* and *Scenedesmus obliquus* (Chang et al., 1990). However, the transcription of the $tRNA^{glu}$ gene was not changed in *C. reinhardtii* (Jahn, 1992) nor was the level of glutamyl-$tRNA^{glu}$ in barley affected by light (Berry-Lowe, 1987). This suggests that formation of glutamyl-$tRNA^{glu}$ can not be the limiting factor for ALA synthesis in the dark. The fact that in higher plants the same glutamyl-tRNA synthetase charges $tRNA^{glu}$ for protein and ALA synthesis (Bruyant and Kannangara, 1987) supports the hypothesis that inhibition of glutamyl-tRNA synthetase by Pchlide and heme is not a specific site of the regulation. Pontoppidan and Kannangara (1994) showed that proto(heme) inhibits glutamyl-tRNA reductases purified from *Synechocystis sp.* and barley, respectively. Levels of mRNA encoding glutamyl-tRNA reductase were elevated in response to light (Ilag et al., 1994). Expression analysis of the small gene family revealed that mRNA species accumulate differently in light and in plants organs (Bougri and Grimm, 1996; Tanaka et al., 1996). Glutamyl-tRNA reductase activity was increased in greening cucumber cotyledons in comparison to etiolated tissue (Masuda et al., 1996). Increased mRNA and activity levels for GSA aminotransferase were determined in response to light in *C. reinhardtii* cultures and *Arabidopsis* (Kannangara and Gough, 1978b; Ilag et al., 1994; Matters and Beale, 1994), while its RNA levels did not significantly alter in greening barley seedlings (Grimm, 1990). This suggests that post-transcriptional steps may be involved in regulation of light induced ALA formation. At present it is difficult to judge whether a single enzymatic step in ALA synthesis is rate limiting or whether the control is shared among all enzymes involved.

Another important control point in chlorophyll biosynthesis is the reduction of Pchlide. The

enzyme catalyzing this light-dependent reaction is present in etioplasts of dark-grown seedlings, whereas light-grown seedlings contain only traces of POR (Apel, 1981; Batschauer and Apel, 1984; Griffits et al., 1985). Recently different control points of *por* gene expression were investigated (Armstrong et al., 1995; Reinbothe and Reinbothe, 1996). Two *por* genes were found in barley, designated *por A* and *por B*, that are differently expressed in response to light. Expression of *por A* is depressed by light (Apel, 1981; Batschauer and Apel, 1984; Holtorf et al., 1995), while the gene (*por B*) is active in both etiolated and illuminated seedlings (Holtorf et al., 1995). Reinbothe et al. (1995) demonstrated that the POR A precursor is imported to plastids only in the presence of Pchlide. Fully developed chloroplasts have reduced POR A import when the concentration of Pchlide is reduced.

The mechanism of distribution of Proto IX at the branch point for heme and chlorophyll formation might also be an important control point in tetrapyrrole biosynthesis. As mentioned above, in plants heme synthesis takes place in both plastids and mitochondria. For the synthesis of mitochondrial heme Protogen IX has to be transported from plastids into the mitochondria (Fig. 2). Until now export mechanisms of Proto(gen) IX into the mitochondria remain unclear. It is not known how Protogen is released from the plastids. Is there an active export mechanism or simple diffusion through the outer membrane? Regulation of the channelling of Proto IX to the heme or Chl-synthesizing branches was investigated by Papenbrock et al. (1999). It has been demonstrated that the activity of Fe-chelatase and the level of its RNA showed a maximum just at the transition from light to dark and oscillated with a phase approximately opposite to that of Mg-chelatase activity. It is suggested that the activities of Mg- and Fe-chelatase contribute to a coordinated allocation of Proto IX to either chlorophyll or heme synthesis under photoperiodic cycles.

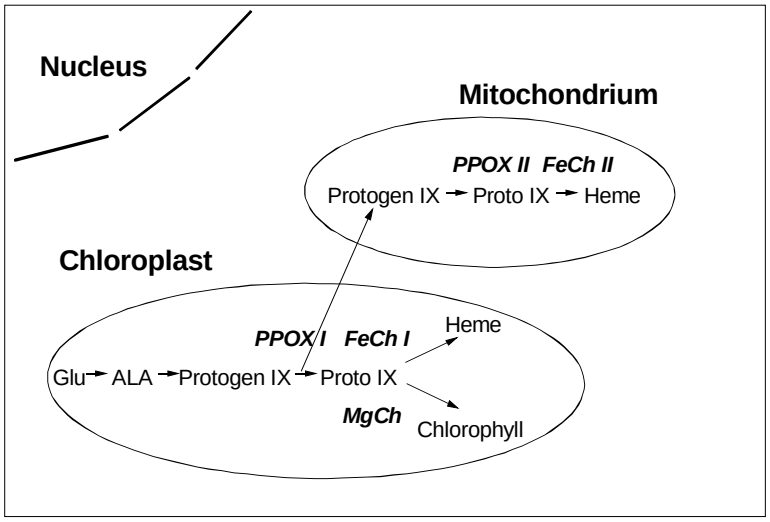


Figure 2: Cellular organization of tetrapyrrole biosynthesis (according to Smith et al., 1993, Grimm, 1999, modified). Early steps of the pathway from glutamate (Glu) via 5-aminolevulinate (ALA) to protoporphyrinogen IX (Protogen IX) are exclusively located in plastids. Chlorophyll synthesis proceeds with Mg chelatase (MgCh) and following enzymes in the plastids. The two final steps of the heme synthesizing branch protoporphyrinogen oxidase (PPOX) and ferro chelatase (FeCh) occur in plastids and mitochondria.

1.3 Deregulation of tetrapyrrole biosynthesis

Deficiencies in tetrapyrrole biosynthesis result in the accumulation of intermediates, which are photoactively oxidized, leading to the generation of highly reactive oxygen species that results in photodynamic damage. These toxic effects are evident in human patients affected by metabolic diseases called porphyrias, all resulting from deficiencies of one of the enzymes of porphyrin biosynthesis (Moore, 1993). The deleterious effect of accumulated tetrapyrroles are also evident in plants treated with a variety of herbicides that act via inhibition of PPOX (Duke et al., 1991; Matringe et al., 1989).

Pigment-deficient mutants and antisense technology have been used to examine the mechanisms of metabolic control or to analyze biochemically the enzymatic steps which are affected by a mutation or by expression of antisense mRNA. The use of mutants is limited due to their pleotropic phenotype, particularly if the mutated genes are not identified. In contrast, antisense technology allows to examine the regulatory function of a single enzymatic step. The antisense strategy is characterized by insertion of a cDNA sequence of the enzyme in reverse orientation under control of a cauliflower mosaic virus (CaMV) 35S promoter or a tissue-specific promoter into an expression vector and introduced by *Agrobacterium* mediated

transformation into plants. The antisense gene can control the transcript level in the nucleus and the translation efficiency of the target mRNA in the cytoplasm.

Tobacco plants transformed with an antisense GSAAT gene are an example of this approach. This enzyme catalyses the last step in 5-aminolevulinate synthesis. Antisense transformants show a general or tissue-specific reduction in chlorophyll (Höfgen et al., 1994). Lower pigment contents corresponded to reduced activity of GSAAT. It was demonstrated that the reduced GSA aminotransferase activity did not affect gene expression of other tetrapyrrolic enzymes (Härtel et al., 1997). Therefore, the reduced Chl content is clearly due to the reduced GSAAT activity.

In our group transgenic plants expressing antisense mRNA for UROD (Mock and Grimm, 1997) and CPO were generated and analyzed (Kruse et al., 1995b). In these transformants reduced activity of UROD or CPO resulted in accumulation of porphyrin intermediates causing photo-oxidative damage (Kruse et al., 1995; Mock and Grimm, 1997; Mock et al., 1998). These plants grow almost normally under dim light or with short light periods but develop extensive necrotic lesions on the leaves under high light intensity. The UROD antisense plants accumulate Urogen and Uro, the transformants with reduced CPO activity-Coprogen and Copro up to 500 fold of the level found in wild type plants. The extent of necrosis correlates with the amount of porphyrins accumulated in the leaves. Both types of transformants contain only slightly reduced levels of Chl and heme. In conclusion, the transgenic plants appear not to suffer from a deficiency in Chl, but rather from the accumulating substrate of each target protein.

To reduce the level of protoporphyrinogen oxidase, an activating antisense expression technology in *Arabidopsis* based on the yeast GAL4 transactivation system was used (Molina et al., 1999). Plants expressing the antisense mRNA for PPOX I showed growth retardation and necrotic leaf lesions.

The subsequent cellular responses were investigated in UROD and CPO antisense transgenic plants (Mock et al., 1998). In comparison to control plants, the transformants had increased levels of antioxidant mRNA, particularly those encoding superoxide dismutases (SOD), catalases and glutathione peroxidase. The cellular antioxidative response triggered by accumulation of porphyrins resembles that of plants treated with photodynamic herbicides (Knörzer et al., 1996).

UROD and CPO antisense tobacco and *Arabidopsis* plants expressing antisense *PPOX I* mRNA revealed an increased resistance against pathogens attack in comparison to wild type controls. (Mock et al., 1999; Molina et al., 1999). These transgenic plants express increased levels of pathogenesis-related proteins and synthesize high levels of free and conjugated salicylic acid. In consequence transgenic plants were more resistant either to tobacco mosaic virus (Mock et al., 1999) or to *Peranospora parasitica* (Molina et al., 1999). It was concluded that porphyrinogenesis as a result of deregulated tetrapyrrole synthesis induces a set of defense responses that resemble the hypersensitive reaction observed after pathogen attack (Mock et al., 1999).

1.4 Protoporphyrinogen IX Oxidase is the last common enzyme in chlorophyll and heme biosynthesis

Protoporphyrinogen oxidase (EC 1.3.3.4) catalyzes the oxygen-dependent oxidation of non-fluorescent protoporphyrinogen IX to fluorescent protoporphyrin IX, the last common precursor of both hemes and chlorophylls. Molecular oxygen is the obligatory electron acceptor in the reaction catalyzed by the eucaryotic type of PPOX oxidases. In the facultative anaerob *Escherichia coli* (*E. coli*), and presumably also in anaerobic photosynthetic bacteria, the respiratory chain can function as the electron acceptor. Since the conversion of Protogen IX to Proto IX can also occur chemically at neutral pH, the existence of an enzyme catalyzing this reaction was initially in doubt. Studies on protoporphyrinogen oxidase were mostly dedicated to elucidate the molecular basis of the human disease variegate porphyria. Patients who have decreased level of PPOX activity are characterized by both neuropsychiatric symptoms and skin lesions (Brenner and Bloomer, 1980; Deybach et al., 1981). The discovery that PPOX is the molecular target of diphenyl ether-type (DPEs) herbicides stimulated research on this enzyme. DPEs herbicides are very potent inhibitors of protoporphyrinogen oxidase activity (Matringe et al., 1989; Witkowski and Halling, 1989). Their light-dependent phototoxicity involves intracellular peroxidation promoted by accumulation of Protogen IX, substrate of PPOX.

The enzyme was initially characterized from yeast (Poulson and Polglase, 1975) and mammalian liver (Poulson, 1976). Both enzymes require dioxygen for their activity, and no other electron acceptor supports the enzyme reaction under anaerobic conditions. The oxidation of Protogen in facultative aerobe *E. coli* and obligate anaerobe *Desulfovibrio gigas*

required nitrate and fumarate as electron acceptors (Jacobs and Jacobs, 1975; 1976; Klemm and Barton, 1985). Protoporphyrinogen oxidase has been purified partially or completely from a number of organisms. In *Disulfovibrio gigas* PPOX was purified to the apparent homogeneity from the plasma membrane by Klemm and Barton (1987). The enzyme has a molecular weight of 148 kDa and was found to have three different subunits (12, 18.5 and 57 kDa) which are linked by disulfide bonds. PPOX was purified with a 68 % yield from bovine liver mitochondria (Siepker et al., 1987). The enzyme contains FAD as a cofactor. It has an apparent Mr 57 kDa. The activity of the isolated enzyme was markedly stimulated by fatty acids such as oleic acid. PPOX purified from mouse liver mitochondria has a molecular weight of approximately 65 kDa (Dailey and Karr, 1987) and has a noncovalently bound cofactor FMN (Proulx and Dailey, 1992). From yeast mitochondrial membranes protoporphyrinogen oxidase was purified to homogeneity and found to be a 55-kDa polypeptide (Camadro et al., 1994). The purified enzyme contains stoichiometric amounts of the cofactor FAD. Studies with rabbit antibodies raised against yeast protoporphyrinogen oxidase indicated that the enzyme is synthesized as a high molecular weight precursor (58 kDa) that is rapidly converted *in vivo* to the mature (55 kDa) membrane-bound form. In plants, PPOX activity was found in both mitochondria and chloroplasts (Jacobs and Jacobs, 1987; Matringe et al., 1989; Smith et al., 1993). The two enzymes have similar properties (Camadro et al., 1991; Jacobs and Jacobs, 1987; Matringe et al., 1989). Matringe et al. (1992b) showed that protoporphyrinogen oxidase is an integral protein of both the thylakoid and the envelope membranes of spinach chloroplasts. PPOX has also been purified to homogeneity from barley etioplasts and mitochondria (Jacobs and Jacobs, 1987). The enzymes from both organelle fractions had a Km of 0,5 µM and were labile to mild heat and acifluorfen. A 55 kDa protein with protoporphyrinogen oxidizing activity was purified from lettuce etioplasts (Camadro et al., 1993). Fluorescence spectra of the purified enzyme revealed the presence of a flavin covalently bound to the polypeptide chain (Decker, 1993).

Protoporphyrinogen oxidizing activity was found in the microsomal and plasma membrane fractions prepared from seven-day-old, etiolated barley leaves (Lee et al., 1993). The plasma membrane-associated protoporphyrinogen oxidizing activity was not sensitive to inhibition by acifluorfen-methyl. It had a lower affinity for protoporphyrinogen IX (172 µM) than did the etioplastic enzyme (26 µM). Yamoto et al. (1994) purified a protoporphyrinogen oxidizing enzyme from the soluble fraction of tobacco cell lines. Amino acid sequences from this

soluble protoporphyrinogen-oxidizing enzyme corresponded to the acid/base catalysis and heme binding regions of plant peroxidases (Yamoto et al., 1995).

1.5 Isolation of PPOX genes from different organisms

Mutagenesis of *E. coli* K12 (Sasarman et al., 1968) by neomycin and the selection of dwarf colonies have provided a useful way for isolating heme-deficient mutants. By this way *E. coli* mutant deficient in PPOX activity was obtained. The mutant was designated *hemG* (Sasarman et al., 1979). This mutant grows very poorly, even on a rich medium. Genes involved in protoporphyrinogen IX oxidase activity has been identified first from *E. coli* by a mini-Mu *in vivo* cloning procedure (Sasarman et al., 1993) and designated *hemG*. The *hemG* gene restored normal growth to the *hemG* mutant, and the transformed cells display strong protoporphyrinogen oxidase activity. Sequencing of the *hemG* gene identified an open reading frame of 546 nucleotides (181 amino acid). It is the minimal fragment which is able to complement the mutant. Independently, *hemG* was cloned by complementation of an *E. coli* VSR751 strain defective in PPOX (Nishimura et al., 1995a). From *Bacillus subtilis,* Hansson and Hederstedt (1992) cloned and sequenced an open reading frame (ORF) encoding a 53 kDa protein with protoporphyrinogen oxidase activity. The gene encoding a protoporphyrinogen oxidase in *Bacillus subtilis* was designated *hemY*. Both the *E. coli hemG* and the *Bacillus hemY* gene encode peptides, which did not share any sequence similarity. They represent two distinct protoporphyrinogen oxidizing systems, the oxygen dependent of the *HemY*-type and the bacterial multi-component system. The structural gene for protoporphyrinogen IX oxidase from yeast (*Saccharomyces cerevisiae)* was identified by functional complementation of a *hem14-1* yeast mutant which is deficient in enzyme activity and resembles the HemY protein (Camadro and Labbe, 1996). The *E. coli hemG* mutant could be complemented with eucaryotic cDNA sequences encoding the HemY like protein. Functional complementation of *hemG* mutant of *E. coli* appears to be extremely productive for the characterization of protoporphyrinogen oxidase from mammals (Dailey and Dailey, 1996b; Nishimura et al., 1995b) and mice (Dailey et al., 1995; Taketani et al., 1995), and also those from plants, *Arabidopsis thaliana* (Ward and Volrath, 1995; Narita et al., 1996). A cDNA for the plastidal PPOX from chicory was isolated by using the tobacco cDNA for PPOX I as molecular probe (Adomat and Böger, 2000).

1.6 Peroxidizing herbicides. Classification and mechanism of action

The use of herbicides to control undesirable vegetation has become a universal practice. Many herbicides with biocidal side effects are being phased out, due to toxicological problems and environmental impacts. Such side effects could be due to a high use rate (more than 1 kg a.i./ha.) of conventional herbicides. During the past 15 years scientists have concentrated their efforts on molecular design of herbicides with higher efficacy and specificity. Protoporphyrinogen IX oxidase is the target enzyme in the porphyrin pathway for the most powerful peroxidizing herbicides. Since some of these herbicides are effective at a very low dosage, below a few grams per hectare or less, this class of herbicides has become an attractive subject of modern agrochemistry as they provide an excellent tool for weed control. The first commercial inhibitors of protoporphyrinogen oxidase were diphenyl ethers (DPEs) (Matsunaka, 1976).

A survey of the known inhibitors of protoporphyrinogen oxidase has resulted in their classification into three main chemical classes: the diaryl ethers, the phenyl heterocycles, and the heterocyclic carboxamides (Table1). The first class, the diaryl ethers, is subdivided into the diphenyl ethers (e.g., oxyfluorfen, nitrofluorfen, acifluorfen) and the heterocyclyl phenyl ethers (e.g., AH 2.430). The diphenyl ethers are the most prominent class of protox inhibitors. Many diphenyl ethers are commercial products.

Structural analysis of DPEs herbicides showed that their bicylic structure allows a competitive inhibition of protoporphyrinogen oxidase by filling the complementary space of the binding site for the natural substrate (Matringe et al., 1992a; Nandihalli et al., 1992). They compete with Protogen in *in vitro* assay with PPOX containing protein extracts from yeast, mammalian and plant mitochondria and plant chloroplasts (Camadro et al., 1991). Inhibition of protoporphyrinogen oxidase leads to accumulation of its substrate, Protogen IX. It is assumed that excess of Protogen leaks out of the plastid and is oxidized to Proto 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 main physiological markers for a photodynamic action of peroxidizing herbicides are the immediate halt of chlorophyll biosynthesis, a strong degradation of chlorophylls and carotenoids, rapid degradation of membranes leading to evolution of saturated short-chain hydrocarbons like ethane, accumulation of Proto IX. (Böger and Wakabayashi, 1995). All parameters listed above can be easily detected. Taking into consideration the knowledge about

photoreactivity of porphyrins and also physiological markers obtained by treatment with peroxidizing herbicides, a herbicide-mediated radical peroxidation process has been proposed as mode of action of peroxidizing herbicides (Fig. 3).

Recently using the flavinic nature of protoporphyrinogen oxidase a new class of PPOX inhibitors that act via a mechanism very different from that of diphenyl ether-type herbicides was characterized. The reactivity of protoporphyrinogen oxidase toward the 2,2´-diphenyleneiodonium derivatives, the known inhibitors of several flavoproteins, was investigated (Arnould et al., 1997). It was demonstrated that diphenyleneiodonium inhibited the membrane-bound yeast PPOX competitively with molecular oxygen.

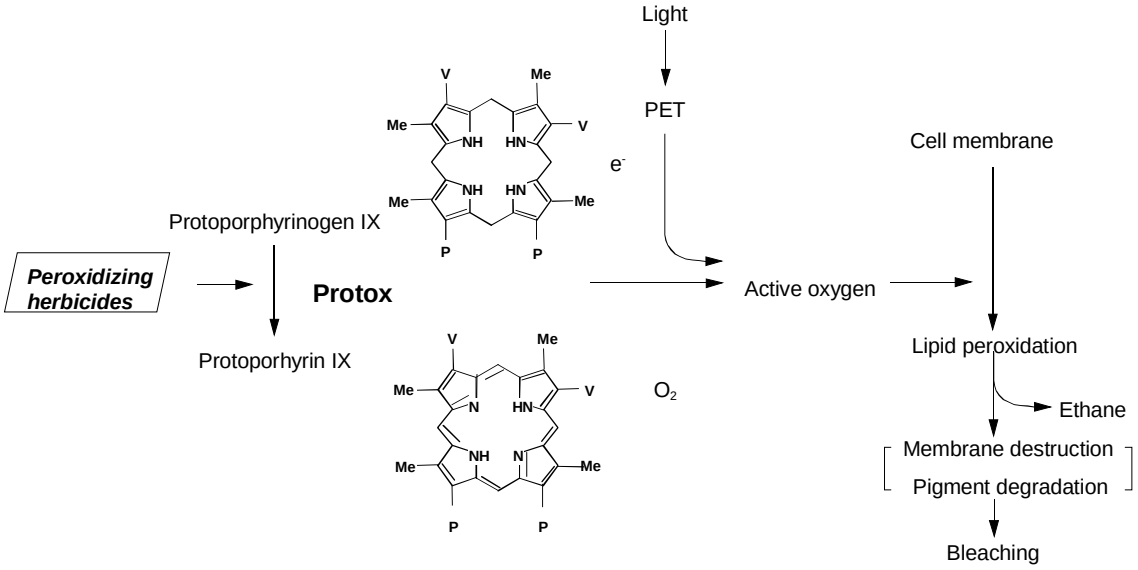
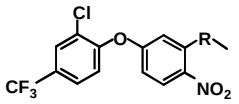
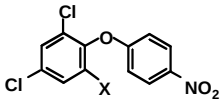
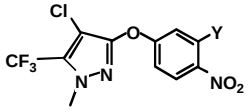
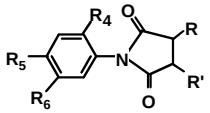
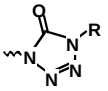


Figure 3: Mode of action of peroxidizing herbicides: herbicide-mediated lipid peroxidation and destruction of photosynthetic membranes and pigments (according to Wakabayashi and Böger, 1999)

Table 1: Classification of peroxidizing herbicides according to Anderson et al. (1994). Three major classes with subclasses defined for each class are presented. Specific compounds shown as examples of the various subclasses. Patent citations for specific compounds are given.

<table>
<tr><td colspan="5">Diaryl ethers</td></tr>
<tr><td colspan="3">Diphenyl ethers</td><td colspan="2">Heterocyclyl phenyl ethers</td></tr>
<tr><td colspan="3"> 
R=OC_2H_5, Oxyfluorfen
Rohm and Haas
R=H, Nitrofluorfen
R=COOH, Acifluorfen
Rohm and Haas
 
X=H, Nitrofen
Rohm and Haas
X=Cl, Chloronitrofen
Mitsui
X=F, Fluoronitrofen
Mitsui</td><td colspan="2"> 
Y=H, AH 2.430
Monsanto
Y=COOH, AH 2.431
Monsanto</td></tr>
<tr><td colspan="5">Phenyl heterocycles</td></tr>
<tr><td colspan="4">N-phenyl heterocycles</td><td rowspan="2">C-phenyl heterocycles</td></tr>
<tr><td>Imides</td><td>Tetrazolones</td><td>Triazolones</td><td>Uracils</td></tr>
<tr><td> 
USP 4,484,941
(Sumitomo)</td><td> 
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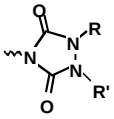
(FMS)</td><td> 
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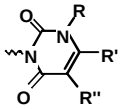
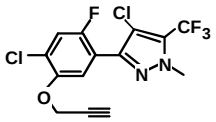
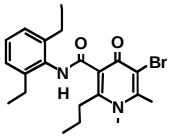
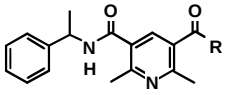
(FMS)</td><td> 
USP 5,169,430
(Unirogal)</td><td> 
Monsanto</td></tr>
<tr><td colspan="5">Heterocyclic carboxamides</td></tr>
<tr><td colspan="2">Pyridone carboxamides</td><td colspan="3">Pyridine carboxamides</td></tr>
<tr><td colspan="2"> 
DLH-1777, (Daicel)</td><td colspan="3"> 
LC 81 601, (Phone-Poulenc)</td></tr>
</table>

1.7 Mechanisms of herbicide resistance

Natural tolerance against peroxidizing herbicides varies among plants species. Some plants, such as rice and soybean, are known to be naturally more tolerant against protoporphyrinogen

oxidase inhibitors, while most other plant species are highly susceptible. (Lee et al., 1991; Pornprom et al., 1994). These differences in herbicide tolerance could be sometimes explained by variation 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 (Jacob et al., 1996).

Several strategies have been developed for obtaining resistant plants towards the peroxidizing herbicides directed against PPOX. One way to obtain herbicide resistance can be an alteration of the herbicide binding site in the catalytic cleft of the enzyme preventing stable binding of specific herbicides. Screens for resistant spontaneous and induced mutants have been a useful tool. Mutant seedlings and cell cultures have been selected with a medium containing PPOX inhibitor. A photomixotrophic tobacco cell culture was selected upon stepwise increasing concentrations of the herbicides S23142 (Ichinose et al., 1995) or ET 62311 (Horikoshi and Hirooka, 1999; Horikoshi et al., 1999). Analysis of the mutants to elucidate the mechanism of resistance revealed that in the first case the cells increased the activity of protoporphyrinogen oxidase and had 10 times increased level of mitochondrial *PPOX* mRNA (Watanabe et al., 1998). In the second case a point mutation, leading to the substitution of Ala by Met at amino acid 231, was found in the *PPOX* cDNA which could cause the resistance of mutated cell culture. A single-point mutation (Val389Met) of PPOX I of *C. reinhardtii* conferred herbicide resistance to the *RS-3* mutant strain (Randolf-Anderson et al., 1998). It is known that PPOX originated from microorganisms is only weakly inhibited by the known tetrapyrrole-dependent photodynamic herbicides (Dailey et al., 1994). Expression of the less herbicide susceptible *Bacillus subtilis* PPOX (*HemY*) in the cytoplasm and in the chloroplasts of transgenic tobacco plants leads to a slight resistance against the herbicide oxyfluorfen (Choi et al., 1998). The resistance of plant species to herbicides can also be developed by other strategies, such as reduced uptake, sequestration of the herbicides, a rapid metabolic destruction of the herbicides, or of Protogen IX and Proto IX. Jacobs and Jacobs (1993) described a Protogen IX degradation mechanism that prevents Proto IX accumulation in plant cells. Actually, the destruction of Protogen IX was less active in young leaves of cucumber, a plant highly

susceptible to the herbicide, while higher levels of Protogen IX destruction were found in leaves of broadleaf mustard and radish, two plants that exhibited herbicide tolerance (Jacobs et al., 1996).

Overproduction of a herbicide-sensitive target enzyme is an other possibility to introduce herbicide resistance into plant cells. In this thesis I present transgenic tobacco plants which are resistant against DPE herbicide acifluorfen due to the overexpression of *Arabidopsis thaliana* PPOX I (Lermontova and Grimm, 2000).

1.8 Formation of reactive oxygen species and antioxidative defense

Different growth and abiotic stress conditions, like elevated levels of air pollutants such as ozone and SO_2, high UV doses, salinity and cellular senescence as well as pathogen attack, herbicide application and accumulation of tetrapyrrole intermediates can induce the generation of reactive oxygen species in plants (Foyer and Mullineaux, 1994; Inze and van Montagu, 1995; Streb and Feirabend, 1996; Alscher et al., 1997; Mock et al., 1998). Böger and Sandmann (1990) suggested the following mechanism of Proto IX-induced formation of ROS. Accumulation of excessive amounts of Proto IX in the light leads to the generation of singlet oxygen (light reaction 1) or of superoxide anions in case of suitable reaction occurs or reductant is available (light reaction 2).

Light reaction:

1) $\text{Proto IX} \xrightarrow{\text{light}} \text{proto IX*} \xrightarrow{O_2} {}^1O_2$

2) $\text{Proto IX} \xrightarrow{\text{light}} \text{proto IX*} \xrightarrow{\text{reductant}} \text{proto IX}^{\bar{\cdot}}$

The Proto IX radical may form a hydroxy radical $HO^{\cdot}$ via superoxide in three steps as indicated below. Dismutation of superoxide formed in the first reaction results in accumulation of hydrogen peroxide (H_2O_2) (reaction 2). Hydroxyl radical could be generated from a transition metal (Me) catalyzed reaction of H_2O_2 (reaction 3). Hydroxyl radical initiates a radical-chain reaction with fatty acids (e.g. linolenic acid) leading to the respective radical (α-linolenic acid radical; α-lin$^{\cdot}$) (reaction 4). This radical keeps the chain reaction going. In parallel, singlet oxygen formed in the light reaction 1 may lead to direct formation of peroxo compounds of α-linolenic acid.

Formation of starter radical:

1) proto IX $^{\cdot-}$ + O_2 ⟶ proto IX + $O_2^{\cdot-}$

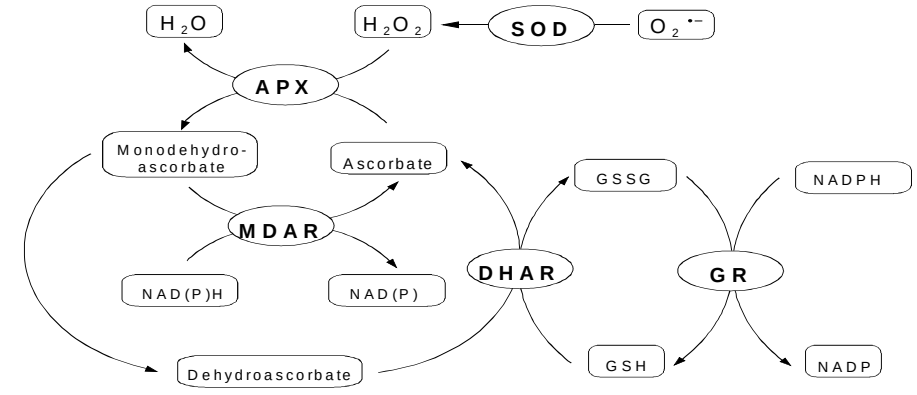
2) $2O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2$

3) $H_2O_2 \xrightarrow{Me^+ \; Me^{n+1}} OH^- + HO^{\cdot}$

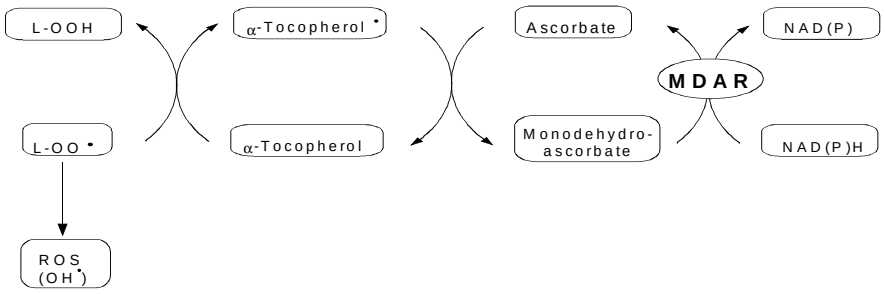
4) α-lin + $HO^{\cdot}$ ⟶ α-lin$^{\cdot}$ + H_2O

Under relatively normal circumstance, plants are able to cope with a wide range of environmental changes because they posses a number of protective or scavenging systems consisting of enzymatic and low molecular weight antioxidants. They function continuously or are latent until required. These include enzymatic scavengers such as SOD, which catalyze the dismutation of $O_2^{\cdot-}$ to H_2O_2; catalase, which decompose H_2O_2 to water; hydrophilic radical scavengers such as ascorbate, urate and glutathione (GSH); lipophylic radical scavengers such as tocopherols, flavonoids, carotenoids and ubiquinol; enzymes involved in the regeneration of oxidized forms of the small molecular antioxidants (GSH reductase, dehydroascorbate reductase) or enzymes responsible for the maintenance of protein thiols (thioredoxin reductase) and the cellular machinery that maintains a reducing environment (e.g., glucose-6-phosphate dehydrogenase, which regenerates NADPH). The main pathway of detoxification of ROS is localized in chloroplast and was named Halliwell-Asada pathway (Fig. 4a; Foyer and Halliwell, 1976; Nakano and Asada, 1980) This pathway scavenges hydrogen peroxide, formed as a product of SOD action, in several steps including enzymes and nonenzymatic compounds. In the first step ascorbate peroxidase (APX) catalyzes the reduction of H_2O_2 to water using ascorbate (AsA) as electron donor. Oxidation of ascorbate by ascorbate peroxidase leads to formation of a monodehydroascorbate radical (Nakano and Asada, 1981). It either spontaneously disproportionates to give ascorbate and dehydroascorbate (DasA), or is reduced to ascorbate by NAD(P)H (Winkler et al., 1994). The latter reduction is catalyzed by the flavoenzyme monodehydroascorbate reductase (MDAR) (Hossain et al., 1984). For the reduction of dehydroascorbate to ascorbate, dehydroascorbate reductase (DHAR) requires GSH as reductant (Hossain and Asada 1984). GSSG is then NADPH-dependently reduced by glutathione reductase (GR) (Halliwell and Foyer 1978). Thus, the ascorbate-glutathione cycle receives its reductant directly from photosynthesis via NADPH.

Several low molecular weight antioxidants in higher plants act as a radical scavengers. The lipophylic α-tocopherol interrupts radical chain reaction during lipid peroxidation by reducing polyunsaturated fatty acid peroxyl radicals of membrane lipids and is recycled via ascorbate (Fig. 4b). It was previously demonstrated that ascorbate and α-tocopherol play an important role in cellular protection against oxidative damage by ROS. Their ratio is an important factor in plant susceptibility against herbicides (Sies, 1993; Finckh and Kunert, 1985). It was previously shown that plants with a ratio of ascorbate to tocopherol between 10 to 15:1 (wt/wt) were most tolerant against the phytotoxic effect of peroxidizing herbicides. Variation from the optimized level resulted in higher susceptibility to herbicides. It is evident that the balance of antioxidants in plants is crucial for effective protection against oxidative damage.



(a) Ascorbate-glutathione pathway (Halliwell-Asade pathway)



(b) Ascorbate/a-tocopherol pathway

Figure 4: Pathways of antioxidative defense in higher plants (according to Elstner, 1990; Kreuz et al., 1996).

1.9 Research objectives

Investigations of the chlorophyll biosynthetic pathway on the physiological, biochemical and molecular levels are the research subjects of our group. The tetrapyrrole biosynthesis and the role of tetrapyrroles on plant metabolism were intensively investigated over many years. Nevertheless, impact of single enzymatic steps, regulation of the gene expression coding for some enzymes involved in tetrapyrrole biosynthetic pathway has to be elucidated and analyzed in more details.

Thus, the aim of my research project was to investigate the tetrapyrrole biosynthetic pathway on the level of the conversion of Protogen IX to Proto IX. This reaction is catalyzed by protoporphyrinogen oxidase, the last common enzyme in heme and chlorophyll biosynthesis.

1) At the time when I started my work, cDNA sequences for PPOX were known only from bacteria, yeast and mammals, and no plant sequences were available. My aim was identification, cloning and sequencing of genes encoding the enzyme PPOX from tobacco. Based on the data previously obtained, which demonstrated activity of PPOX in two organelles, chloroplasts and mitochondria, I expected to find two genes encoding for two different isoforms of PPOX.

a) After identification of two cDNA sequences, it was important to show the subcellular localization of the respective isoenzymes for the further investigation of the role of both isoform in tetrapyrrole biosynthesis.

b) Expression of some genes involved in tetrapyrrole biosynthesis is regulated by light and depends on the developmental stage of plants and leaves. To study the regulation of PPOX expression, the expression of mRNA for PPOX I and II was analyzed in a leaf gradient and under diurnal and circadian growth conditions.

c) For the enzymological studies and for the antibodies production, PPOX I and PPOX II should be overexpressed in *E. coli*, because purification of PPOX from plant sources was found to be rather difficult

2) PPOX is the target of peroxidizing herbicides. My aim was to generate plants resistant toward peroxidizing herbicides by different strategies.

a) The first approach to obtain herbicide resistance, was the generation of transgenic plants carrying the cDNA sequences for PPOX I and II in sense orientation with the aim to generate the herbicide resistance by the overproduction of the target enzyme.

b) The second approach was mutagenesis of the cDNA sequence encoding PPOX I in random mutagenesis strain XL-Red with the aim to modify the herbicide binding site of PPOX for preventing the stable binding of the herbicides by the enzyme.

3) To elucidate the role of PPOX I in the chlorophyll biosynthesis and in plant metabolism, the enzyme activity of PPOX I in tobacco plants was reduced by expression of antisense mRNA for this enzyme. Molecular and biochemical analysis of transgenic plants should be performed to study the effect of PPOX deficiency under low and high light growth conditions.

2 Material

2.1 Chemicals, enzymes and kits

All reagents and enzymes used were of the highest quality. Most chemicals were obtained from the following companies: Roth (Karlsruhe), Serva (Heidelberg), Merck (Darmstadt), Appligene (Darmstadt), Sigma (St. Louis MO, USA). The ^{32}P labeled radiochemicals were obtained from Amersham Pharmacia Biotech (Braunschweig). Sequencing and PCR oligonucleotide primers were synthesized at Metabion (München), Amersham Pharmacia Biotech (Braunschweig). The DNA size markers were obtained from Life Technology (Karlsruhe), MBI Fermentas (Vilnius, Lithuania), Amersham Pharmacia Biotech (Braunschweig) and protein size markers from Amersham Pharmacia Biotech (Braunschweig). The PCR Purification, Gel Extraction, Nucleotide Removal and Plasmid Isolation Kits used were obtained from Qiagen (Hilden).

2.2 Primers and oligonucleotides

For expression in E. coli

PPXI Kpn I	5´-GAC GGT ACC TCA TTT GTA TGC ATA CCG AGA C- 3´
PPXI Sma I	5´-GAT CCC GGG TCA TTT GTA TGC ATA CCG AGA C-3´
PPXI Bam HI s	5´-TAT GGG GAT CCA CAA CAA CTC CCA TCG CCA ATC-3´
PPXI Nco I m	5´-GAC CCA TGG TTG CCA AAG ATT ACA CAG TTC-3´
PPXI Bam HI as1	5´-GAC GGA TCC TCA TTT GTA TGC ATA CCG AGA C-3´
PPXI Bam HI as2	5´- GAC GGA TCC TTT GTA TGC ATA CCG AGA C-3´
PPXI Nco I p	5´-GAC CCA TGG CAA CAA CTC CCA TCG CCA ATC AT-3´
PPXII Kpn I	5´-GAC GGT ACC TCA GCA ATG TCT TTT GGA GTC-3´
PPXII Sma I	5´-GAT CCC GGG TCA GCA ATG TCT TTT GGA GTC-3´
PPXII Bam HI s	5´-TAT TGG GAT CCG CTC CTT CTG CCG GAG AAG ATA AAC-5´
PPXII Bam HI as1	5´-GAC GGA TCC TCA GCA ATG TCT TTT GGA GTC-3´
PPXII Bam HI as2	5´-GAC GGA TCC GCA ATG TCT TTT GGA GTC-3´
PPXII Sph I	5´-GAC GCA TGC TTG CTC CTT CTG CCG GAG AAG-3´
PPXII Nhe I	5´-TGT ACG CTA GCG CTC CTT CTG CCG GAG AAG ATA AAC-3´
PPXII Eco RI	5´-AGG CTG AAT TCG CTC CTT CTG CCG GAG AAG ATA AAC-3´

Sequencing primers

PPXI 1	5´-GCA GTA GAT TGT GGA TTG AAG G-3´

PPXI 2	5´-GTT GGC TCT CCG TCT TAG AC-3´
PPXI 3	5´-AGC AGT TCG TGC GTC GTA ATC-3´
PPXI 4	5´-CAC GAA TAG CTT CTT GAG G-3´
PPXI 5	5´-AGC TGG ACT GTG TTA TAG TTG G-3´
PPXI 6	5´-CAT AAG CAC CTT CAA CAC ACC-3´
PPXI 7	5´-CGA GAC ACC AGA AGG AGT AG-3´
PPXI 8	5´-GCT ACC ACC AGT TTC TTC C-3´
PPXI 9	5´-TGT GCG AGT ATG GCC ACA AGC-3´
PPXI 10	5´-CAG TCG TTA TGT TGC CAC CG-3´
PPXII 1	5´-AGA AAA GCA ACA ATT TCC-3´
PPXII 2	5´-GAC TGA ATC ATA GTT ATG CCC G-3´
PPXII 3	5´-CTC AGT CAT ACT TGG AGC TA-3´
PPXII 4	5´-CGG TAC ATA ATC AAC CTC AGG-3´
PPXII 5	5´-TGT GGT GGT GAT CCT GAC TC-3´

2.3 Vectors

pBluescript SK, KS (Stratagene, La Jolla, USA)

BinAR (Höfgen and Willmitzer, 1992)

pCR^{TM}II TA-Cloning Vector (Invitrogen, Leek, NL)

pDS56-SphI vector (Stüber et al., 1984)

pGEX2T (Pharmacia Biotech, Freiburg)

pQE 60 vector (Quiagen, Hilden).

2.4 Organisms

Bacteria

Agrobacterium tumefaciens GV 2260 (Deblaere et al., 1985)

Escherichia coli BL21 (Studier et al., 1990)

Escherichia coli DH5α (Gibco BRL, Gaithersburg, USA)

Escherichia cili SG13009 (Gottesmann et al., 1981)

Escherichia coli XL1-Red (Stratagene, La Jolla, CA)

Plants

Nicotiana tabacum cv. Samsun NN (IPK Gatersleben)

Pisum sativum L. cv. frühe Harzerin Saatzucht (Quedlinburg, FRG)

2.5 Media

Bacterial media

LB: 10 g Bacto Tryptone; 5 g Yeast extract; 5 g NaCl for 1L pH 7,0 with NaOH
solid medium: add 15 g Bacto Agar for 1L medium
soft medium: add 7 g Bacto Agar for 1L medium

SM: 50 ml 1 M Tris/HCl pH 7,5; 5 ml 2 % Gelatine; 2 g $MgSO_4$; 5,8 g NaCl

SOC: 20 g Bacto Tryptone; 5 g Yeast extract; 0,5 g NaCl; 2,5 mM KCl; 10 mM $MgCl_2$; 20 mM Glucose; pH 7,5 with NaOH

2YT: 16 g Bacto Tryptone; 10 g Yeast extract; 5g NaCl; pH 7,0 with NaOH
solid medium: add 15 g Bacto Agar

Plant growth media

MS: 2,3 g MS medium; 6 g BiTek Agar; pH 5,7 with NaOH

2MS: 4,4 g MS medium; 20 g Sucrose; 6g BiTek Agar; pH 5,7 with NaOH

2MG: 4,4 g MS medium; 16 g Glucose; 6 g biTek Agar; pH 5,7 with NaOH

Laboratory tools

Appligene, Heidelberg: Vacuum blotter

Beckman, München: Spectrophotometer DU 7400

Bio-Rad, München: Protein gel-electrophoresis units, GelAir Dryer

Heraeus, Hanau: Biofuge, Growth chamber

Invitrogen, Leek, NL: Electroporator

Jasco, Groß-Umstadt: Fluorescence detector FP-920

Perkin Elmer, Langen: Fluorimeter, PCR Termo Cycler

Schütt Labortechnic GmbH, Göttingen: DNA gel-electrophoresis units, Protein gel-transfer apparatus, Homogeniser RTR 2020 (Heidolph), Waring mixer 8011-G (Bender and Hobein)

Sorvall Instruments, Bad Homburg: Zentrifuge RC5C

Stratagene, La Jolla, USA: UV-Stratalincer 2400

Waters, HPLC equipment: Eschborn: 717 plus Autosampler, 600 and 600S Controller, HPLC columns, 474 Scanning Fluorescence Detector

3 Methods

3.1 Plant growth and treatment regimes

To study the developmental expression of two isoforms of protoporphyrinogen oxidase, tobacco plants (*Nicotiana tabacum* cv. Samsun NN, IPK Gatersleben) were grown in the greenhouse for 6 weeks. Supplemental illumination was provided by 400 W higher pressure sodium vapour lamps to ensure a light intensity of 300 µmol m^{-2} s^{-1} over a 16 h photoperiod. Leaves of six-weeks-old plants were harvested from the top to the base.

To analyze the expression levels of protoporphyrinogen oxidase under diurnal and circadian growth another set of plants were grown in a growth chamber in a 12/12 h light/dark cycle at 24°C for four weeks at a light intensity of 75 µmol m^{-2} s^{-1}. Plantlets were harvested every 4 h over a 24 h time period starting 1 h after onset of the light phase. Another set of plants was transferred from dark/light conditions to continuous light or darkness. Samples were collected starting 48 h after the first harvest. Plant material was immediately frozen in liquid nitrogen and stored at -80°C.

Six to eight days old pea seedlings (*Pisum sativum* L. cv. frühe Harzerin, Saatzucht Quedlinburg, FRG) were used for isolation of mitochondria and plastids.

To analyze plants overexpressing protoporphyrinogen oxidase I, 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 at 25 C°. Leaves were harvested from 4-6-week-old plants, frozen in liquid nitrogen and stored at -80 C°. All experiments were carried out with primary transformants. Individuals of the primary transformants were obtained by vegetative propagation.

For basic analysis of tobacco plants expressing antisense mRNA for protoporphyrinogen oxidase I, plants were grown under greenhouse conditions with circa 300 µmol m^{-2} s^{-1} during 16 h.

To study the effect of light dosage in plants with reduced PPOX I levels, wild type (*Nicotiana tabacum* var. Samsun NN) and transgenic tobacco plants were grown in the greenhouse for 4-6 weeks and then transferred to the growth chambers with low light (70 µmol m^{-2} s^{-1}) and high light (500 µmol m^{-2} s^{-1}) conditions with a 12 h light/ 12 h dark cycle at 25 C°. Material was harvested four days after the transfer to the different light conditions, frozen in liquid nitrogen and subsequently either immediately analyzed, or stored at -80 C°.

Plants overexpressing PPOX II were grown either under standard greenhouse conditions or in growth chambers with low light (70 µmol m^{-2} s^{-1}) and high light (500 µmol m^{-2} s^{-1}) intensities for 4-6 weeks.

3.2 PCR-amplification

The polymerase chain reaction (PCR) was used to amplify a segment of DNA that lies between two regions of a known sequence for subcloning into different vectors. Moreover PCR reaction was used for the screening of tobacco plants for insertion of the transgene, or PCR fragments were used as molecular probes in Northern and Southern hybridization. As DNA templates, 1 to 10 ng of plasmid DNA or genomic DNA were used in a 100 µl of total reaction volume. Two oligonucleotides with the final concentration 1 µM were used as primers for a series of synthetic reactions that are catalyzed by DNA polymerase (mainly by *Tag* polymerase). Amplification efficiency is dependent on the length of the DNA fragment which is to be amplified, on the length and specificity of the primers, the number of cycles and annealing temperature. These factors were taken into account for the design of PCR conditions. Example time program are presented below: 25-30 cycles with the denaturation at 94 °C for 1 min, annealing at 58 °C for 2 min and extension at 72 °C for 3 min.

3.3 Basic cloning methods

Insert preparation: PCR products were purified via the " QIAquick PCR Purification Kit" (Qiagen) or were separated on agarose gel, excised from the gel as an agar block and eluted by " the QIAquick Gel Extraction Kit".

Purified fragments were digested by appropriate restriction enzymes. For subcloning DNA fragments into different vectors after restriction digestion, DNA fragments were separated on agarose gel and the fragment of interest was eluted from the gel.

Restriction enzymes and nucleotides were removed by " QIAquick Nucleotide Removal Kit". Purified fragments were used for the ligation reaction.

Vector preparation: Plasmid DNA was digested with certain restriction enzymes. The vector was purified from oligonucleotides or DNA fragments via the nucleotide removal Kit or from the agarose gel. The vector was usually dephosphorylated by "Shrimp"-Phosphatase. After the separation of DNA from enzyme and buffer by Phenol/ Chloroform extraction, the vector was used for the ligation.

Restriction digestion, ligation and DNA electrophoresis were performed according to the methods described by Sambrook et al. (1989).

Transformation of E. coli: The transformation of *E. coli* was performed using the heat-shock procedure (Sambrook et al., 1989) or by electroporation (Inoue et al., 1990).

Preparation of plasmid DNA: Plasmid DNA extraction and purification was done using the Qiagen Plasmid Kit.

3.4 Isolation of cDNA clones for protoporphyrinogen IX by functional complementation of *E. coli* mutant defective in *hemG*

A Lambda-ZAP II tobacco (SR1) cDNA library (Stratagene) from tobacco was amplified and pBluescript was excised from the phage DNA. *E. coli* strain R751 defective in the *hemG* gene (Nishimura et al., 1995a) was electroporated with plasmid DNA containing the library. The cells were plated on LB agar containing 100 mg/ml ampicillin. Strain R751 grew very poorly in the absence of heme. Complemented *E. coli* cells were selected on the basis of normal size colonies. Plasmid DNA was isolated from these colonies and reintroduced into the *hemG* mutant to confirm the recovery from poor growth. The cDNA fragments were sequenced by the dideoxy chain termination method (Sanger et al., 1977) on both strands using fluorescent labeled primers for the application to the ALF DNA Analysis System (Pharmacia Biotech). Results were analyzed with the PC/GENE program (Intelli Genetics Inc., Mountain View, CA).

3.5 Random mutagenesis in XL1-red strain

The plasmid pBS KS encoding the tobacco chloroplastic PPOX enzyme (pBS KS), was used to transform the random mutagenesis strain XL1-Red (Stratagene, La Jolla, CA). The transformants were plated on LB medium containing 100g/ml ampicillin and were incubated 24 or 48 h at 37 °C. Lawns of transformed cells were scraped from plates and inoculated into the liquid LB medium with ampicillin. Cultures were grown for 5h at 37°C, then plasmids were isolated by using of Qiagen Plasmid Kit (Qiagen). Alternatively, the transformed XL-Red cells were inoculated directly into the liquid medium (without plating on LB Agar plates). Cultures were incubated 24h at 37°C and then plasmids were isolated. The mutated plasmid DNAs were transformed into *hemG* mutant R751 and plated on LB medium with 100mg/ml

ampicillin and the same medium with 100 µM acifluorfen. The plates were incubated for 3days at 37°C. Plasmid minipreps were isolated from all colonies, which were able to grow in the presence of acifluorfen. All plasmids were transformed again into the *hemG* mutant to ensure that the resistance is plasmid-borne. *PPOX I* cDNA fragments from resistant clones were recloned into nonmutated pBS KS plasmid and again transformed into the *hemG* mutant. Finally, resistant clones were sequenced.

3.6 Construction of tobacco protoporphyrinogen IX oxidase expression vectors

The synthetic sense primer *PPXI Nco I m* and the antisense primer *PPXI Bam HI as1* were used to amplify by PCR an open reading frame encoding a putative mature protoporphyrinogen IX oxidase beginning with the amino acid valine at position 53 of the entire peptide sequence. In addition, 1593 bp cDNA fragment encoding a protein precursor was amplified using primers: *PPXI Nco I p* and *PPXI Bam HI as1*. The DNA fragments generated were purified, cleaved with *Nco I* and *Bam HI* and inserted into *Nco I*/*Bam HI* cleaved pQE 60 vector (Quiagen). Additionally, to increase solubility of the recombinant protein, the Glutathione S-transferase (GST) Gene Fusion System (Pharmacia) was used. A cDNA fragment encoding a protein precursor was amplified using primers: *PPXI Bam HI s* and *PPXI Sma I*. The purified PCR product was digested with *Bam HI/Sma I* restriction enzymes and cloned into the pGEX-2T expression vector digested with the same restriction enzymes.

A 1512 bp cDNA fragment encoding a second isoenzyme was amplified by PCR using the sense primer *PPXII Sph I* and the antisense primer *PPXII Bam HI as1*. The purified PCR fragment was digested with *Sph I*/*Bam HI* and subcloned into the *Sph I*/*Bam HI* sites of the pDS56-SphI vector (Stüber et al., 1984). In parallel, a GST Gene Fusion expression system was used. A cDNA fragment was generated by PCR using *PPXII Bam HI s* and *PPXII Sma I* sense and antisense primers, respectively. After purification, the PCR fragment was cleaved with *Bam HI* and *Sma I* and inserted into the pGEX 2T vector cleaved by *Bam HI/Sma I*.

The cDNA fragments were inserted in frame behind the ATG initiation codon present in the expression vectors. The *E. coli* expression strains SG13009 (Gottesmann et al., 1981), BL21 (Studier et al., 1990) and JM105 were transformed with these expression vectors. Recombinant protein synthesis was induced upon addition of IPTG (1mM final concentration) during exponential growth at OD_{600nm}= 0.4 for 4 h at 37°C. Total bacterial cell extracts were

sonicated and separated by centrifugation into soluble and pelletable protein fraction and analyzed on a 10% SDS-polyacrylamide gel.

3.7 Purification of recombinant proteins

One liter of IPTG induced bacteria cell culture containing expression plasmids for PPOX I or PPOX II was centrifuged at 500g for 15 min at 4°C. Lysis with lysozyme and detergent was performed according to Sambrook et al. (1989). The cell lysate was centrifuged at 12000g for 15 min at 4°C. Soluble fractions were removed and proteins were purified from inclusion bodies (Sambrook et al., 1989). Pellets containing inclusion bodies proteins were solubilized in SDS gel-loading buffer and separated by SDS-polyacrylamide gel electrophoresis. Proteins on SDS-polyacrylamide gels were visualized using Coomassie Blue R-250. Slices with PPOX I or PPOX II were cut from the gel. Proteins were eluted from the gel by Electro-Elutor (Bio-Rad). Circa 500 µg of PPOXI and 300µg of PPOX II were used to immunize rabbits.

3.8 Substrate preparation

The protoporphyrinogen, substrate of protoporphyrinogen oxidase, was reduced from protoporphyrin IX by sodium amalgam. All procedures for the reduction of protoporphyrin were carried out in dim light. Sodium amalgam was prepared as described by Vogel (1974). A solution of protoporphyrin IX (1 mM) was prepared in 10 mM KOH containing 20 % ethanol and flushed with argon gas. Sodium amalgam was ground to a fine powder in a mortar and pestle immediately before use, and added to protoporphyrin IX solution. The flask was vigorously shaken for 2-5 min until the fluorescence of protoporphyrin IX disappeared. After filtration of protoporphyrinogen IX solution, 50 mM DTT in 500 mM Tris was added to get a final concentration 0,2 mM DTT in 2 mM Tris. The solution was portioned and stored at –80°C.

3.9 Production of radiolabelled precursor proteins and organellar import studies

In vitro transcription and translation of the transcripts in the rabbit reticulocyte lysate in the presence of ^{35}S-methionine were performed according to the manufacturers' protocols (Stratagene and Promega). Mitochondria were isolated from pea plantlet essentially as described (Knorpp et al., 1994). Mitochondrial import assays were described in details

elsewhere (Grohmann et al., 1996). *In vitro* transport into pea chloroplasts was carried out according to (Grossman et al., 1982) with minor modifications (Grimm et al., 1989).

3.10 Enzyme assay of PPOX in bacterial extract

Bacterial cell cultures containing recombinant plasmids with cDNA sequences for PPOX I and PPOX II were grown to OD_{600} 0,4-0,6. The IPTG was added (to1mM) and bacteria were grown for 2.5-3 h. One ml aliquots of the *E. coli* cell cultures expressing one of the two recombinant isoenzymes of protoporphyrinogen IX oxidase, respectively, were centrifuged. The pellet was resuspended in 800 µl of assay buffer (50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 5 mM DTT, 0.03% Tween 80). Bacteria were lysed by sonication and, after dilution with assay buffer, incubated with 2 µM protoporphyrinogen IX at 30°C in the dark for 5 and 10 min according to Smith et al. (1993). Protoporphyrinogen IX was always freshly prepared from protoporphyrin IX (Fluka) using sodium amalgam as described above. The formation of protoporphyrin IX was monitored fluorimetrically at $405nm_{ex}$ and $632nm_{em}$ on a LS 50 B Fluorescence Spectrophotometer (Perkin-Elmer). Autoxidation of protoporphyrinogen IX was monitored by incubating substrate with heat-inactivated enzyme. Inhibitory effects of herbicides (e.g. acifluorfen) were assayed under the same reaction conditions using concentrations as indicated.

3.11 Vector constructs for plant transformation

A cDNA fragment of ca. 1,600 bp encoding *Arabidopsis thaliana* PPOX I (Accession No.: D83139; Ward and Volrath, 1995; Narita et al., 1996) was amplified by polymerase chain reaction 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 corresponding restriction sites of the multiple cloning site in the Bin AR vector (Höfgen and Willmitzer, 1992), a pBIB derivative. This vector contains the selectable kanamycine resistance gene marker, the *Agrobacterium tumefaciens* (*A. tumefaciens*) T-DNA left and right border sequences and the PPOX I sequence in sense orientation between the 5´ CaMV 35S promoter and the 3´ OCS transcription termination sequence.

A ca. 1.600 bp DNA fragment encoding tobacco PPOX I (Accession number: Y13465; Lermontova et al., 1997) was cut with *Bam HI*/*Sal I* from pBS KS inserted in antisense orientation into the Bin AR vector digested with the same restriction enzymes.

A cDNA fragment of 1939 bp encoding the mitochondrial isoform of tobacco protoporphyrimogen oxidase (Lermontova et al., 1997) was excited with *Bam HI* and *Sal I* from pBS SK and inserted into the Bin AR vector digested with the same restriction enzymes.

The resulting plasmids were transformed into the *A. tumefaciens* strain GV 2260 and transferred into tobacco plants (*Nicotiana tabacum* cv. SNN) by leaf disc transformation (Horsch et al., 1985). The insertion of copies of the transgene into the plant genome was confirmed by kanamycine resistance of regenerated explants and by genomic Southern blot hybridization or PCR amplification using the specific probes or oligonucleotide primers, respectively.

3.12 Transformation of tobacco plants

Over night cultures containing the vector for plant transformation were centrifuged 10 min at 3.000 g and the pellet was resuspended in 20 ml of 2YT medium. Sterile *N. tabacum* leaves were cut in 1 cm^2 pieces and incubated several minutes with the bacterial suspension. After removal of excessive liquid on sterile Whatman-paper, leaf pieces were placed on Petri-dishes with MS medium without antibiotics. Plates were incubated for 2 days in darkness. Then, leaves were transferred to MG medium with certain antibiotics and hormones until approximately 1-cm seedlings were regenerated. Regenerated seedlings were cut and placed on 2MS medium with selectable antibiotic (kanamycin) until the roots were formed.

3.13 DNA analysis

The methods for DNA extraction and for Southern blotting were carried out according to standard procedures (Sambrook et al., 1989). Aliquots of 8 µg DNA per sample were analyzed. For Southern hybridization DNA was digested with appropriate restriction enzymes, separated on 0,8 % agarose gel in Tris-borate buffer (Sambrook et al., 1989), transferred on Hybond N+ membrane (Amersham) and hybridized with cDNA fragments radioactively labeled with [^{32}P]dCTP by random priming (RadPrime DNA Labeling System; Amersham).

3.14 RNA analysis

Total RNA was extracted as described by Chomczinsky and Sacchi (1987), and analyzed by Northern blotting. Ten µg of RNA per sample were electrophoretically separated on 1% formaldehyde-agarose gels followed by capillary blotting on nylon membranes (Hybond N+, Amersham). Equal loading of samples was controlled by hybridization of RNA with cDNA probe for actin or 18S rRNA. Filters were probed with cDNA inserts that were radiolabelled by random priming, and washed under high stringency conditions.

3.15 Extraction of total leaf protein

Plant material (100 mg) was ground under liquid nitrogen and suspended in 1 ml solubilization buffer (56 mM Na_2CO_3, 56mM dithiothreitol, 2% SDS, 12% sucrose, 2mM EDTA). After 10 min incubation at 70^{o}C cell debris were removed by centrifugation. Protein concentration was determined according to Bradford (1976). Bovine serum albumin was used as a standard protein. Aliquots of the supernatant (10 to 20 µg protein) were used for SDS-polyacrylamide gel electrophoresis.

3.16 Western blot analysis

After SDS-polyacrylamide gel electrophoresis proteins were transferred onto nitro-cellulose membranes by the “semidry”-electro transfer apparatus by means of a three buffer system: Anode buffer I (0,3 M TRIS pH 10,4; 20% (v/v) methanol), Anode buffer II (25 mM TRIS pH 10,4; 20% (v/v) methanol) and Kathode buffer (40 mM 6-Aminocaproic acid pH 9,4). Protein was transferred 1 h at 0,8 mA/ cm². The filters were blocked with 3% milk-powder and 0,1% Tween 20 in 1x TBS (50 mM Tris/HCl pH 8; 150 mM NaCl) for 45 min. After the blocking step, filters were incubated for 1 h with the primary antibodies dissolved in blocking solution. The titer of antibodies was initially determined, when antiserum was used for the first time. After washing 3x10min with washing buffer (0,1% Tween 20, 1xTBS) membranes were incubated 1 h with the peroxidase-conjugated secondary antibodies diluted 1:10000 in blocking solution. A washing step 3x10 min with washing buffer subsequently followed. For the detection of recombinant proteins the ECL-System (Western blotting analysis system, Amersham) was used. It is based on chemoluminescense. Oxidation of luminol by a peroxidase conjugated to the secondary antibodies produces blue light. The light output is

increased and prolonged by the presence of an enhancer so that it can be detected on a blue-light sensitive film.

3.17 Analysis of tetrapyrrole intermediates

3.17.1 Determination of 5-aminolevulinic acid (ALA) synthesizing capacity

The ALA synthesizing capacity was measured as described by Papenbrock et al. (1999). Three leaf discs of the 4th leaf of the plant were harvested for each sample, incubated in 20mM phosphate buffer, pH 7.5 containing 40mM levulinic acid in the light for 6 h and then frozen in liquid nitrogen. Samples were homogenized, resuspended in 1 ml 20 mM K_2HPO_4/KH_2PO_4, pH 6.8 and centrifuged. The 500 µl supernatant was mixed with 100 µl ethylacetoacetate, subsequently boiled for 10 min and cooled down for 5 min. An equal volume of modified Ehrlich´s reagent was added and the absorption of the chromophore was determined at λ 553 and 525 nm with the spectrophotometer (Mauzerall and Granick, 1956). Standard curves were used for calculating the amounts of accumulated ALA.

3.17.2 Porphyrin analysis

Plant tissue (100 mg) was ground in 1 ml 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 ml. Porphyrins were separated by HPLC on a RP 18 column (Novapak C18, 4 µm particle size, 4.6X150 mm; Waters Chromatography) at a flow rate of 1 ml/min. Porphyrins were eluted with solvent B (90% methanol, 0.1 M ammonium acetate, pH 5.2) to solvent A (10% methanol, 0.1 M ammonium acetate pH, 5.2) as shown in table 2. The column eluate was monitored by a fluorescence detector (model 474, Waters) at λ_{ex}405nm and λ_{em}625nm. Protoporphyrin IX was identified and quantified using authentic standards (Kruse et al., 1995a).

Table 2: The gradient of buffer A and B for the separation and elution of porphyrins on a RP 18 column.

Time	Flow	Solvent A	Solvent B
	1ml	100 %	0 %
5 min	1ml	0 %	100 %
21 min	1ml	0 %	100 %
23 min	1ml	100 %	0 %
29 min	1ml	100 %	0 %

3.17.3 Determination of chlorophyll contents

Chlorophyll was extracted from 100 mg leaf tissue with 80 % alkaline acetone until the pellet became white (1-1,5 ml extract). Samples were diluted 1:10 in acetone and absorption was recorded at 664, 646 and 750 nm in a spectrophotometer. Chlorophyll contents were calculated according to Porra et al. (1989).

3.18 Enzyme assay of protoporphyrinogen oxidase in plants

Plastids were isolated from 8g of leaf material of 5-week-old tobacco plants. The leaves were homogenized with a Waring blender for 10s in 40 ml homogenization buffer (0.5 M sorbitol, 0.1 M Tris-HCl, pH 7.5, 1 mM dithiothreitol, 0.1% bovine serum albumin) and the homogenate was filtered through nylon gauze (100 µm). Plastids were collected by centrifugation at 5.000g for 10 min and resuspended in 2 ml assay buffer containing 0,1 M Tris-HCl, pH 7.5, 5 mM DTT, 1 mM EDTA and 0.03% Tween 80. The incubation mixture containing 70 µl of the chloroplast suspension (about 0.5 mg protein) and 130 µl of assay buffer. The reaction was started by adding 20 µl of approximately 130 µM protoporphyrinogen IX substrate. After a 5 min incubation, the reaction was stopped by adding 1ml of ice-cold methanol-DMSO (8:2, v/v). The mixture was centrifuged, and the resulting supernatant was flushed with nitrogen. A boiled chloroplast suspension was used in a parallel assay to correct for formation of protoporphyrin IX by autooxidation (Guo et al., 1991). For the inhibition experiment, the chloroplast suspension was mixed with an equal amount of assay buffer containing 2 µM acifluorfen and pre-incubated for 5 min before the reaction was started. Porphyrins were directly analyzed by HPLC as described above.

The biochemical analysis of enzyme activities and the determination of tetrapyrrole endproducts and precursors were performed at least in triplicate on each independent sample.

3.19 Analysis of antioxidants

3.19.1 Determination of ascorbate

Ascorbate content was determined according to Law et al. (1983) with some modifications. One hundred mg of leaf powder were homogenized with 1ml of 5 % sulphosalicylic acid. The suspension was centrifuged. The supernatant was adjusted to pH 5.5-6.5 by 150 mM phosphate buffer (pH 6.4) and 4 M NaOH. Aliquots of samples (200 µl) were then sequentially mixed with 100 µl water, 200 µl 10 % TCA, 200 µl 44 % H_3PO_4, 200 µl 4 % bipyridyl (dissolved in 70 % ethanol) and 100 µl of 3 % $FeCl_3$, shaking the tube thoroughly after each step. The reaction was incubated 60 min at 30^oC and the resulting absorbance was determined at 525 nm. For measuring the content of total ascorbate the oxidized fraction was reduced by adding 50 µl of 10 mM DTT and incubating for 15 min at room temperature. Surplus of DTT was removed by mixing with 50 µl of 0.5% N-ethylmaleimid. Except the addition of 100 µl of water, the same procedure was then carried out as described above for the reduced ascorbate. Values of absorbance were recalculated into ascorbate content per fresh weight by means of a calibration curve, using different concentrations of ascorbate and dehydroascorbate standards, respectively.

3.19.2 Analysis of glutathione

Glutathione was analyzed according to Creissen et al. (1999) with some modifications. Plant material (100 mg) was homogenized in liquid nitrogen. The extraction was performed by 1 ml of 0.1 N HCl. After 10 min centrifugation at 4^oC supernatants were collected in new Eppendorf tubes. Cleared supernatants were neutralized by adding an equal volume of 0.1 M NaOH. Aliquots (50µl) were mixed with 20 µl of 1M Tris pH 8 and 190 µl of water. Samples were incubated 1 h at room temperature for neutralization. For derivatisation 25 µl of 10 mM monobrombimane (MB) were added to the samples. Samples were incubated at room temperature for 15 min and subsequently combined with 705 µl of 5 % acetic acid. For determining total (reduced and oxidized) thiols, extracts were first reduced by the addition of 10 µl 10 mM DTT (2.5 mM final concentration). Separation of MB-derivatised thiols was achieved on a C18 reverse-phase column (Novapak C18, 4µm particle size, 4.6X250 mm;

Waters Chromatography) using a Waters HPLC system. The elution protocol (RT, 1 ml/min) employed linear gradients of solvents A (100 mM potassium acetate) and B (100 % methanol) as follows: 0 min 0 % B; 20 min, 18,5 % B; 22 min, 100 % B; 30 min, 100 % B; 33 min 0 % B; 36 min 0 % B; reinject. The MB derivatives of thiols were fluorimetrically detected (exitation 380 nm; emission 480 nm). Identification and quantification of GSH were performed by comparing retention time of known amounts of standards derivatised with MB.

3.19.3 Analysis of tocopherol

Tocopherol was extracted from 100 mg of leaf powder by adding 1ml of methanol containing 10 µM KOH. The homogenate was centrifuged at maximum speed and the supernatant was used for HPLC analysis. Tocopherols were separated on a HPLC system equipped with a C18 column (Novapak C18, 4µm particle size, 4.6X150 mm; Waters Chromatography) by using a gradient of solvent A (30 % methanol, and 10 % 0.1 M ammonium acetate, pH 5.2) and solvent B (100 % methanol) as follows: a linear gradient from 6 % A/94 % B at 0 min to 1 % A/99 % B at 10 min until 23 min with the same ratio of solution A and B. Standards were purchased from Merck (Darmstadt, Germany) and used to quantify and qualify the tocopherol eluted by HPLC.

3.20 Determination of thiobarbituric acid-reactive substances (TBA-RS)

TBA-RS as indicator of lipid peroxidation were measured according to Heath and Packer (1968). 0.1 g of leaf powder was extracted in 1 ml of 0.25 % TBA dissolved in 10 % TCA. After heating for 30 min at 95 °C, insoluble material was removed by centrifugation for 15 min at 15,000 g and the resulting supernatant was photometrically measured. The absorbance read at λ532 nm corrected for turbidity at λ600 nm gave the value for membrane damage on a fresh weight basis.

3.21 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 leaf 5 and 6 (as counted from the top of the plants) and incubated at 25°C in darkness for 20 h in 5 ml 1 % sucrose 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 dark incubation. As an indication for cellular damage electrolyte leakage was measured with a conductivity meter according to Orr and Hess (1981).

In a separate experiment four to six week-old plants were sprayed with a volume of 20 ml of a 10µM acifluorfen solution (in 10mM TRIS/HCl, pH 7.7, 0.05 % Tween 80) at the beginning of the dark period. For the analysis of protoporphyrin IX content leaf material was harvested 18h (12h dark incubation and 6h illumination), 3, 5 and 7 days after foliar spraying.

4 Results

4.1 Isolation and analysis of cDNA clones encoding protoporphyrinogen IX oxidase

Enzymatic activity of protoporphyrinogen oxidase has been measured in both plastids and mitochondria (Jacobs et al., 1991; Matringe et al., 1992b), indicating that two different genes coding for plastidal and mitochondrial isoforms of protoporphyrinogen oxidase could exist. For the identification of *PPOX* genes from tobacco we used the following strategies. The plasmid pBluescript SK- containing the tobacco cDNA library was excised from the amplified λ-ZAP vector. The plasmid library with approximately 1 x10^6 individual cDNA sequences was used to transform the *E. coli hemG* mutant R751 lacking protoporphyrinogen oxidase activity (Nishimura et al., 1995a). This mutant growth poorly even in rich medium. After transformation the bacteria cells were spread on LB agar plates. Bacterial clones were selected by colony size and growth rate. Only a few colonies grew on LB medium without heme supplementation. These colonies were picked and plasmid DNA was purified from them. The complementation capacity was confirmed by retransformation of the *hemG* mutant R751 with the isolated plasmid DNA. The putative cDNAs for tobacco PPOX were sequenced.

The first cDNA sequence comprised a total of 1644 nucleotides, including 29 and 216 nucleotides of 5´and 3´untranslated regions, respectively. It encoded a protein of 548 amino acids with a calculated molecular mass of 59,138 Da. The second cDNA sequence contained an open reading frame of 1515 nucleotides, a 5´untranslated region of 38 nucleotides and a 3´untranslated region of 386 nucleotides. The cDNA encoded a protein of 505 amino acids with a calculated molecular mass of 55,407 Da. The two cDNA sequences were designated *PPOX I* and *PPOX II*, respectively (Fig. 5; deposited in the EMBL gene data bank with accession numbers Y13465 and Y13466). The other sequenced cDNAs were identical either to *PPOX I* or *PPOX II*.

The coding regions of both full-length cDNA sequences were not in frame with the coding sequence of the *LacZ* gene indicating that complementation of the *hemG* mutant was not due to synthesis of the fusion LacZ-PPOX translation products. It is proposed that the synthesis of the proteins begins at the first or at an internal ATG codon of the cDNA sequences.

PPOX I

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1          TGAAGCGCGGTCTACAAGTCAGGCAGTCATGACAACAACTCCCATCGCCAATCATCCTAA
1                                      M  T  T  T  P  I  A  N  H  P  N

61         TATTTTCACTCACCAGTCGTCGTCATCGCCATTGGCATTCTTAAACCGTACGAGTTTCAT
21           I  F  T  H  Q  S  S  S  S  P  L  A  F  L  N  R  T  S  F  I

121        CCCTTTCTCTTCAATCTCCAAGCGCAATAGTGTCAATTGCAATGGCTGGAGAACACGATG
41           P  F  S  S  I  S  K  R  N  S  V  N  C  N  G  W  R  T  R  C

181        CTCCGTTGCCAAAGATTACACAGTTCCTTCCTCAGCGGTCGACGGCGGACCCGCCGCGGA
61           S  V  A  K  D  Y  T  V  P  S  S  A  V  D  G  G  P  A  A  E

241        GCTGGACTGTGTTATAGTTGGAGCAGGAATTAGTGGCCTCTGCATTGCGCAGGTGATGTC
81           L  D  C  V  I  V  G  A  G  I  S  G  L  C  I  A  Q  V  M  S

301        CGCTAATTACCCCAATTTGATGGTAACCGAGGCGAGAGATCGTGCCGGTGGCAACATAAC
101          A  N  Y  P  N  L  M  V  T  E  A  R  D  R  A  G  G  N  I  T

361        GACTGTGGAAAGAGACGGCTATTTGTGGGAAGAAGGTCCCAACAGTTTCCAGCCGTCCGA
121          T  V  E  R  D  G  Y  L  W  E  E  G  P  N  S  F  Q  P  S  D

421        TCCTATGTTGACTATGGCAGTAGATTGTGGATTGAAGGATGATTTGGTGTTGGGAGATCC
141          P  M  L  T  M  A  V  D  C  G  L  K  D  D  L  V  L  G  D  P

481        TAATGCGCCCCGTTTCGTTTTGTGGAAGGGTAAATTAAGGCCCGTCCCCTCAAAACTCAC
161          N  A  P  R  F  V  L  W  K  G  K  L  R  P  V  P  S  K  L  T

541        TGATCTTGCCTTTTTTGATTTGATGAGCATTCCTGGCAAGTTGAGAGCTGGTTTTGGTGC
181          D  L  A  F  F  D  L  M  S  I  P  G  K  L  R  A  G  F  G  A

601        CATTGGCCTCCGCCCTTCACCTCCAGGTCATGAGGAATCAGTTGAGCAGTTCGTGCGTCG
201          I  G  L  R  P  S  P  P  G  H  E  E  S  V  E  Q  F  V  R  R

661        TAATCTTGGTGGCGAAGTCTTTGAACGCTTGATAGAACCATTTTGTTCTGGTGTTTATGC
221          N  L  G  G  E  V  F  E  R  L  I  E  P  F  C  S  G  V  Y  A

721        TGGTGATCCCTCAAAACTGAGTATGAAAGCAGCATTTGGGAAAGTTTGGAAGTTGGAAGA
241          G  D  P  S  K  L  S  M  K  A  A  F  G  K  V  W  K  L  E  E

781        AACTGGTGGTAGCATTATTGGAGGAACCTTTAAAGCAATAAAGGAGAGATCCAGTACACC
261          T  G  G  S  I  I  G  G  T  F  K  A  I  K  E  R  S  S  T  P

841        TAAAGCGCCCCGCGATCCGCGTTTACCTAAACCAAAAGGACAGACAGTTGGATCATTCAG
281          K  A  P  R  D  P  R  L  P  K  P  K  G  Q  T  V  G  S  F  R

901        GAAGGGTCTCAGAATGCTGCCGGATGCAATCAGTGCAAGATTGGGAAGCAAATTAAAACT
301          K  G  L  R  M  L  P  D  A  I  S  A  R  L  G  S  K  L  K  L

961        ATCATGGAAGCTTTCTAGCATTACTAAGTCAGAAAAAGGAGGATATCACTTGACATACGA
321          S  W  K  L  S  S  I  T  K  S  E  K  G  G  Y  H  L  T  Y  E

1021       GACACCAGAAGGAGTAGTTTCTCTTCAAAGTCGAAGCATTGTCATGACTGTGCCATCCTA
341          T  P  E  G  V  V  S  L  Q  S  R  S  I  V  M  T  V  P  S  Y

1081       TGTAGCAAGCAACATATTACGTCCTCTTTCGGTTGCCGCAGCAGATGCACTTTCAAATTT
361          V  A  S  N  I  L  R  P  L  S  V  A  A  A  D  A  L  S  N  F
```

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1141      CTACTATCCCCCAGTTGGAGCAGTCACAATTACATATCCTCAAGAAGCTATTCGTGATGA
381         Y  Y  P  P  V  G  A  V  T  I  T  Y  P  Q  E  A  I  R  D  E

1201      GCGTCTGGTTGATGGTGAACTAAAGGGATTTGGGCAGTTGCATCCACGTACACAGGGAGT
401         R  L  V  D  G  E  L  K  G  F  G  Q  L  H  P  R  T  Q  G  V

1261      GGAAACACTAGGAACGATATATAGTTCATCACTCTTCCCTAACCGTGCCCCAAAAGGTCG
421         E  T  L  G  T  I  Y  S  S  S  L  F  P  N  R  A  P  K  G  R

1321      GGTGCTACTCTTGAACTACATTGGAGGAGCAAAAAATCCTGAAATTTTGTCTAAGACGGA
441         V  L  L  L  N  Y  I  G  G  A  K  N  P  E  I  L  S  K  T  E

1381      GAGCCAACTTGTGGAAGTAGTTGATCGTGACCTCAGAAAAATGCTTATAAAACCCAAAGC
461         S  Q  L  V  E  V  V  D  R  D  L  R  K  M  L  I  K  P  K  A

1441      TCAAGATCCTCTTGTTGTGGGTGTGCGAGTATGGCCACAAGCTATCCCACAGTTTTTGGT
481         Q  D  P  L  V  V  G  V  R  V  W  P  Q  A  I  P  Q  F  L  V

1501      TGGTCATCTGGATACGCTAAGTACTGCAAAAGCTGCTATGAATGATAATGGGCTTGAAGG
501         G  H  L  D  T  L  S  T  A  K  A  A  M  N  D  N  G  L  E  G

1561      GCTGTTTCTTGGGGGTAATTATGTGTCAGGTGTAGCATTGGGGAGGTGTGTTGAAGGTGC
521         L  F  L  G  G  N  Y  V  S  G  V  A  L  G  R  C  V  E  G  A

1621      TTATGAAGTTGCATCCGAGGTAACAGGATTTCTGTCTCGGTATGCATACAAATGAAACCT
541         Y  E  V  A  S  E  V  T  G  F  L  S  R  Y  A  Y  K  *

1681      GTGTTGGGGGTAGTCCAAACCTTGTTAGTAGTACGATCATGCCTTGGGAAAATTGGCATG
1741      TGCCTAAAAGTTTTGCTCATTAGAGTTATTTTAGCCTTGGTAAATGATTTGTACTTGATA
1801      TCAGTCGTTTTCTTTGAGATAAAATGTTCCTGTTCAGGAAATATAATGTATATCAATTTT
1861      AAACACTTGAATGTTGAGAAAAAAAAAAAAAA
```

PPOX II

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1         AGGAATTCCTCCCATTTGGAGATTATCGAAACCAGGATATGGCTCCTTCTGCCGGAGAAG
1                                                    M  A  P  S  A  G  E

61        ATAAACACAGTTCTGCGAAGAGAGTCGCAGTCATTGGTGCAGGCGTCAGTGGGCTTGCTG
20        D  K  H  S  S  A  K  R  V  A  V  I  G  A  G  V  S  G  L  A

121       CAGCATACAAGTTGAAAATCCATGGCTTGAATGTGACAGTATTTGAAGCAGAAGGGAAAG
40        A  A  Y  K  L  K  I  H  G  L  N  V  T  V  F  E  A  E  G  K

181       CTGGAGGGAAGTTACGTAGCGTGAGCCAAGATGGCCTGATATGGGATGAAGGGGCAAATA
60        A  G  G  K  L  R  S  V  S  Q  D  G  L  I  W  D  E  G  A  N

241       CTATGACTGAAAGTGAAGGTGATGTTACATTTTTGATTGATTCTCTTGGACTCCGAGAAA
80        T  M  T  E  S  E  G  D  V  T  F  L  I  D  S  L  G  L  R  E

301       AGCAACAATTTCCACTTTCACAAAACAAGCGCTACATTGCCAGAAATGGTACTCCTGTAC
100       K  Q  Q  F  P  L  S  Q  N  K  R  Y  I  A  R  N  G  T  P  V

361       TGTTACCTTCAAATCCAATTGATCTGATCAAAAGCAATTTTCTTTCCACTGGATCAAAGC
120       L  L  P  S  N  P  I  D  L  I  K  S  N  F  L  S  T  G  S  K

421       TTCAGATGCTTCTGGAACCAATATTATGGAAGAATAAAAAGCTCTCCCAGGTGTCTGACT
140       L  Q  M  L  L  E  P  I  L  W  K  N  K  K  L  S  Q  V  S  D
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481       CACATGAAAGTGTCAGTGGATTCTTCCAGCGTCATTTTGGAAAGGAGGTTGTTGACTATC
160       S  H  E  S  V  S  G  F  F  Q  R  H  F  G  K  E  V  V  D  Y

541       TAATTGACCCTTTTGTTGCTGGAACGTGTGGTGGTGATCCTGACTCGCTTTCAATGCACC
180       L  I  D  P  F  V  A  G  T  C  G  G  D  P  D  S  L  S  M  H

601       ATTCATTTCCAGAGTTGTGGAATTTAGAGAAAAGGTTTGGCTCAGTCATACTTGGAGCTA
200       H  S  F  P  E  L  W  N  L  E  K  R  F  G  S  V  I  L  G  A

661       TTCGATCTAAGTTATCCCCTAAAAATGAAAAGAAGCAAGGGCCACCCAAAACTTCAGCAA
220       I  R  S  K  L  S  P  K  N  E  K  K  Q  G  P  P  K  T  S  A

721       ATAAGAAGCGCCAGCGGGGATCTTTTTCCTTTTTGGGCGGAATGCAAACACTTACTGATG
240       N  K  K  R  Q  R  G  S  F  S  F  L  G  G  M  Q  T  L  T  D

781       CAATATGCAAAGATCTCAGAGAAGATGAACTTAGACTAAACTCTAGAGTTCTGGAATTAT
260       A  I  C  K  D  L  R  E  D  E  L  R  L  N  S  R  V  L  E  L

841       CTTGTAGCTGTACTGAGGACTCTGCGATAGATAGCTGGTCAATTATTTCTGCCTCTCCAC
280       S  C  S  C  T  E  D  S  A  I  D  S  W  S  I  I  S  A  S  P

901       ACAAAAGGCAATCAGAAGAAGAATCATTTGATGCTGTAATTATGACGGCCCCACTCTGTG
300       H  K  R  Q  S  E  E  E  S  F  D  A  V  I  M  T  A  P  L  C

961       ATGTTAAGAGTATGAAGATTGCTAAGAGAGGAAATCCATTTCTACTCAACTTTATTCCTG
320       D  V  K  S  M  K  I  A  K  R  G  N  P  F  L  L  N  F  I  P

1021      AGGTTGATTATGTACCGCTATCTGTTGTTATAACCACATTTAAGAGGGAAAACGTAAAGT
340       E  V  D  Y  V  P  L  S  V  V  I  T  T  F  K  R  E  N  V  K

1081      ATCCCCTTGAGGGCTTTGGGGTTCTTGTACCTTCCAAGGAGCAACAACATGGTCTCAAGA
360       Y  P  L  E  G  F  G  V  L  V  P  S  K  E  Q  Q  H  G  L  K

1141      CACTAGGCACCCTCTTCTCTTCTATGATGTTTCCAGATCGGGCACCAAACAATGTTTATC
380       T  L  G  T  L  F  S  S  M  M  F  P  D  R  A  P  N  N  V  Y

1201      TCTATACTACTTTTGTTGGTGGAAGCCGAAATAGAGAACTTGCAAAAGCCTCAAGGACTG
400       L  Y  T  T  F  V  G  G  S  R  N  R  E  L  A  K  A  S  R  T

1261      AGCTGAAAGAGATAGTAACTTCTGACCTTAAGCAGCTGTTGGGTGCTGAGGGAGAGCCAA
420       E  L  K  E  I  V  T  S  D  L  K  Q  L  L  G  A  E  G  E  P

1321      CATATGTGAATCATCTATACTGGAGTAAAGCATTTCCATTGTACGGGCATAACTATGATT
440       T  Y  V  N  H  L  Y  W  S  K  A  F  P  L  Y  G  H  N  Y  D

1381      CAGTCCTAGATGCAATTGACAAAATGGAGAAAAATCTCCCTGGATTATTCTATGCAGGTA
460       S  V  L  D  A  I  D  K  M  E  K  N  L  P  G  L  F  Y  A  G

1441      ACCACAGGGGGGGATTGTCAGTTGGCAAAGCATTATCTTCTGGATGCAATGCAGCTGATC
480       N  H  R  G  G  L  S  V  G  K  A  L  S  S  G  C  N  A  A  D

1501      TTGTTATATCATATCTTGAATCCGTCTCAACTGACTCCAAAAGACATTGCTGAAATCTAT
500       L  V  I  S  Y  L  E  S  V  S  T  D  S  K  R  H  C  *
1561      TTTCTCATGCAGCTTTCTAGATTGCTAAAATCGCTTAACTTTATCGCACAAGGTGCAACT
```

Figure 5: Nucleotide and derived amino acid sequences of the two isoforms of tobacco protoporphyrinogen oxidase. The sequence data reported here have been deposited in the EMBL gene bank database under accession numbers Y 13465 and Y 13466.

The aligned peptide sequences derived from protoporphyrinogen IX oxidase I and II (Fig. 6) showed 27.2% identical amino acid residues. The N-terminal end of both peptides differed in length. It was not assessable, whether each protein contains a plastidal or mitochondrial target sequence. Alignment of both proteins with known sequences revealed significant similarity to protoporphyrinogen IX oxidases which were identified by *E. coli hemG* complementation. The tobacco protoporphyrinogen IX oxidase I and II show sequence identity to the *A. thaliana* PPOX of 71.2% / 24.6% (Narita et al., 1996), *B. subtilis* HemY of 28.1 %/ 25.1 % (Hansson et al., 1992), mouse PPOX of 24.6% / 24.6% (Dailey et al., 1995), human PPOX of 23.5% / 21.4%(Nishimura et al., 1995) and S. *cerevisiae* PPOX of 19.3%/19.8% (Camadro et al., 1996). Sequence comparison indicated the close relation of the *Arabidopsis* protein to protoporphyrinogen IX oxidase I. On the basis of the sequence comparison to other eucaryotic homologous proteins we could not clearly establish which tobacco isoform is the mitochondrial enzyme. An alignment of all known protoporphyrinogen oxidase sequences indicated only a few conserved residues which are most likely involved in structural function and substrate and cofactor binding. A glycine rich motif G-X-G-X-X-G was previously proposed to be a dinucleotide binding site of well characterized flavoproteins (e.g. monoamine oxidase, putrescine oxidase, dimethylaniline oxidase) (Wierenga et al., 1986).

```
NTPPOXI     MTTTPIANHPNIFTHQSSSSPLAFLNRT--SFIP-FSSISKRNSVNCNGWRTRCS    52
ATHPPOX     ME------------------LSLLRPTTQSLLPSFSKPNLRLNV-YKPLRLRCS    35
NTPPOXII    MAPSAGEDK------HSSAKRVA------------------------------    17
BACHEMY     MS----DGKKHV-------------------------------------------     8
MUSPO       MGRTV--------------------------------------------------     5
            *.

NTPPOXI     VAKDYTVPSSAVDGGPAAEL--DCVIVGAGISGLCIAQVMS--ANYPN----LMV    99
ATHPPOX     VAGGPTVGSSKIEGGGGTTITTDCVIVGGGISGLCIAQALA--TKHPDAAPNLIV    88
NTPPOXII    ------------------------VIGAGVSGLAAAYKLK--IH----GLNVTV    41
BACHEMY     ------------------------VIIGGGITGLAAAFYMEKEIKEKNLPLELTL    39
MUSPO       ------------------------IVLGGGISGLAASYHLIRGPSPP----KVIL    32
                                     ..*.*..** . .           . .

NTPPOXI     TEARDRAGGNITTVE-RDGYLWEEGPNSFQPSDPMLT---MAVDC-GLKDDLVLG   149
ATHPPOX     TEAKDRVGGNIITRE-ENGFLWEEGPNSFQPSDPMLT---MVVDS-GLKDDLVLG   138
NTPPOXII    FEAEGKAGGKLRSVS-QDGLIWDEGANTMTESEGDVT---FLIDSLGLREKQQFP    92
BACHEMY     VEASPRVGGKIQTVK-KDGYIIERGPDSFLERKKSAP---QLVKDLGL-EHLLVN    89
MUSPO       VEGSKRLGGWIRSIRGSDGAIFELGPRGIRPTGPLGARTLLLVSELGLESEVLPV    87
             *.  . ** . .    .* . . *. ..       .     ..  ** ..

NTPPOXI     DPNAP----RFVLW--------KGKLRPVPSKLTDLAFFDLMSIPGKLRAGFGAI   192
ATHPPOX     DPTAP----RFVLW--------NGKLRPVPSKLTDLPFFDLMSIGGKIRAGFGAL   181
NTPPOXII    LSQNK----RYIAR--------NGTPVLLPSNPIDLIKSNFLSTGSKLQMLLEPI   135
BACHEMY     NATGQ----SYVLVNRTLHPMPKGAVMGIPTKIAPFVSTGLFSLSGKARAAMDFI   140
MUSPO       RGDHPAAQNRFLYVGGTLHPSPSG------------LRGLLRPSPPFSKPLFWA   129
                     ...          .*               ....        .
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NTPPOXI     GLR----PSPPGHEE-SVEQFVRRNLGGEVFEPLIEPFCSGVYAGDPSKLSMKAA   242
ATHPPOX     GIR----PSPPGREE-SVEEFVRRNLGDEVFERLIEPFCSGVYAGDPSKLSMKAA   231
NTPPOXII    LWKNKKLSQVSDSHE-SVSGFFQRHFGKEVVDYLIDPFVAGTCGGDPDSLSMHHS   189
BACHEMY     ------LPASKTKDDQSLGEFFRRRVGDEVVENLIEPLLSGIYAGDIDKLSLMST   189
MUSPO       GLREL-LKPRGKEPDETVHSFAQRRLGPEVASLAMDSLCRGVFAGNSRELSIRSC   183
                      . ..  * .*. * ** .  ....  *. .*.  .**.

NTPPOXI     FGKVWKLEETGGSIIGGTFKA-IKERSSTPKAPRDPRLPKPKGQTVGSFRKGLRM   296
ATHPPOX     FGKVWKLEQNGGSIIGGTFKA-IQERKNAPKAERDPRLPKPQGQTVGSFRKGLRM   285
NTPPOXII    FPELWNLEKRFGSVILGAIRSKLSPKNEKKQGPPKTSANKKRQRGSFSFLGGMQT   244
BACHEMY     FPQFYQTEQKHRSLILGM-------KKTRPQGSGQQLTAKKQGQ-FQTLSTGLQT   236
MUSPO       FPSLFQAEQTHRSILLGLL---LGAGQSPQPDSSLIRQARAERWSQWSLRGGLEV   235
            *   .. *.   *.. *          .   .       .       ..  *.

NTPPOXI     LPDAISARL-GSKLKLSWKL-----SSITKSEKGGYHLTYETPEGVVSLQSR--S   343
ATHPPOX     LPEAISARL-GSKVKLSWKL-----SGITKLESGGYNLTYETPDGLVSVQSK--S   332
NTPPOXII    LTDAICKDLREDELRLNSRVLELSCSCTEDSAIDSWSIISASPHKRQSEEESFDA   299
BACHEMY     LVEEIEKQLKLTKVYKGTKVTKLSHSG------SCYSLELDNGVTLDA-----DS   280
MUSPO       LPQALHNHLASKGVTV---LSGQPVCGLSLQPEGRWKVSLGDS----SLEA--DH   281
            * ...   *  . .    .    .       . . .  ..    .

NTPPOXI     IVMTVPSYVASNILRPLSVAA--ADALSNFYYPPVGAVTISYPQEAIRDERLVDG   396
ATHPPOX     VVMTVPSHVASGLLRPLSESA--ANALSKLYYPPVAAVSISYPKEAIRTECLIDG   385
NTPPOXII    VIMTAPLCDVKSMKIAKRGNPFLLNFIPEVDYVPLSVVITTFKRENVKYP-----   349
BACHEMY     VIVTAPHKAAAGMLSELPA----ISHLKNMHSTSVANVALGFPEGSVQ----MEH   327
MUSPO       IISAIPASELSKLLPAEAAP--LARILSTIKAVSVAVVNLQY-----RGACLP--   327
            .. . *      .            . .    ... *   .     .

NTPPOXI     ELMGFGQLHPRT---QGVETLGTIYSSSLFPNRAPKGRVLLLNYIGGAKNPEILS   448
ATHPPOX     ELKGFGQLHPRT---QGVETLGTIYSSSLFPNRAPPGRILLLNYIGGSTNTGILS   437
NTPPOXII    -LEGFGVLVPSKEQQHGLKTLGTLFSSMMFPDRAPNNVYLYTTFVGGSRNRELAK   403
BACHEMY     EGTGFVI-----SRNSDFAITACTWTNKKWPHAAPEGKTLLRAYVGKAGDESIVD   377
MUSPO       -VQGFGHLVPSSEDP---TVLGIVYDSVAFPEQDGNPPSLRVTVMLGGYWLQKLK   378
               **             . .  ...  .*. .    *  . .  .     .

NTPPOXI     KTESQLVEVVDRDLRKMLIKPKAQ--DPLVVGVRVWPQA-IPQFLVGH-LDTLST   499
ATHPPOX     KSEGELVEAVDRDLRKMLIKPNST--DPLKLGVRVWPQA-IPQFLVGH-FDILDT   488
NTPPOXII    ASRTELKEIVTSDLKQLL--GAEG--EPTYVNHLYWSKA-FP--LYGHNYDSVLD   451
BACHEMY     LSDNDIINIVLEDLKKVM--NING--EPEMTCVTRWHES-MPQYHVGHK-QRIKE   426
MUSPO       AAGHQLSPELFQQQAQEAAATQLGLKEPPSHCLVHLHKNCIPQYTIGH-CQKLDS   432
             .  ..   .  .  .          .*         .. .**   **  . . .

NTPPOXI     AKAAMNDNGLEGLFLGGNYVSGVALGRCVEGAYEVASEVTGFLSRYAYK-----   548
ATHPPOX     AKSSLTSSGYEGLFLGGNYVAGVALGRCVEGAYETAIEVNNFMSRYAYK-----   537
NTPPOXII    AIDKM-EKNLPGLFYAGNHRGGLSVGKALSSGCNAADLVISYLESVSTDSKRHC   504
BACHEMY     LREALAS-AYPGVYMTGASFEGVGIPDCIDQGKAAVSDALTYLFS---------   470
MUSPO       AMQFLTAQRLP-LTLAGASYEGVAVNDCIESGRQAAVAVLG--------TESNS   477
               . .    .  .*.  .*...   .. . ...  . .
```

Figure 6: Sequence alignment of the tobacco protoporphyrinogen oxidase I (NTPPOXI) and II (NTPPOXII) with the homologous enzymes of the *A. thaliana* (ATHPPOX), *B. subtilis* (BACHEMY) and mouse (MUSPO). The glycine rich motif is underlined. The position of identical amino acid residues in all sequences is indicated by asterisks, the position of well conserved residues by dots.

4.2 Translocation of protoporphyrinogen IX oxidase isoenzymes to plastids and mitochondria.

Particular attention was paid to the subcellular localization of both isoenzymes. Messenger RNA complementary to the cDNA sequences for protoporphyrinogen IX oxidase I and II was *in vitro* synthesized and translated. The translation products were incubated with intact plastids and mitochondria purified from young green pea plants. Reference precursor proteins, whose exclusive import into one of the organelles has been shown before, were additionally offered to plastids and mitochondria and served as marker for purity of organelle preparation and selective import. After finishing the translocation experiments, organelles were treated with proteases. Proteins protected from proteolysis were applied to polyacrylamide gels. A processed protoporphyrinogen IX oxidase I with an apparent molecular mass of 53 kDa was equally distributed between stroma and thylakoid fraction (Fig. 7, left panel). Coproporphyrinogen oxidase, the preceding enzyme in the pathway (Kruse et al., 1995a), was translocated to plastids and exclusively accumulated in the stroma. Protoporphyrinogen IX oxidase II and the 76 kDa NADH:ubiquinone oxidoreductase (Rasmusson et al., 1998) were attached to plastids, but did not resist to proteinase treatment of intact plastids indicating that these proteins were not imported.

The translocation experiment with intact mitochondria was also terminated with protease treatment. Protoporphyrinogen IX oxidase II and NADH:ubiquinone oxidoreductase were protected against protease and integrated in mitochondria (Fig. 7, right panel). A processing of both proteins could not be detected assessed from the mobility of both translation and import products on a 10% polyacrylamide gel. Difference in size between precursor and mature NADH:ubiquinone oxidoreductase could not be resolved in the gel system. This is likely due to the high molecular mass of the protein (Rasmusson et al., 1998). Protoporphyrinogen IX oxidase I and coproporphyrinogen oxidase were bound to mitochondria, but obviously not protected from protease treatment. Lack of these radioactively-labeled proteins in the protease treated mitochondrial fraction is an additional indication that the plastidal contamination in this preparation can be neglected.

Immunological detection of the protoporphyrinogen IX oxidase in subcellular fractions confirmed the correct targeting of the *in vitro* translated and imported isoenzyme I to plastids and II to mitochondria (Fig. 8).

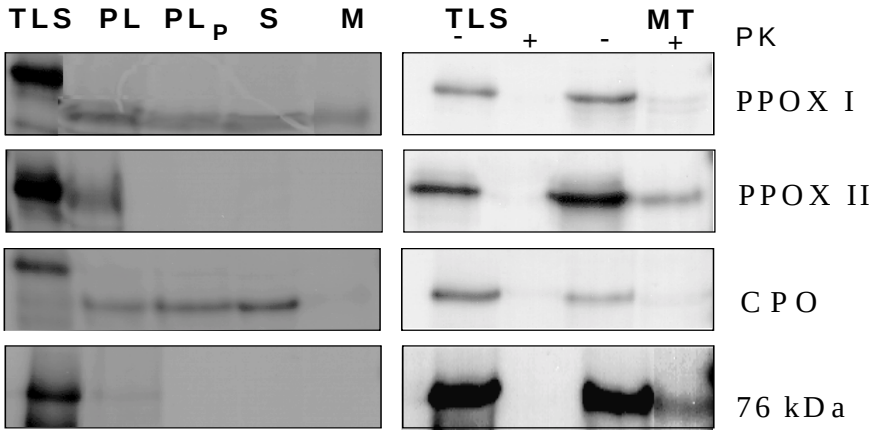


Figure 7: Post-translational uptake of *in vitro* synthesized precursor proteins. ^{35}S-labelled precursor of protoporphyrinogen IX oxidase I (PPOX I) and II (PPOX II) were synthesized by *in vitro* transcription/translation (TLS) and offered to intact pea chloroplasts (left panel) or mitochondria (right panel). Left panel: Following the import reaction, plastids were either analyzed directly (PL) or after treatment with proteases (PL_P). Plastids were reisolated and fractionated into stroma (S) and membrae (M). Right panel: Aliquots of the translation products (TLS) and of mitochondria after the import assay were incubated with (+) and without (-) proteinase K (PK) and applied to polyacrylamide gels. In both experiments, coproporphyrinogen oxidase (CPO) as a plastidal marker and a nuclear encoded mitochondrial subunit of NADH:ubiquinone oxidoreductase (76 kDa) were included as a reference. This experiment was performed by Dr. E. Kruse.

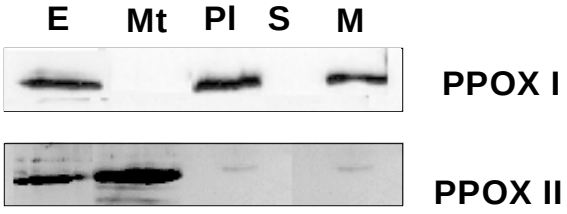


Figure 8: Immunological determination of the subcellular localization of the protoporphyrinogen IX oxidase isoforms. Proteins from total leaf extract (E), purified mitochondria (Mt) and plastids before (Pl) and after separation into stroma (S) and membrane (M) fraction were subjected to Western blot analysis using monospecific antibodies raised against recombinant the plastidal (PPOX I) and the mitochondrial (PPOX II) protoporphyrinogen IX oxidase.

4.3 In vivo expression studies

Southern blot hybridization with genomic tobacco DNA (Fig. 9) suggests that genes encoding plastidal and mitochondrial protoporphyrinogen IX oxidase are apparently encoded in small

subfamilies. Different patterns of hybridizing DNA bands were obtained with the two cDNA probes. The low similarity of both cDNA sequences excluded cross hybridization.

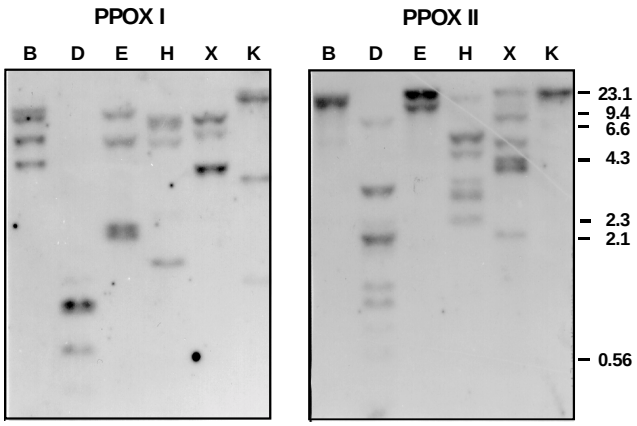


Figure 9: Southern blot analysis of PPOX in tobacco. 10 µg of genomic DNA was digested with *Bam* HI (B), *Dra* I (D), *Eco* RV (E) *Hind* III (H), *Xba* I (X) and *Kpn* I (K) and subjected to Southern blot hybridization using ^{32}P-labeled cDNA inserts encoding the plastidal (PPOX I) and mitochondrial (PPOX II) isoenzyme.

Northern blot analysis revealed protoporphyrinogen IX oxidase transcripts of circa 1,800 bases. The development dependent expression of genes encoding isoform I and II was determined in 6-week-old tobacco plants (Fig. 10). These RNA species transiently accumulated in expanding premature leaves (maximum in leaf 5, counting from the top to the bottom of the plant) before the level drastically decreased towards the oldest leaves. In roots RNA for the mitochondrial protoporphyrinogen IX oxidase was present, whereas the RNA level for the plastidal enzyme was below the detection limit. The amount of transcripts encoding protoporphyrinogen IX oxidase I and II did not alter during greening of 8-days-old etiolated tobacco seedlings (data not shown).

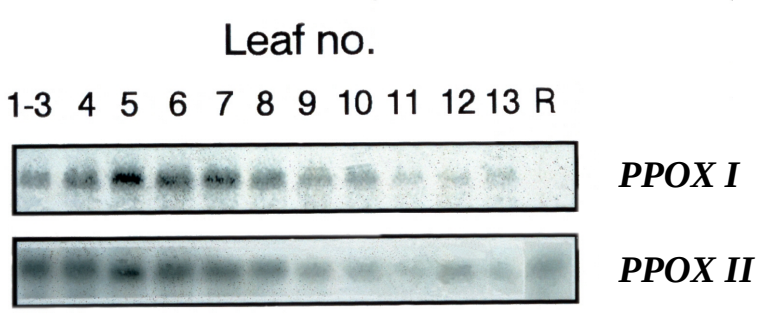


Figure 10: Accumulation of protoporphyrinogen IX oxidase transcripts during leaf development and in roots. Roots and leaves were harvested from six-week-old tobacco plants including the youngest leaves (1-3) and the oldest fully expanded leaf (12). Total RNA was extracted, 10 µg were subjected to Northern blot analysis using ^{32}P-labeled cDNA encoding both isoenzymes (PPOX I and PPOX II).

We also analyzed the RNA content within a period of 24 hour in tobacco plants grown 12h:12h light/dark regime and subsequently under constant low light (70 µmol photons m^{-2} s^{-1}) and dark conditions. RNA content encoding both isoenzymes oscillated under diurnal conditions with a maximum in the dark period (Fig. 11). The RNA displayed an inverse rhythmic periodicity under the light/dark regime in comparison to photosynthetic gene products (Kloppstech, 1986), which accumulate during illumination. Rhythmicity of protoporphyrinogen IX oxidase RNA expression could not be observed during constant growth conditions. This RNA was reduced to a basis level in constant darkness. The RNA expression is apparently not controlled by the endogenous clock. But the light/dark rhythm seems to synchronized the protoporphyrinogen IX oxidase gene activity leading to an oscillating expression pattern of the RNA species during diurnal growth. Experiments which are described in this section (4.3) were performed with the help of Dr. E. Kruse.

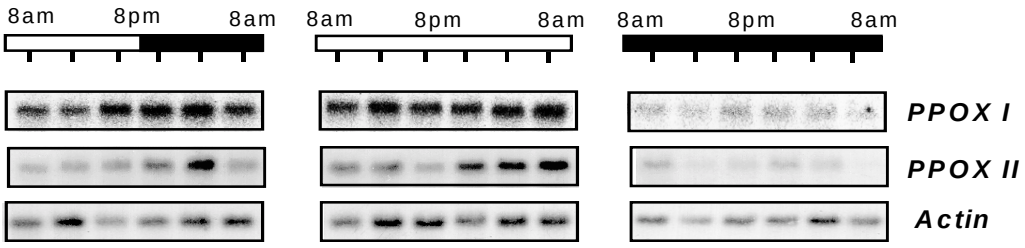


Figure 11: Steady state levels of protoporphyrinogen IX oxidase transcripts under diurnal and circadian growth conditions. Tobacco plants were grown for 4 weeks in a 12/12 light/dark cycle. Plants were harvested every four hours starting 1 hour after the onset of the light phase. Another set of plants was subsequently transferred to continuous light or darkness for 48 h. Leaf material was collected for the same four hour time intervals. Total RNA was isolated and used for Northern blot analysis as described in the legend of Fig. 10.

4.4 Expression in *E. coli*

4.4.1 Expression of PPOX I

Purification of protoporphyrinogen IX oxidase from plant sources is difficult because of the low content of the enzyme in plants (Camadro et al., 1995). The enzyme activity is reduced once this membrane-bound protein is solubilized with suitable detergents. Nevertheless, PPOX has been partially purified from barley (Jacobs and Jacobs, 1987), lettuce (Camadro et al., 1994) and chicory (Adomat and Böger, 2000). We attempted to overproduce active

protoporphyrinogen oxidase from tobacco in *E. coli*. For overexpressing of putative mature tobacco PPOX I, which is beginning with the amino acid valine at position 53 of the entire peptide sequence, the expression vector pQE 60 was used. When bacteria carrying the recombinant plasmid were induced with IPTG at 37 °C, an additional protein of circa 43 kDa was detected in cell extract of *E. coli* (Fig. 12, left panel) Bacteria carrying the non-recombinant plasmid, did not contain this protein. Surprisingly, the molecular mass of the obtained protein did not correspond to the mass of the deduced amino acid sequence. Sequencing of the recombinant *PPOX I* in the expressing vector showed a nucleotide substitution at position 1256 leading to a change of a codon for glycine to a stop codon. Probably this mutation was obtained during PCR amplification of the cDNA for the construction of the expression vector. The truncated PPOX I protein was used for the production of antibodies. To check the solubility of this protein, the bacterial culture was sonicated and centrifuged to yield bacterial supernatant and pellet. These fractions were separated by SDS-PAGE. The main portion of the recombinant protein was detected in the pellet fraction (Fig. 12, left panel, lane 2). PPOX I is a membrane bound protein. It was assumed that during overexpression in *E. coli* it could be found attached to the bacterial membranes. Subsequent experiments showed that the protein is part of inclusion bodies (data not shown). Recombinant truncated PPOX I was partially purified from inclusion bodies and loaded on SDS-PAGE, the band of interest was excised from the gel after visualization with Coomassie Blue stain. The protein was eluted from the gel by electro eluter. Approximately 500 µg of protein, obtained from two gels, were used for rabbit immunization (Fig. 12, left panel, lane 4).

In a second attempt expression of the entire mature protein was achieved by cloning of PCR amplified DNA fragment, obtained with another *Tag* polymerase, into the same expression vector. After induction of the bacterial culture with IPTG, the protein band was hardly detectable on a Coomassie Blue stained gel. The synthesis of the plastidal enzyme was immunologically demonstrated (Fig. 12, left panel, lane 5). The main amounts of expressed protein were detected again in inclusion bodies. Expression of several recombinant proteins showed that their association to inclusion bodies, in most cases, leads to the loss of activity due to incorrect protein folding. Proteins formed in inclusion bodies are hardly soluble and can only be solubilized with denaturants such as 6 M Gu HCl or 8 M urea. This makes protein purification and refolding difficult. Modified expression conditions such as lower temperature,

lower and higher concentration of IPTG, induction at different OD, dark growth did not help to increase the solubility of recombinant PPOX I proteins.

To increase the solubility of recombinant PPOX I, the glutathione S-transferase (GST) gene fusion system was used (Fig. 12, right panel). Four hours after induction of protein expression with IPTG, an additional band around 85 kDa was detected by SDS-PAGE in the pellet fraction. Changes of bacterial growth conditions unfortunately also did not lead to higher solubility of the expressed protein.

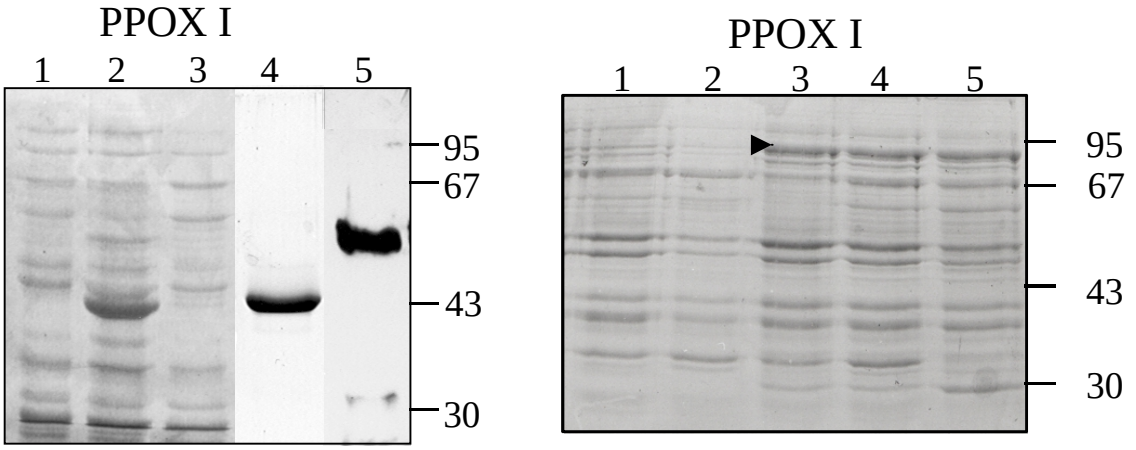


Figure 12: Protein analysis of tobacco protoporphyrinogen oxidase I overexpressed in *E. coli*. Left panel: Cells carrying the recombinant pQE 60 plasmid containing *PPOX I* cDNA were grown at 37 °C to an OD_{600}nm of 0.6, induced by IPTG and incubated for 3 h at 37 °C. Thereafter, cells were collected by centrifugation, sonicated and separated by centrifugation in to pellet and soluble fractions, which were loaded on SDS- PAGE (lane 2 and 3). A protein extract of bacteria containing pQE 60-vector (lane1) was used as control. Lane 4 shows a purified protein prepared for rabbit immunization. Expression of nonmutated putative mature protein was detected by Western blot analysis with antibodies obtained against truncated PPOX I (lane 5). Right panel: Bacterial cells carrying the recombinant pGEX 2T plasmid with cDNA for PPOX I were grown and induced under the same condition as described above. After induction with IPTG, cells were grown for 4, 6 and 24 hours at 37 °C. A protein extract from bacteria containing the pGEX 2T –vector was loaded as control (lane 1). Bacterial cells collected 4 h after induction were sonicated. Soluble and pellet fractions were loaded on the SDS-PAGE (lane 2 and 3). Lanes 4 and 5 showed an expression of recombinant PPOX I, 6 and 24 h after induction with IPTG.

4.4.2 Expression of PPOX II

For overproduction of the mitochondrial isoform of PPOX, the expression vector pDS 56/Sph I was used. One hour after induction of the recombinant gene expression with IPTG an additional band appeared on the SDS-gel (Fig. 13, left panel). Molecular mass of the recombinant mitochondrial isoenzyme (55 kDa) separated by SDS-PAGE was in good agreement to the mass of its deduced amino acid sequence. The main part of the synthesized protein was found in inclusion bodies. The protein from inclusion bodies was purified as described above for PPOX I and circa 300 µg of the recombinant protein were used for antibodies production. Overexpression of PPOX II in the pGEX 2T expression vector resulted in accumulation of a nonsoluble protein with a molecular mass of circa 80 kDa in bacterial extract harvested 4 hours after induction with IPTG (Fig. 13, right panel).

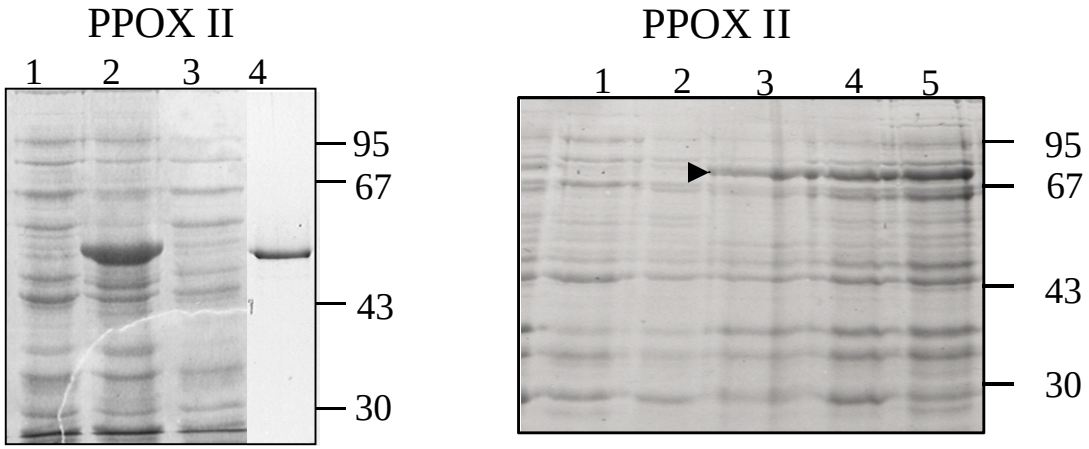


Figure 13: Protein analysis of tobacco protoporphyrinogen oxidase II after overexpression in *E. coli*. Expression of PPOX II was performed in expression vector pDS56/ SpH (left panel) and pGEX 2T (right panel). The lanes in the left and right panel correspond to the same lanes described for Fig. 12.

4.4.3 Activity of recombinant protoporphyrinogen IX oxidase I and II

Protoporphyrinogen IX oxidase activity was determined from bacterial extract containing the recombinant isoenzymes I and II. Bacterial extracts were collected after different time periods of induced synthesis of the two recombinant protoporphyrinogen IX oxidases. A significant activity of the plastidal enzyme could not be shown relative to the heat denatured control (data

not shown). The mitochondrial enzyme was synthesized in an active form in both selected expression systems. Comparison of protoporphyrinogen oxidase activity in *E. coli* cell extract with and without recombinant plasmid revealed an approx. 14-fold increase in the conversion of protoporphyrinogen IX to protoporphyrin IX in the presence of the recombinant plasmid. This activity could be inhibited by acifluorfen at a saturating substrate concentration (Fig. 14). 100 nM acifluorfen reduced the enzyme activity to more than 50%. This level of inhibition indicates that the tobacco mitochondrial protoporphyrinogen IX oxidase seems to be more sensitive to acifluorfen than the recombinant enzyme of *Arabidopsis* (Narita et al., 1996) or the native enzyme from barley etioplasts (Lee et al., 1993) and human tissues (Corrigall et al., 1994; Dailey and Dailey, 1996 b), but less prone than the yeast enzyme (Camadro et al., 1994).

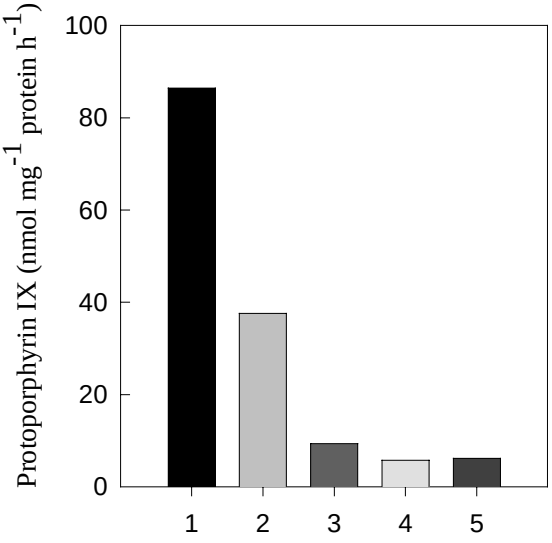


Figure 14: Activity of the recombinant isoform II of PPOX. An *E.coli* clone expressing the mitochondrial PPOX (PPOX II) was lysed. Enzyme activity of the bacterial homogenate (1), in the presence of 100 nM (2), 1 µM (3) and 10 µM (4) acifluorfen and endogenous PPOX activity of the *E.coli* strain SG 13009 harboring the plasmid pDS 56 (5) was measured as described in Methods.

4.5 Generation of transgenic tobacco plants expressing PPOX I or PPOX II gene in sense and antisense orientation

A direct approach to determine the significance of an enzyme for the control of the metabolite flux in a pathway is to decrease or to increase the amount of the target enzyme in transgenic plants by expressing genes in antisense or sense orientation. We inserted cDNA sequences coding for PPOX I from *Arabidopsis* in sense and from tobacco in antisense orientation into the plant expression vector under control of a cauliflower mosaic virus (CaMV) 35S promoter.

A cDNA fragment encoding tobacco PPOX II was inserted in sense orientation into the same vector. Constructs were introduced into plants by *Agrobacterium* mediated leaf discs transformation. As an example, the plasmid map of a vector carrying *PPOX I* in sense orientation is presented in Figure 15.

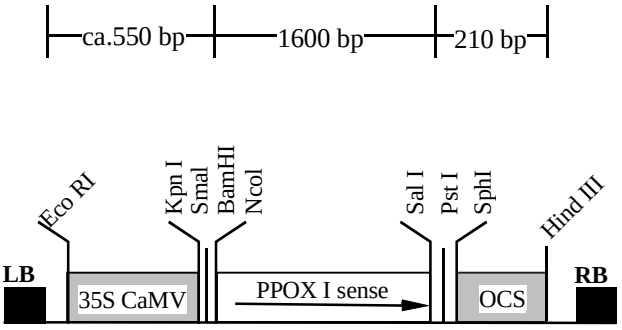


Figure 15: Restriction map of sense construct for PPOX I in BinAR vector. A cDNA fragment coding for *Arabidopsis thaliana* PPOX I was amplified by PCR. After digestion with Bam HI and Sal I fragment was inserted in the same restrictions sites of the multiple cloning site of BinAR vector.

4.6 Characterization of PPOX I overexpressing plants

4.6.1 Selection of transformants

The generation of transgenic plants, overexpressing a herbicide target enzyme, is one way to produce herbicide resistant plants. We inserted the full-length cDNA sequence encoding the *Arabidopsis thaliana* PPOX I into the binary plant vector BinAR which was transformed into the tobacco plants as described above. Approximately 125 individual transgenic lines which grew 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 increase of PPOX I levels in comparison to control plants. These lines were phenomenologically not distinguishable from the wild type plants. They displayed the same growth rate as control plants. None of the lines displayed bleached or necrotic leaf lesions as result of transgene expression.

4.6.2 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 five weeks in soil. Three discs of leaf 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 in comparison to control plants (Fig. 16) 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.

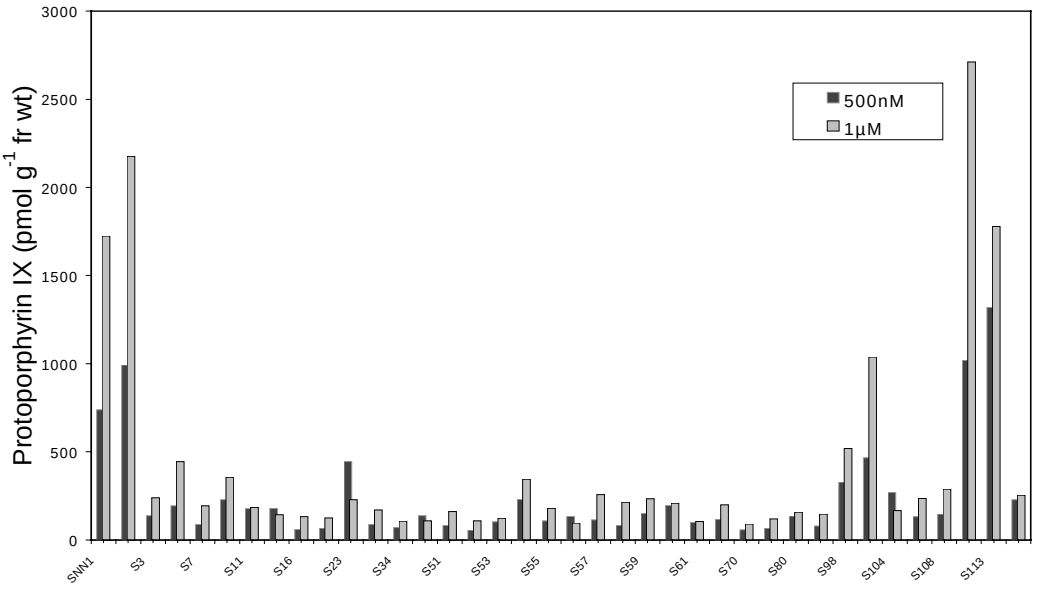


Figure 16: Acumulation of protoporphyrin IX in leaf discs of tobacco wild type and PPOX I overexpressing transgenic plants after 20 h dark incubation with buffer containing 500 nM and 1 µM of acifluorfen. Leaf discs were harvested from leaf 5 of five-week-old tobacco plants and incubated in the presence of acifluorfen. Protoporphyrin IX was extracted and analyzed by HPLC.

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 result of membrane damage after herbicide treatment (Fig. 17). Peroxidative degradation of porphyrins in the cytoplasm affects membrane

integrity. The degree of ion leakage is used to quantify phytotoxicity of the peroxidizing herbicide. Becerril and Duke (1989) showed that ion leakage of cell membranes correlates with the content of accumulated protoporphyrin IX. In Figure 17A protoporphyrin IX contents are presented from discs of leaf 5 and 6 after a 20 h dark incubation in various concentrations of acifluorfen in the range from 0.5 to 100 µM. The control plants accumulated at least three times more protoporphyrin IX than the transgenic plants at all tested 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 10 µM acifluorfen were required to elevate protoporphyrin IX levels in the leaves of the transformants (Fig. 17B). Membrane permeability as indicated by conductivity measurements did not significantly change in leaf disks 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. 17C).

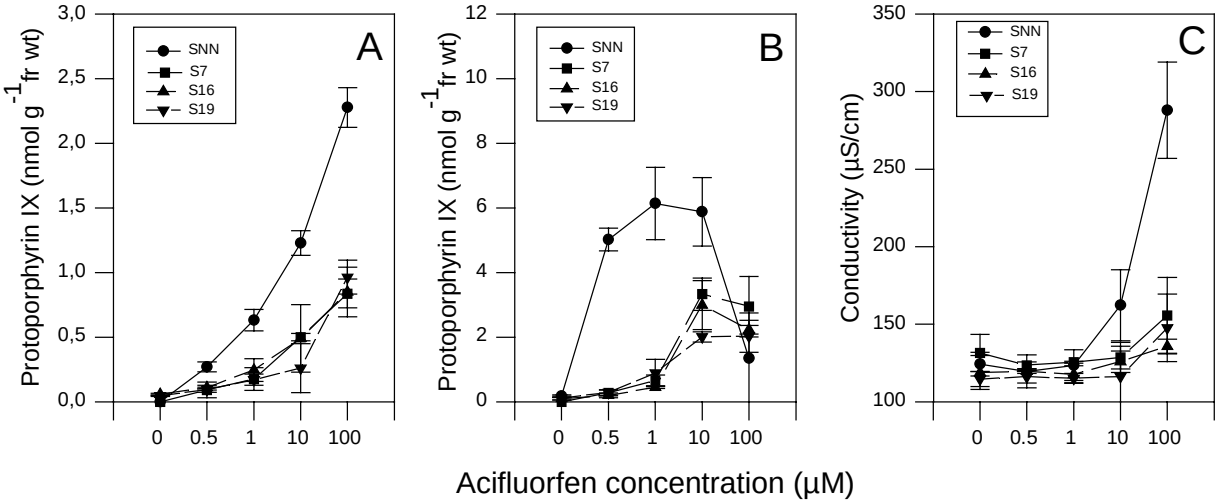


Figure 17: Effects of the peroxidizing herbicide acifluorfen on protoporphyrin IX content and membrane integrity. Discs of leaves 5 and 6 (counting from the top of the plant) of 4-week-old wild-type and PPOX I-over-expressing plants (line S7, S16, and S19) were incubated at 25°C with various concentration of acifluorfen either 20 h in darkness (A) or 20 h in darkness and 6 h in light (B, C). Protoporphyrin IX was extracted and quantified by HPLC (A, B). Electrolyte leakage was measured from leaf discs with a conductivity meter (C).

4.6.3 Physiological and molecular analysis of plants overexpressing PPOX I protein

Chlorophyll content, as end product of tetrapyrrole biosynthesis was measured in leaves of selected transgenic lines. 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 3). Line S7 contained on average 13% less chlorophyll and line S19 contained ca. 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 endproducts. A lower metabolic rate would lead to less accumulating porphyrin in the presence of the inhibitor. The capacity of ALA synthesis was similar or slightly higher in the transgenic lines in comparison to the wild type (Table 3).

Table 3: ALA synthesizing capacity and chlorophyll content in leaves of wild type (SNN) and transgenic tobacco plants overexpressing *Arabidopsis* PPOX I

	ALA	Chlorophyll	
	Leaf no. 4	Leaf no. 4	Leaf no. 6
	nmol g^{-1} fresh wt	µg g^{-1} fresh wt	
SNN	403.19 ± 63.12	947.22 ± 31.96	1050.13 ± 78.76
S7	409.73 ± 25.29	824.53 ± 8.60	921.52 ± 11.72
S16	440.34 ± 51.79	925.13 ± 91.18	1059.07 ± 183.98
S19	424.80 ± 44.74	1049.94 ± 100.60	1170.23 ± 5.38

Southern blot analysis of genomic DNA isolated from wild type and PPOX I overexpressing plants showed the integration of only one copy of the transgene into the tobacco genome in case of transgenic lines 7, 16 and 19 and several copies in case of lines 34, 42 and 52 (Fig. 18). Use of the *Arabidopsis* cDNA fragment led to strong hybridization signals with the transgene and weak signals with the endogenous tobacco PPOX I gene. Increased hybridization

temperature from 60 to 65°C resulted in hybridization of radioactive probe only with the transgenes (data not shown).

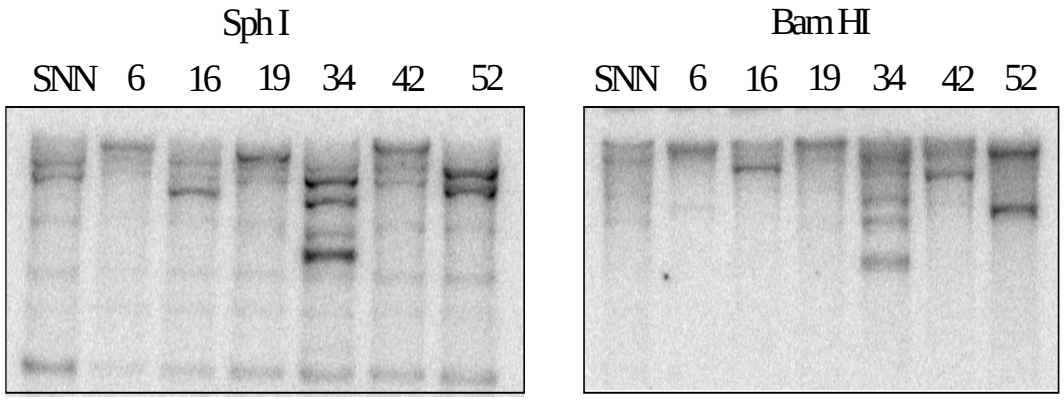


Figure 18: Southern blot analysis of 10 µg genomic DNA isolated from tobacco wild type (SNN) and PPOX I overexpressing transgenic lines 7, 16, 19, 34, 42, 52. DNA was cut with Bam HI (right) and Sph I (left) and subjected to Southern blot hybridization using an *Arabidopsis* ^{32}P-labeled cDNA insert as probe.

Total RNA was extracted from leaf 2, 4, and 6 of tobacco wild type and transgenic plants. Northern blot analysis was performed with radioactively labeled cDNA probes encoding the *Arabidopsis thaliana PPOX I* and tobacco *PPOX II* (Fig. 19). 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 towards 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. 19, upper panel).

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 immuno-detectable protein band for PPOX I revealed enormous differences between the levels of wild type and transgenic plants (Fig. 19, lower panel). 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 over-expressing plants in comparison to the wild type.

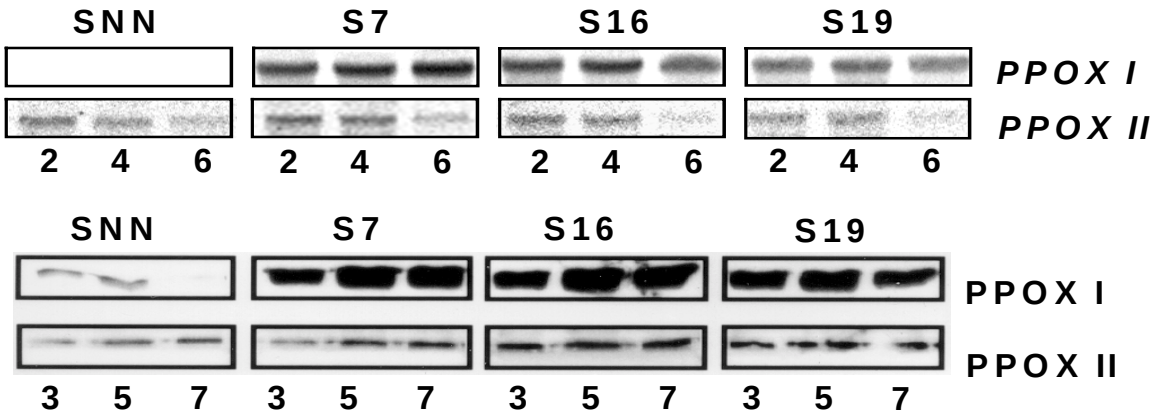


Figure 19: Expression studies in wild type (SNN) and PPOX I overexpressing plants (line S7, S16, S19). Upper panel: Northern blot analysis of PPOX I and PPOX II mRNA levels in leaf 2, 4 and 6 of wild type (SNN) and PPOX I sense plants. Equal amounts of RNA (10 µg) were separated on formaldehyde-containing agarose gels. Equal loading of RNA was proven by ethidium bromide staining. RNA was blotted onto nylon membrane. The filters were hybridized with fragments of *Arabidopsis PPOX I* and tobacco *PPOX II* cDNA probes.
Lower panel: Western blot analysis of PPOX I sense and control plants. Total protein extracts were prepared from leaf 3, 5, 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.

4.6.4 Determination of PPOX enzyme activity

Activity of PPOX was determined from chloroplast suspensions prepared from four-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 determines the amount of fluorescent protoporphyrin IX at the beginning and after different time points. Furthermore, the amount of protoporphyrin IX which is enzymatically formed can be measured by subtracting the amount of protoporphyrin IX formed in the heat-denatured sample. The PPOX activity was increased in the 3 selected lines in comparison to the wild

type. Extracts of lines S7 and S16 displayed a six-fold, line S19 a five-fold higher PPOX activity than the wild type (Fig. 20).

Addition of 1µM acifluorfen to the enzyme assays completely abolished the PPOX activity of control extracts. In spite of a relatively high concentration of acifluorfen in the enzyme assays, the protoporphyrinogen oxidation capacities of the plastids extracts from the transformants were in a range similar to the activities found in non-inhibited wild type extracts. PPOX I activity of line S16 was inhibited by acifluorfen to 6%, of lines S7 and S19 to 17 and 13% remaining activity, respectively.

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

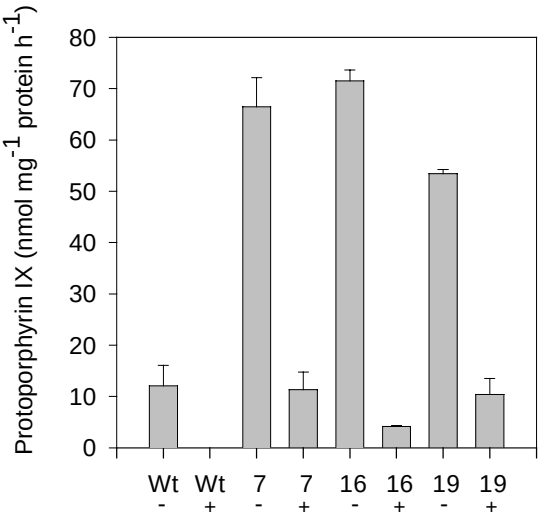


Figure 20: PPOX I enzyme activity in crude chloroplast extracts of PPOX I overexpressing (line S7, S16 and S19) and wild type (Wt) tobacco plants. Crude chloroplast extracts were prepared as described in the Methods. Enzyme activities were measured without (-) and with (+) 1 µM of acifluorfen.

4.6.5 Germination and growth of transgenic tobacco seeds expressing PPOX I in the presence of acifluorfen.

To test the resistance of PPOX I overexpressing plants towards peroxidizing herbicides a number of experiments were performed. Different ways of herbicide application were

followed by measurements of physiological and biochemical parameters which could directly or indirectly show the extent of inhibition of target enzyme by the herbicides.

Seeds of transgenic plants, overexpressing the PPOX I protein, could germinate and grow at herbicide concentrations which are toxic for seeds of control tobacco plants. In pre-experiments the lethal concentration of acifluorfen of wild type tobacco seeds was determined. Seeds were sterilized and germinated on sterile Murashige-Skoog agar medium containing 50, 100, 200 and 300 nM of acifluorfen in the light. A the sublethal concentration of 100 nM of 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 21. Wild type seeds did not germinate in the presence of the herbicide at this concentration.

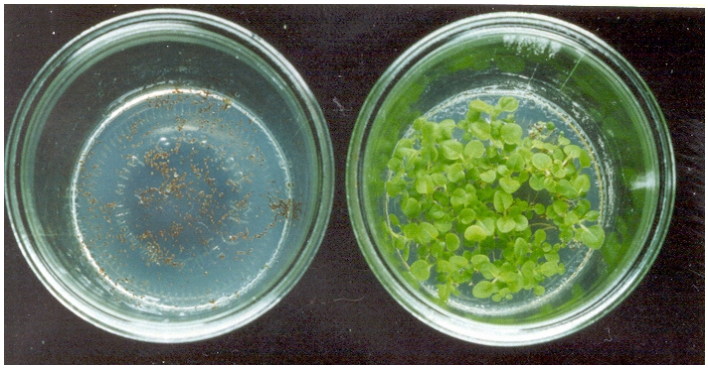


Figure 21: Germinating seeds from tobacco wild type (SNN) and the T1 generation of the PPOX I overexpressing line S19 on MS medium containing 300 nM acifluorfen.

4.6.6 Treatment of tobacco PPOX I overexpressing and control plants with acifluorfen

Five-week-old PPOX I over-expressing and wild type plants were sprayed with 20 ml of 10 µM acifluorfen at the beginning of the dark period**.** Leaf discs were harvested from leaf 3 and 5 for porphyrin analysis 18 hours (12 h dark and 6 h light), 3, 5 and 7 days after the treatment. Figure 22 shows a control and a transgenic plant (line S7) 3 days after the single acifluorfen treatment. The selected transgenic plants hardly showed any necrotic lesions after the application of acifluorfen, while necrotic areas of entirely desiccated tissue became visible on

wild-type leaves. The differences between transgenic and control plants reflect the lower photosensitization in the transgenic leaf tissue. Protoporphyrin IX contents of PPOX I over-expressing and wild type plants were compared 18 h after herbicide application (Fig. 23). Porphyrin contents in transgenic plants were observed to be lower than in wild type plants (corresponding to 20–40% of the wild type value) and were below a certain level which seemed to be not phytotoxic to tobacco plants. The amounts of accumulating protoporphyrin IX were decreased in all plants from day 1 to 7 after application of the herbicide due to photooxidative degradation of protoporphyrinogen IX (data not shown).

Figure 22: Comparison of the phenotype of transgenic PPOX I overexpressing line S7 (right) and wild type plant (left) 3 days after acifluorfen treatment. Each plant was sprayed with 20 ml of a 10 µM acifluorfen solution.

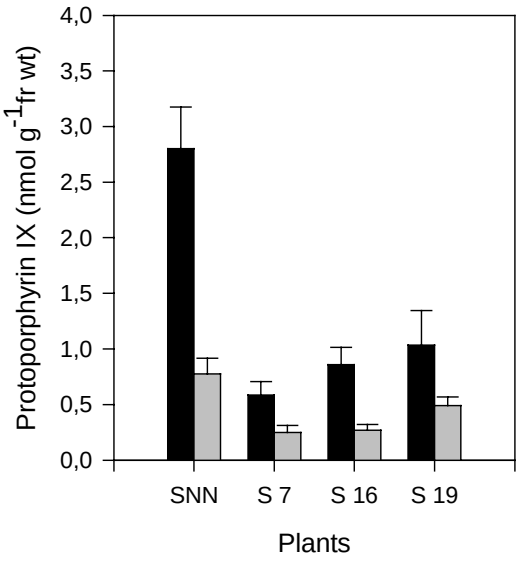


Figure 23: Accumulation of protoporphyrin IX in 5-week-old tobacco wild type (SNN) and PPOX I antisense transgenic plants (S7, S16, S19) after a 18 h treatment with acifluorfen. Each plant was sprayed with 20 ml of a 10 µM acifluorfen solution. Porphyrins were analyzed from leaf 4 (black) and 6 (grey column).

4.6.7 Tolerance of PPOX I overexposing plants against herbicides of other chemical classes

Peroxidizing herbicides functioning via inhibition of protoporphyrinogen IX oxidase consist of several chemical classes (section 1.6). The potency of these herbicides depends on their chemical structure and substitutions of different functional groups. The herbicide acifluorfen, which was mainly used in our experiments, belongs to diphenyl ether type of peroxidizing herbicides. Among herbicides of this chemical class acifluorfen has quite low inhibition capacity. To investigate the resistance of PPOX I overexpressing plants against herbicides of other chemical classes we tested two different herbicides provided by the industrial partner. Six transgenic lines overexpressing protoporphyrinogen oxidase I were investigated. Since molecular masses of these herbicides are unknown, we estimated concentrations relatively to acifluorfen in µg/l. In this experiment we found that herbicide SLR 317742 is about 100 times more potent than SLR 217036. On the basis of this observation, different concentrations of herbicides were used: 3,62 and 36,2 µg/l of SLR 317742, 362 and 3620 µg/l of SLR 217036 in further experiments. Three leaf discs of leaf 5 were incubated for 20 h in darkness with herbicides. As indication of herbicide action the amounts of protoporphyrin IX in leaves of control and transgenic plants were measured. Selected transgenic lines accumulated less protoporphyrin IX at all herbicide concentrations. (Fig. 24) Incubation of leaf discs with 3,62 µg/l of herbicide SLR 317742 resulted in 7 to 25 fold differences between control and transgenic plants in accumulation of protoporphyrin IX. At the higher concentrations of this herbicide applied, difference decreased to 2-4 fold. Herbicide SLR 217036 was not so powerful. At concentrations 100 fold higher than the applied concentrations of SLR 317742, it led to lower amounts of accumulated porphyrins in wild type and transformants. Therefore, at a concentration 362 µg/l of SLR 217036, the leaves of control plants accumulated about 3 times less of protoporphyrin IX than with 3,62 µg/l of SLR 317742. Our data suggest that overexpression of PPOX I leads to higher tolerance of tobacco plants, not only against DPEs herbicides, but also herbicides of other chemical classes. The herbicides SLR 317742 and SLR 217036 are more potent than acifluorfen, since application of acifluorfen at concentrations up to 100 µM (36,2 mg/l)) resulted in lower accumulation of protoporphyrin IX. However, these observations were made in separate experiments. A more

precise quantitation could be achieved through a comparison of all three inhibitors in a single experiment.

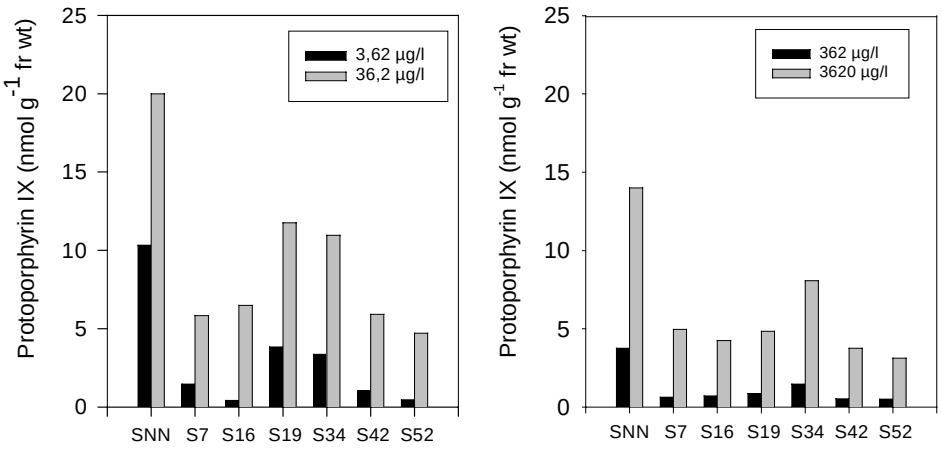


Figure 24: Accumulation of protoporphyrin IX in leaf discs of control and PPOX I overexpressing tobacco plants after 20 h dark incubation with herbicides. Left panel: Leaf discs were incubated with 3,62 and 36,2 of herbicide SLR 317742. Right panel: Leaf discs were incubated with 362 and 3620 µg/l of herbicide SLR 217036. After incubation porphyrins were extracted and analyzed by HPLC.

4.7 Characterization of tobacco plants expressing antisense mRNA for PPOX I

4.7.1 Phenotype of transgenic plants transformed with PPOX I antisense genes

A partial cDNA sequence encoding PPOX I from *N. tabacum* (Lermontova et al., 1997) was inserted in reverse orientation behind the CaMV 35S promoter of the binary vector BinAR as described in "Methods". Fifty generated transgenic lines were cultivated in the greenhouse and analyzed for the insertion of the transgene. The transformants were generally characterized by a growth rate slower than or similar to that of control plants. Three selected transgenic lines, which had necrotic lesions already in tissue culture, were transferred into growth chambers with low light (70 µmol m^{-2} s^{-1}) and high light (550 µmol m^{-2} s^{-1}) intensities. Previous analysis of transgenic plants with reduced activity of UROD showed that the phenotype of transformants depends on the light intensity. Transgenic plants grown under low light conditions were better protected from photooxidation induced by accumulating porphyrins than HL-grown plants (Mock and Grimm, 1997). In contrast, PPOX I antisense transformants grown under low light intensity grew significantly slower than the wild type and had strong necrotic lesions (Fig. 25B). The same transgenic lines grown under greenhouse and high light were only slightly damaged and had nearly the same size as control plants (Fig. 25A, B).

The primary analysis of PPOX I antisense plants was done with T1 progenies of three selected transgenic lines (AS6, AS17, AS19) grown in the greenhouse. Analysis of light effects on growth and phenotype of PPOX I transformants was performed with T1 progenies of transgenic line AS19 grown in the greenhouse for 6-7 weeks and then transferred to the growth chambers with different light intensities. Comparative analysis of transgenic plants grown from the beginning under different light conditions was difficult, due to the different size and development of the transformants. Transgenic plants grown in the greenhouse, showed necrotic lesions after exposure to low light intensity within 48 hours (Fig. 25C, D). The strongest necrosis was on the middle leaves of the transgenic plants (from leaf 4 to 8) (Fig. 25C) In contrast, transformants exposed to the high light grew even better than in the greenhouse. They had normal pigmentation without necrotic spots (Fig. 25B, D). Transfer of transgenic plants, adapted for one week to low light conditions, into the growth chamber with high light intensity, led to their recovery during 1-2 weeks. In contrast, high light adapted plants became necrotic after transfer to low light conditions (data not shown).

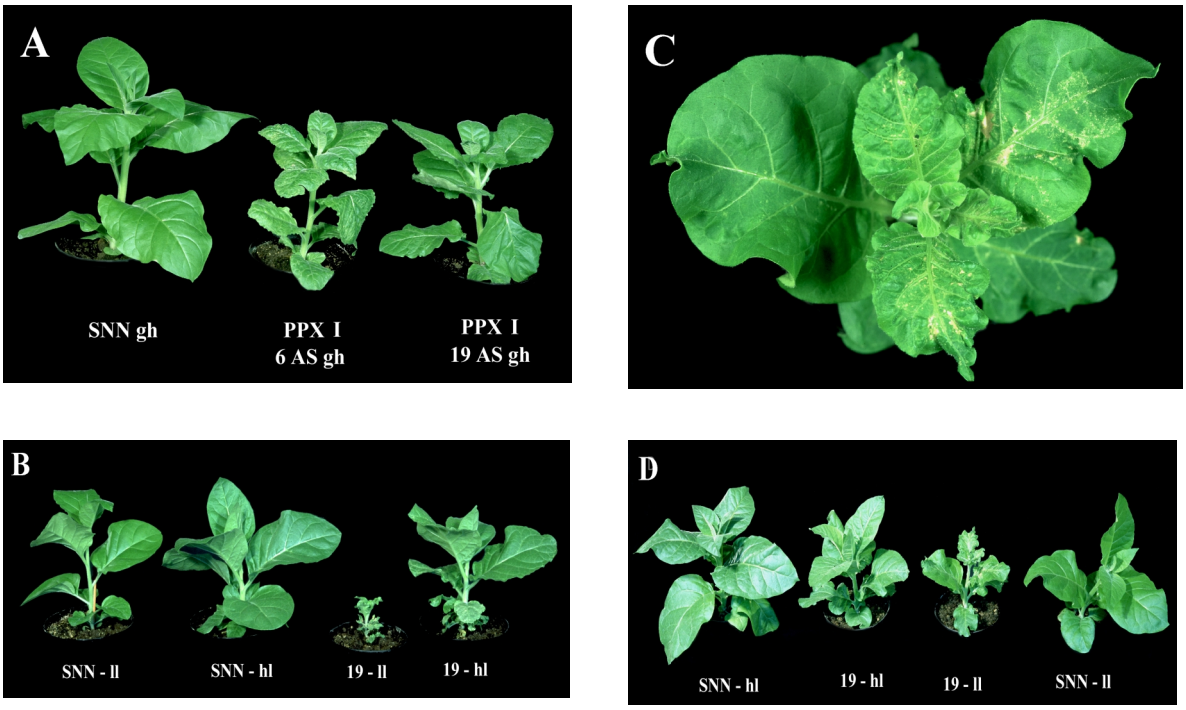


Figure 25: Phenotype of transgenic tobacco plants expressing antisense mRNA for PPOX I. A, Six-week old tobacco wild type (SNN) and transgenic plants grown under greenhouse conditions. B, Comparison of control (SNN) and transgenic plants (AS19) grown under low and high light intensities. C, Top view of transgenic line AS19 4 days after transfer from the greenhouse to low light conditions. D, Control and transgenic plants 4 days after transfer from the greenhouse to low and high light conditions.

4.7.2 Molecular analysis of plants transformed with PPOX I antisense genes

4.7.2.1 Analysis of transgenic plants grown under greenhouse conditions

Genomic Southern blot analysis of transgenic tobacco DNA of primary transformants revealed one to several insertions of the antisense gene into the genome (Fig. 26). It can be assumed that insertion of the PPOX I antisense gene into the genome of tobacco plants does not necessarily lead to accumulation of Proto IX and to the necrotic phenotype. This was the case with transgenic lines 8 and 9, which contain the transgene, but they had unchanged porphyrin level and showed a phenotype similar to the wild type.

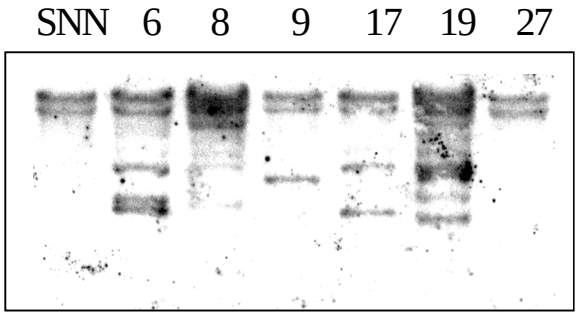
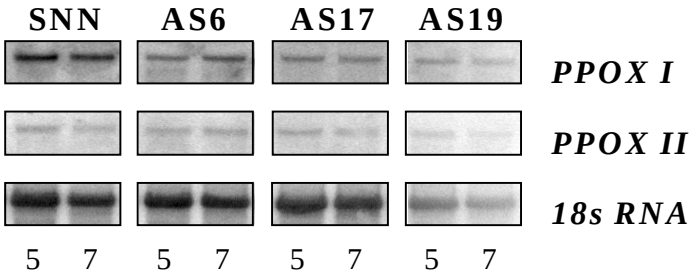


Figure 26: Southern blot analysis of 10 µg genomic DNA isolated from wild-type (SNN) and transformants 6, 8, 9, 17, 19,27. DNA was cut with Bam HI and subjected to Southern blot hybridisation using a ^{32}P-labeled insert of tobacco *PPOX I* as probe.

Total RNA was extracted from leaf 5 and 7 of tobacco wild type and selected transgenic lines AS6, AS17 and AS19, which were grown in the greenhouse for 6 weeks. The steady-state *PPOX I* mRNA content did not vary much during the development of the leaves 5 and 7 (Fig. 27, upper panel) Quantification of steady-state levels of endogenous and antisense mRNA for PPOX I by Northern blot analysis revealed the reduction of mRNA levels in transformants (Fig. 27, upper panel). Analysis of *PPOX II* transcripts showed no difference in *PPOX II* RNA steady-state levels between the corresponding leaves of wild type and transgenic plants (Fig. 27, upper panel).

In addition to RNA analysis, the amount of PPOX I and PPOX II proteins were determined in leaf extracts of transformants and control plants using an antiserum raised against the purified recombinant PPOX I and PPOX II proteins, respectively (Fig. 27, lower panel). The proteins were extracted from leaves 5, 7 and 9 of control and transgenic plants. Selected antisense plants contained reduced amounts of PPOX I protein. In transformants and control plants, the PPOX I protein levels did not vary much with leaf age. The reduction of steady-state levels of PPOX I protein in PPOX I antisense plants correlated with the reduction of total mRNA. The PPOX II protein content remained unchanged in transformants relative to the wild type.



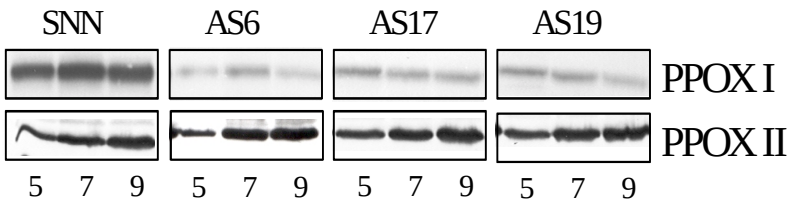


Figure 27: Expression of PPOX I and II in tobacco wild type and PPOX I antisense transformants grown for six weeks under greenhouse conditions. Upper panel: Total RNA was extracted from leaf 5 and 7 of the wild type and transgenic lines AS6, AS17 and AS19. 10 µg of RNA were loaded per lane and separated on 1 % formaldehyde-agarose gel, followed by hybridization to tobacco *PPOX I* and *PPOX II* cDNA probes. 18S RNA levels are displayed as control for equal loading of RNA samples. Lower panel: Total leaf protein was extracted from leaf 5, 7 and 9 of the same transgenic plants. Equal amounts of proteins (15 µg) were loaded on SDS-polyacrylamide gel. After transfer to nitrocellulose filters, immunodetection was performed with antisera raised against the recombinant PPOX I and PPOX II.

4.7.2.2 Molecular analysis of PPOX I antisense plants under greenhouse conditions and after exposure to low and high light intensities

To elucidate the difference in response on low and high light, we extended our comparative and genetic analysis of transgenic plants. Total RNA was extracted from leaves 1-4 and 5-6 of wild-type tobacco and the selected transgenic line AS19. Plants had been grown in greenhouse for 6 weeks prior to bee transferred from the greenhouse for 4 days to the low light and high light conditions. Quantitation of steady-state levels of endogenous and antisense mRNA for PPOX I by Northern blot analysis revealed the reduction of mRNA levels in transformants to the same extent under all selected growth conditions (Fig.28, upper panel).

The amount of PPOX I and PPOX II protein in leaf extracts of transformants and control plants was determined under these different growth conditions using the mentioned above antisera raised against the purified recombinant PPOX I and PPOX II proteins (Fig. 28, lower panel). The protein was extracted from leaves 1-4, 5-6 and 7-8 of wild type and transformants. Under both low and high light conditions the PPOX I protein levels decreased in transformants to the same degree as in the wild type. The steady state levels of PPOX II protein were not altered in the PPOX I antisense plants in comparison to the wild type. These data suggest that the different phenotypical changes under low light and high light intensity in PPOX I antisense plants are not due to differences in expression of PPOX I mRNA and protein.

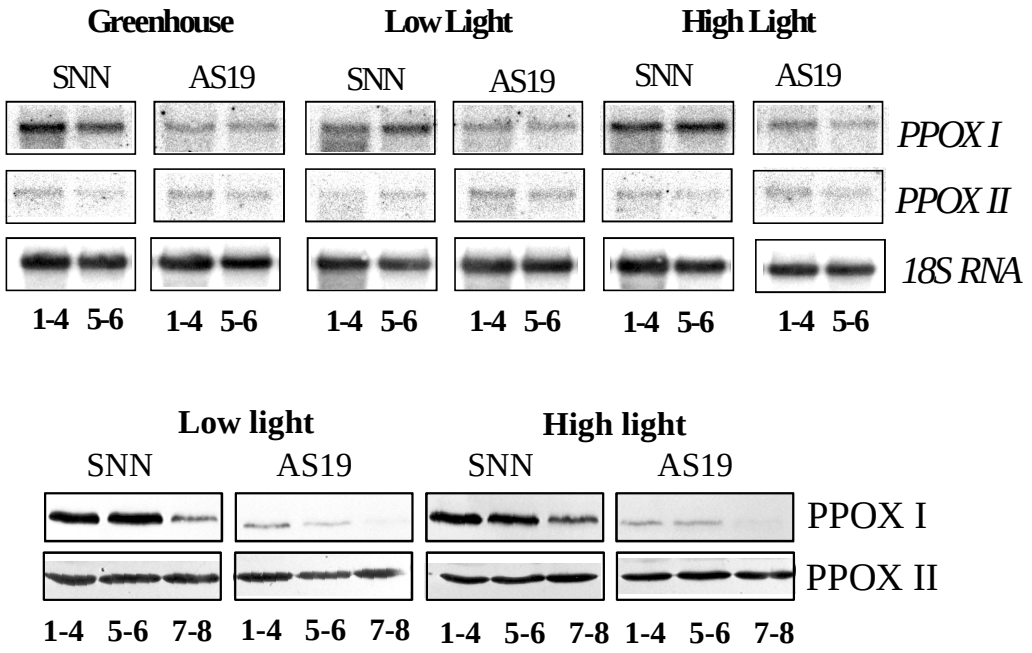


Figure 28: Expression of PPOX I and PPOX II under different light conditions. Tobacco plants were grown for 6 weeks under greenhouse conditions under an average light intensity of 300 µmol m^{-2} s^{-1} followed by a transfer into growth chambers with low light (100 µmol m^{-2} s^{-1}) and high light (550 µmol m^{-2} s^{-1}) intensities for 4 days. After 4 days material from leaves 1-4, 5-6, 7-8 was harvested and pooled together from3 plants. Upper panel: Total RNA was extracted from leaves 1-4 and 5-6 and subjected to Northern blot hybridization with radioactively labeled cDNA fragments for PPOX I and PPOX II. A cDNA probe for 18S rRNA was subsequently hybridized to RNA on the same filters. Lower panel: Total leaf protein was extracted from the leaves 1-4, 5-6 and 7-8 and loaded on a SDS-PAGE. Immunodetection was performed with antisera raised against recombinant PPOX I and PPOX II.

4.7.3 Determination of Proto IX contents in PPOX I antisense transformants

4.7.3.1 Accumulation of Proto IX in PPOX I antisense plants under greenhouse conditions

Reduced PPOX I protein content in transgenic plants was expected to impair porphyrin flow in the metabolic pathway and to lead to the accumulation of protoporphyrinogen IX as substrate of PPOX. It was demonstrated that inactivation of chlorophyll biosynthetic enzymes by expression of antisense genes leads to the accumulation of corresponding substrate in excessive amounts as in case of transgenic plants expressing antisense mRNA for CPO or for UROD (Kruse et al., 1995b; Mock and Grimm, 1997). Methods to distinguish between reduced and oxidized forms of porphyrins are not available. Due to this difficulty to detect the

nonfluorescent Protogen IX, it was chemically oxidized to Proto IX, which was measured by HPLC with fluorescence detection. Porphyrins were extracted from leaf 5, 7 and 9 of tobacco wild type and transgenic lines AS6, AS17 and AS19 and analyzed by HPLC. The levels of accumulated Proto IX were higher in all selected transformants than in control plants (Fig. 29). The biggest difference in Proto IX contents between control and transgenic plants was observed in leaf 5. Proto IX contents in leaf 5 of transgenic lines AS6 and AS19 was increased 5 fold compared to the wild type. Leaves 7 and 9 of transgenic line AS6 accumulated significantly more of Proto IX than the control, while the corresponding leaves of transgenic line AS19 accumulated only slightly more of Proto IX than the control. All leaves of transgenic line AS17 contained only 2 times more of Proto IX than the wild type. The amounts of accumulated porphyrins in PPOX I antisense transgenic plants correlate with the intensity of transgenic phenotype.

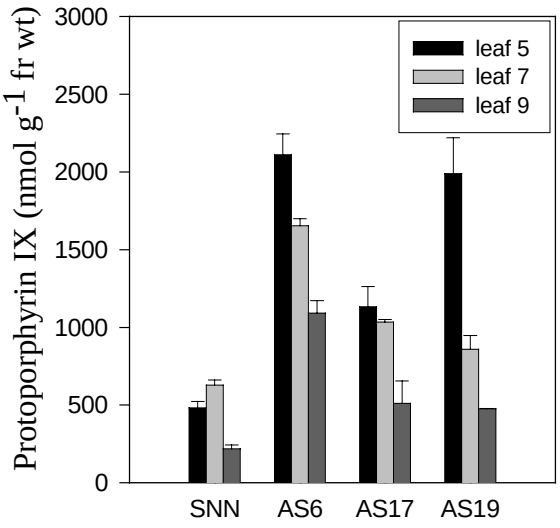


Figure 29: Accumulation of protoporphyrin IX in tobacco wild type and PPOX I antisense plants grown for 6 weeks under greenhouse conditions. Protoporphyrin IX was extracted from leaf 5, 7 and 9 of 5-week-old tobacco wild type (SNN) and transgenic lines AS6, AS17, AS19 and analyzed by HPLC.

4.7.3.2 Accumulation of Proto IX in PPOX I antisense plants under different light intensities

To exclude the possibility that the different effects of low and high light conditions on the generation of the necrotic phenotype of PPOX I antisense plants are due to the different levels of Proto IX: the amounts of accumulated Proto IX under greenhouse conditions and after the

exposure of plants to low and high light intensities were measured. Porphyrins were extracted from leaves 1-4, 5-6 and 7-8 of control and transgenic plants. The Proto IX content in transgenic plants was higher than in the wild type under all growth conditions. The biggest difference was observed in plants grown in the greenhouse, where accumulation of protoporphyrin IX ranged from 4 to 10 fold in transformants, compared with the wild type. Transgenic plants transferred to low and high light conditions accumulated almost the same amounts of protoporphyrin IX in leaves 1-4. In the leaves 5-6 and 7-8 protoporphyrin contents were reduced in LL-grown transgenic plants in comparison to the high light plants. The reduced amount of protoporphyrin IX in leaf 7-8 of LL-grown transformants could be explain with high degree of necrotic lesions.

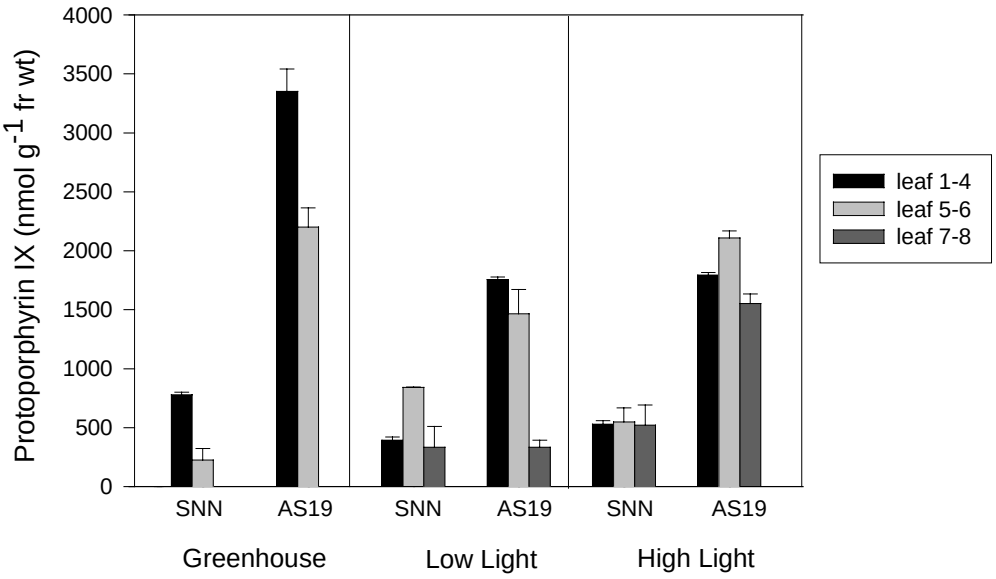


Figure 30: Accumulation of protoporphyrin IX in leaves of 6-7-week-old tobacco wild type and PPOX I antisense plants under different light conditions. The growth conditions of the plants were defined in the legend to Figure 28. Porphyrins were extracted from leaves 1-4, 5-6 and 7-8 and analyzed by HPLC.

4.7.4 Effect of reduced PPOX I enzyme on chlorophyll content in leaves of control and transgenic plants under different light conditions

Chlorophyll content in wild type and transgenic plants was determined under different light conditions to study the consequences of reduced PPOX activity on the availability of tetrapyrrole end products. A significant difference in the chlorophyll content between the wild type and

transgenic line AS19 was observed only under low light conditions. (Fig. 31). The chlorophyll level was reduced in leaves 5-6 and 7-8 of transformants by circa 35% and 23%, respectively, in comparison to control plants. This reduction of the chlorophyll content under low light conditions coincides with photodynamic damage to leaves and results from enhanced chlorophyll degradation.

This suggests that the macroscopic phenotype of LL-grown plants correlates with a reduced level of Proto IX and chlorophyll as result of degradation processes. It is indicated that the phenotype of transformants is primarily associated with the accumulation of photosensitive Proto IX, rather than with lower amounts of the end product chlorophyll.

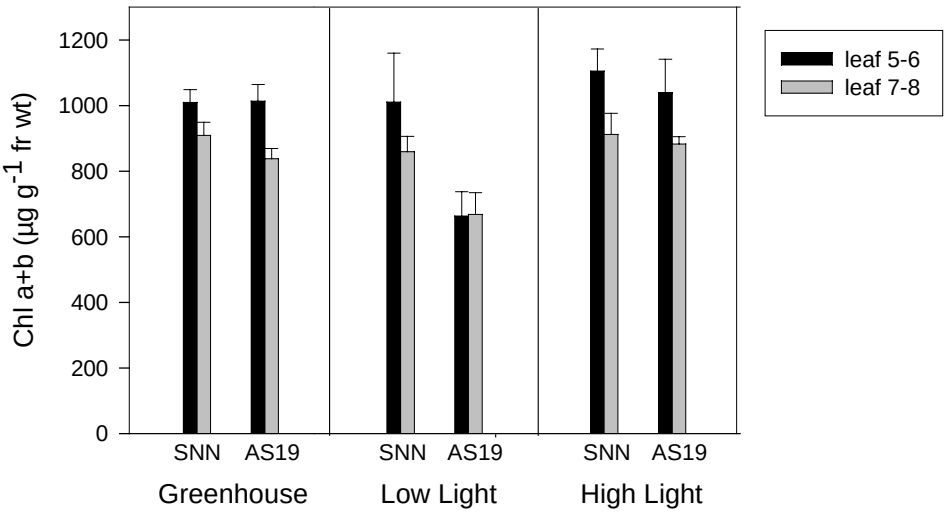


Figure 31: Content of chlorophyll a and b in leaves of wild type and PPOX I antisense plants under greenhouse conditions and after exposure to low and high light intensities. The growth conditions of the plants were defined in the legend to Figure 28. Chlorophylls were extracted and measured as described in the "Methods".

4.7.5 Levels of low-molecular-weight antioxidants: ascorbate and glutathione

Deficiencies in tetrapyrrole biosynthesis result in the accumulation of metabolites which can be photoactively oxidized. They can generate highly reactive oxygen species which cause photodynamic damage. The cellular arsenal for scavenging reactive oxygen species and toxic organic radicals includes a number of small molecules such as ascorbate, glutathione, tocopherol and carotenoids (Foyer, 1993; Hausladen and Alscher, 1993; Hess, 1993; Pallet and Young,

1993). Total and reduced ascorbate and glutathione pools in wild type and transgenic plants were analyzed (Fig. 32). Amounts of total ascorbate and glutathione varied depending on the light conditions and on the leaf age. Under all growth conditions total and reduced ascorbate and glutathione levels declined from leaves 5-6 to older leaves in both control and transgenic plants. Transformants grown under greenhouse conditions had slightly higher amounts of total and reduced ascorbate but significantly higher amounts of total and reduced glutathione in comparison to the wild type (Fig. 32A,B). Under these growth conditions the relative amount of reduced ascorbate was lower in PPOX I antisense plants, but that of reduced glutathione was higher than in the control plants. Transfer of the wild type and transgenic tobacco plants from the greenhouse to low and high light conditions led to a decrease of the total ascorbate and glutathione levels under low light and to an increase (in case of ascorbate) or to the same levels (in case of glutathione) under high light conditions. The total ascorbate content ranged from 1,2 to 1,9 µmol/ g fresh weight under low light conditions and from 3,9 to 5,8 under high light conditions (Fig. 32A). Thus, high light-grown plants contained on average 3 times more of total ascorbate than low light-grown plants. Under such low and high light conditions the relative amounts of total ascorbate did not significantly differ between control and transgenic plants. But the relative yield of the reduced form was significantly lower in transformants under low light conditions compared with the wild type. It decreased from 66% to 33% in leaves 5-6 and from 100% to 78% in leaves 7-8 (Fig. 32C). High light grown transformants were characterized by almost the same amount of total ascorbate than the control plants but had slightly lower amounts of the reduced form. Total glutathione levels were approximately the same in control and transgenic plants transferred to the low and high light conditions, however the total glutathione content in LL-grown plants was approximately only 25% that observed in HL-grown plants. The percentage of reduced glutathione was lower in control and transgenic plants under low light intensities than under the other growth conditions. At the same time LL-grown transformants showed a lower redox state of glutathione in comparison to the wild type: 33% in leaves 5-6 and 32% in leaves 7-8 versus 50% and 56% in the corresponding leaves of control plants. It was assumed that the quantitative differences of low molecular weight antioxidants in PPOX I antisense plants under low and high light conditions could be responsible for the different extent of Proto IX-induced necrotic leaf damage.

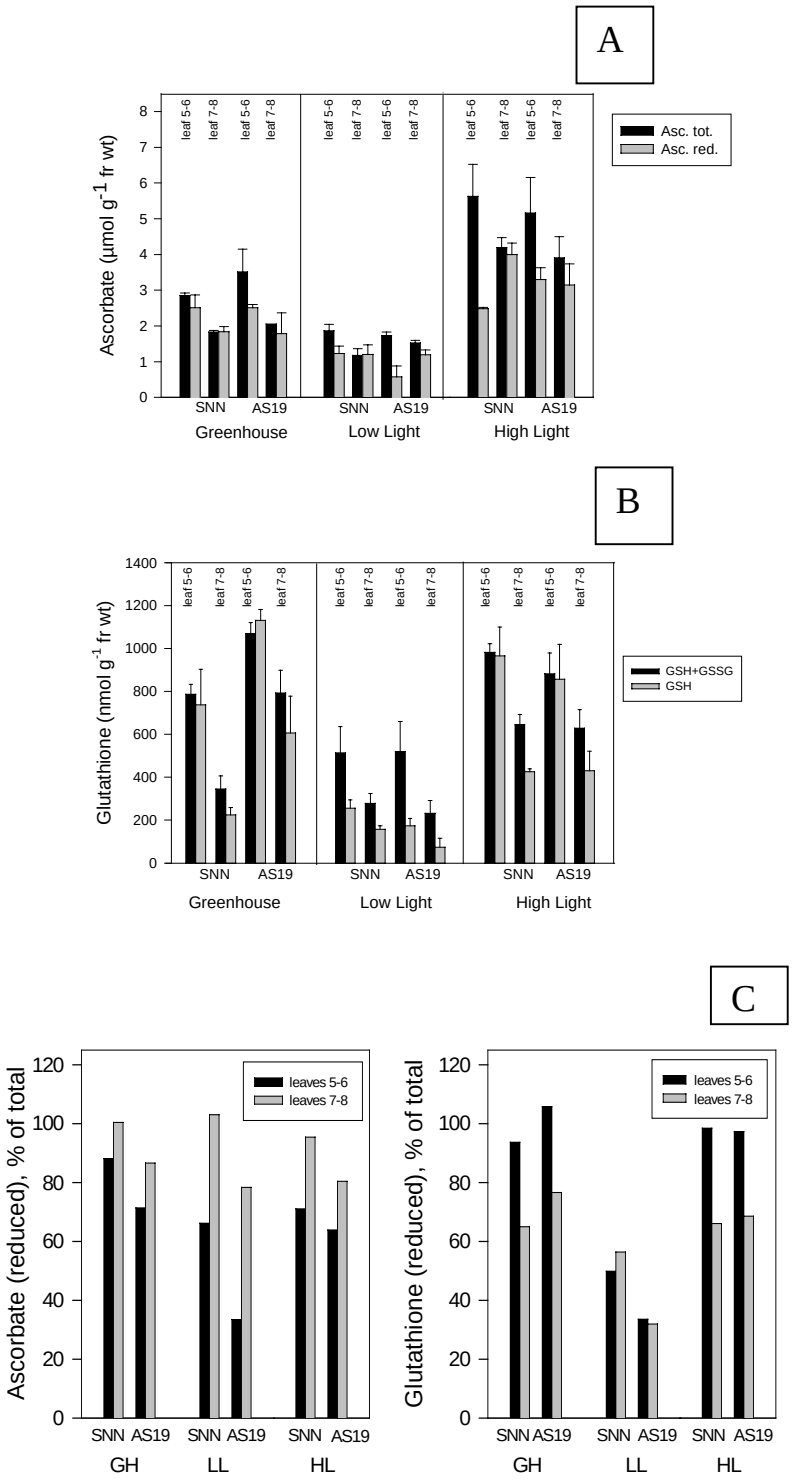


Figure 32: Contents of total and reduced ascorbate and glutathione in leaves of 6-7- week-old tobacco wild type and transgenic line AS19 under greenhouse, low and high light conditions. The growth conditions of the plants were described in the legend to Figure 28. **A.** Ascorbate was extracted from leaves 5-6 and 7-8. Contents of total and reduced ascorbate were analyzed spectrophotometrically. **B.** Level of glutathione in the same leaves as ascorbate was measured by HPLC with fluorescent detection. **C.** Percentage of reduced forms of ascorbate (left panel) and glutathione (right panel) of the total amounts of correspondent antioxidants.

4.7.6 Content of α-tocopherol

Several researchers have proposed that the water soluble ascorbate is an antioxidant-synergist with the lipid soluble tocopherol: tocopherol acts as primary antioxidant, while ascorbate reductively regenerates oxidized tocopherol (Packer et al., 1979). Tocopherol content was analyzed in leaves 1-4, 5-6 and 7-8 from the wild type and transgenic line AS19 under different light conditions. In general, no difference in tocopherol content was observed between control and transgenic plants. Transfer of tobacco plants to the low light intensity leads to dramatic decrease (up to 10 fold) in tocopherol content in both wild type and transformants. Transgenic plants transferred to the high light have on average slightly higher tocopherol content than plants grown in the greenhouse. Therefore, the levels of tocopherol under low light conditions decrease in parallel with the levels of ascorbate and glutathione. Data obtained by Finckh and Kunert (1985) showed that plant species containing low amounts of ascorbate and tocopherol are much more sensitive to peroxidizing herbicide than species with higher levels of antioxidants under the same treatment conditions. This suggests that a transfer of tobacco plants to low light conditions with decreased levels of antioxidants make them more sensitive to accumulated Proto(gen).

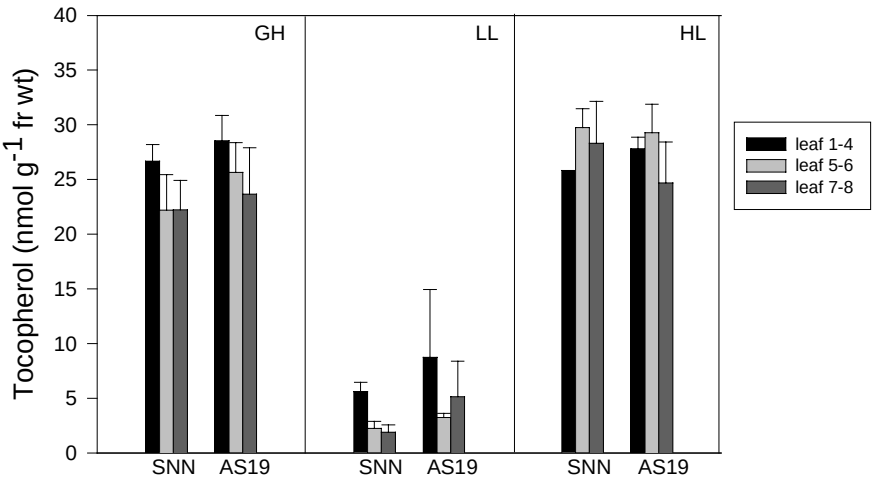


Figure 33: α-Tocopherol content in leaves of wild type (SNN) and transgenic PPOX I antisense (AS19) plants under greenhouse, low and high light conditions. Tocopherol was extracted from leaves 1-4, 5-6 and 7-8 of control and transgenic plants and analyzed by HPLC. The growth conditions of the plants were described in legend to Figure 28.

4.7.7 Accumulation of thiobarbituric acid reactive substances (TBA-RS) in wild type and PPOX I antisense plants under different growth conditions

Generation of reactive oxygen species following the accumulation of porphyrin intermediates leads to lipid peroxidation and membrane destruction. The level of lipid peroxidation products in control and PPOX I antisense plants under low and high light conditions was expressed as TBA-RS (aldehydes, mainly malondialdehyde and endoperoxides; Buege and Aust, 1978). Leaf material of leaves 5-6 and 7-8 of control and transgenic plants, which had been transferred from the greenhouse to low and high light conditions for 4 days, was used for the assay. Levels of TBA-RS were higher in high light grown plants. However the difference in amounts of TBA-RS between control and transgenic plants was much more pronounced under low light conditions. In leaves 5-6 of LL-grown transgenic plants the levels of TBA-RS were increased by 83 % in comparison to the wild type, while older leaves of transformants had the same amounts of TBA-RS as the control. This correlates with the necrotic damage of transgenic plants, which was the strongest on leaves 5-6. Under high light conditions TBA-RS levels were slightly increased in leaves of transgenic plants and ranged 7-10 % higher level than in the control.

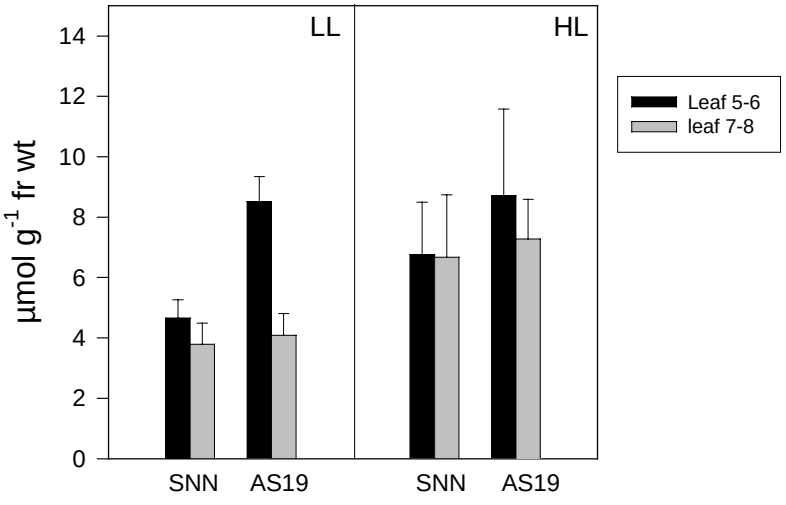


Figure 34: Level of lipid peroxidation products, measured as TBA-RS, in leaves 5-6 and 7-8 of wild type and line AS19 of PPOX I antisense plants under low and high light conditions. The growth conditions for the plants were defined in the legend to Figure 28.

4.8 Characterization of PPOX II overexpressing plants

4.8.1 Phenotype of PPOX II sense plants

The full-length sequence encoding PPOX II from *N.tabacum* (Lermontova et al., 1997) was inserted in sense orientation behind the CaMV 35S promoter of the binary vector BinAR. Primary transformants obtained by *Agrobacterium*-mediated leaf disc transformation of tobacco were grown in the greenhouse and selected for transgene expression by Southern blot analysis. Among circa 50 transformants only one transgenic line S36 showed a chlorotic phenotype under greenhouse, and especially, under high light conditions. It disappeared with aging of the plant. All other tobacco plants containing PPOX II transgenes showed a similar growth rate and green pigmentation as the wild type.

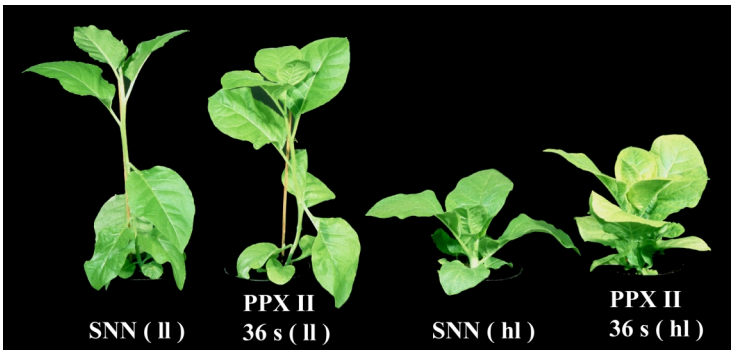


Figure 35: Phenotype of six-week-old control (SNN) and PPOX II sense (36 s) transgenic plants grown under low light (70 µmol m^{-2} s^{-1}) and high light conditions (550 µmol m^{-2} s^{-1})

4.8.2 Physiological and molecular characterization of PPOX II overexpressing plants

Chlorophyll content was determined in the tobacco wild type and transgenic line S36 of PPOX II sense plants grown under low and high light conditions. The reduced levels of chlorophylls (up to 50 % of wild type content) in HL-grown transformants reflect the loss of green pigmentation. In the LL- grown transgenic plants, which had a wild type-like phenotype, the chlorophyll content was only slightly reduced relative to the control.

PPOX II is involved in the biosynthesis of mitochondrial heme, thus overexpression of PPOX II could result in changes of the heme content in transgenic plants. We were not able to measure heme content in tobacco plants due to experimental difficulties. It has been demonstrated that heme inhibits ALA synthesis in intact plastids (Chereskin and Castelfranco, 1982). ALA formed in chloroplast is the common precursor of chlorophyll and heme synthesis in chloroplasts and mitochondria. Therefore, heme that is synthesized in mitochondria may

also be involved in feedback regulation of ALA synthesis. We determined the ALA synthesizing capacity in leaves of wild type and PPOX II overexpressing plants under different light intensities. Strong reduction of ALA synthesizing capacity relative to the control was observed in case of line S36 under high light conditions. We think that under high light conditions increased heme content leads to inhibition of ALA synthesis which results in a reduction of chlorophyll content.

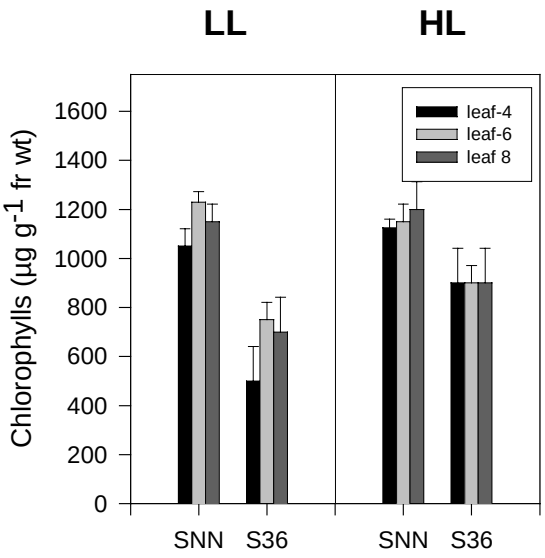


Figure 36: Content of chlorophyll a and b in leaves of wild type and PPOX II sense plants under low and high light conditions. Chlorophylls were extracted from leaves 4, 6 and 8 of tobacco wild type and PPOX II sense plants grown under low light (100 µmol m^{-2} s^{-1}) and high light (500 µmol m^{-2} s^{-1}) conditions

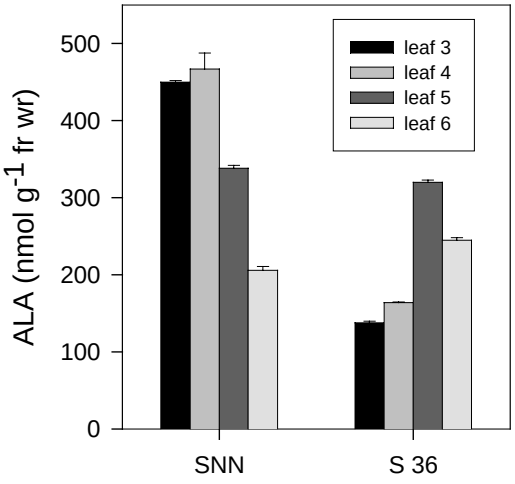


Figure 37: ALA synthesizing capacity in leaves of tobacco wild type (SNN) and PPOX II overexpressing plants. Leaf discs from leaf 3, 4, 5 and 6 of primary transformant S36 and a control tobacco plants were incubated in the presence of levulinate (20 mM) for 4 h in the light. The growth conditions of the plants were defined in the legend to Figure 36. Determination of ALA was performed according description in the „ Methods“.

Southern blot analysis of genomic DNA isolated from PPOX II overexpressing plants showed that all selected transformants contained several copies of the PPOX II transgene (Fig. 38).

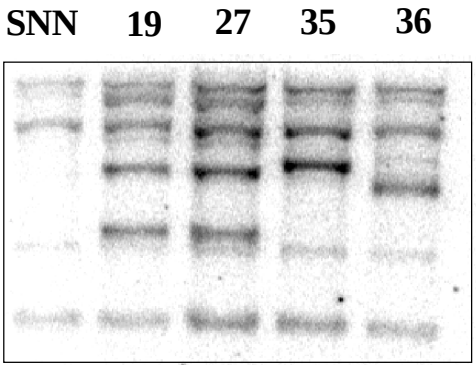


Figure 38: Southern blot analysis of genomic DNA isolated from wild-type (SNN) and PPOX II sense transformants 19, 27, 35 and 36. Genomic DNA was digested with Sph I and subjected to Southern blot hybridisation using a ^{32}P-labeled insert of tobacco PPOX II as probe.

Total RNA was extracted from leaves 3, 5, 7 and analyzed by Northern blot. Increased levels of *PPOX II* transcript was detected in transgenic lines S35 and S36, while *PPOX II* mRNA levels in line S19 remained unchanged. Expression of mRNA for PPOX I is not changed in PPOX II overexpressing plants (Fig. 39).

Western blot analysis was carried on a protein extract from leaf 4 of control and transgenic plants. PPOX II protein level was raised in all selected transgenic plants in comparison to the wild type.

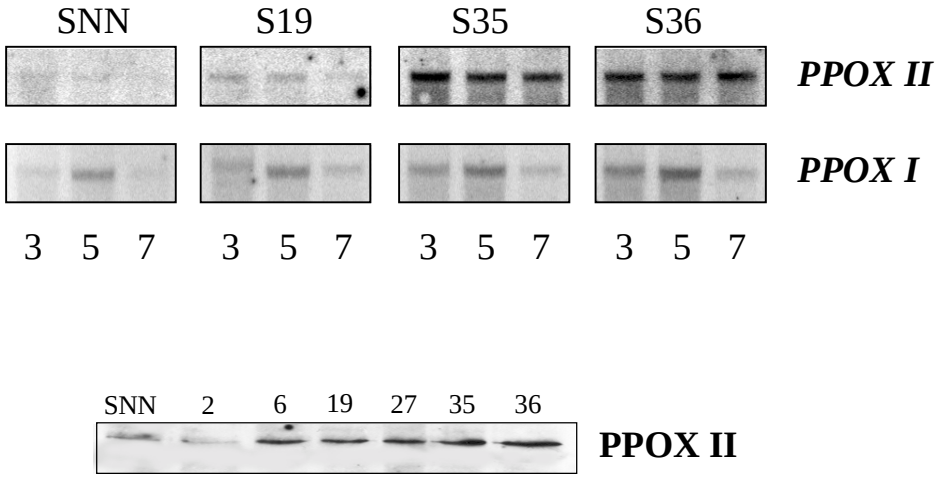


Figure 39: Expression studies in wild type (SNN) and PPOX II overexpressing plants. Upper panel: Northern blot analysis of PPOX II and PPOX I mRNA levels in leaf 3, 5 and 7 of tobacco wild type and PPOX II sense plants. Lower panel: Western blot analysis of PPOX II sense and control plants. Total protein extracts were prepared from leaf 4 and subjected to Western blot analysis with antibodies raised against PPOX II.

4.8.3 Treatment of PPOX II sense plants with acifluorfen

Previously it was demonstrated that the mitochondrial isoform of plant protoporphyrinogen IX oxidase, as well as the plastidal isoform, are sensitive to peroxidizing herbicides (Matringe et al., 1989; Lermontova et al., 1997). Overexpression of the plastidal isoform of PPOX in tobacco leads to herbicide resistance (Lermontova et al., 2000). We predicted that the overexpression of the mitochondrial isoform could also lead to a reduced sensitivity to peroxidizing herbicides. To test the herbicide resistance, we performed almost identical experiments with PPOX II overexpressing plants, as it was described above for PPOX I sense plants.

Germination of seeds from PPOX II transformants on MS medium containing acifluorfen showed that transgenic seeds are almost as sensitive to the herbicide as the wild type (data not shown).

However, leaf discs of LL-and HL-grown PPOX II overexpressing plants after incubation with 500 nM and 1 µM of acifluorfen accumulated almost 50 % less of protoporphyrin IX than the control (Fig. 40). Spraying of PPOX II transgenic plants with 10 µM acifluorfen resulted, similar to the wild type, in necrotic lesions two days after the treatment.

These results show that overexpression of PPOX II leads only to the slight resistance against DPE herbicide acifluorfen, in contrast to the PPOX I overexpressing plants. The one possible explanation for it, could be the different role of two isoforms in tetrapyrrole biosynthesis.

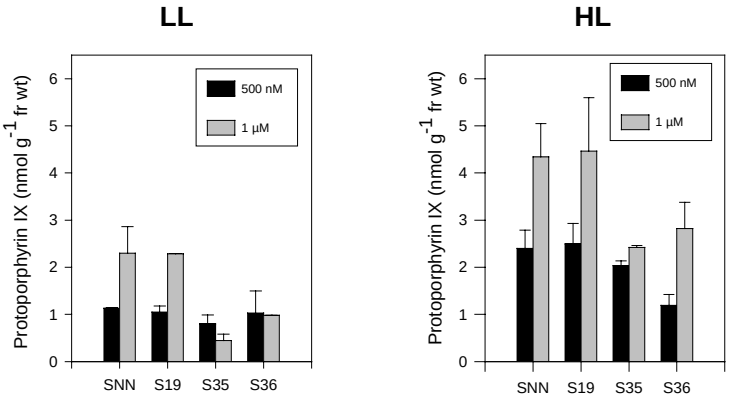


Figure 40: Accumulation of protoporphyrin IX in leaf discs of tobacco wild type and PPOX II overexpressing plants after incubation with acifluorfen. Three leaf discs of leaf 5 were incubated with 500 nM and 1µM of acifluorfen for 20 h in darkness. Porphyrins were extracted and analyzed by HPLC.

4.9 Mutagenesis

4.9.1 Selection for plant PPOX gene resistant to protox-inhibitory herbicides in the E. coli expression system

Several strategies have been developed for obtaining plants resistant towards the peroxidizing herbicides directed against PPOX. One way to obtain resistance is alteration of the herbicide-binding site of the catalytic cleft of the enzyme, thereby preventing stable binding of specific herbicides. We transformed the plasmid pBS KS, containing tobacco PPOX I, into the random mutagenesis strain XL1-Red. After mutagenesis, plasmids were transformed into the *E.coli hemG* mutant. Bacterial clones were selected for herbicide resistance on LB plates in the presence of acifluorfen as described in the "Material and Methods". 250 colonies were finally selected, but only 70 of them remained tolerant towards acifluorfen after retransformation. All 70 clones were sequenced and 4 mutated cDNA sequences were found among them, 2 clones (# 36 and 38) were identical. They have a mutation at amino acid 408 from ACA (Thr) to GCA (Ala). Clone #51 has a substitution of Phe by Leu at position 489 (TTT-CTT). In clone #65 the GTA codon for Val was converted to the GCA codon for Ala (Fig. 41).

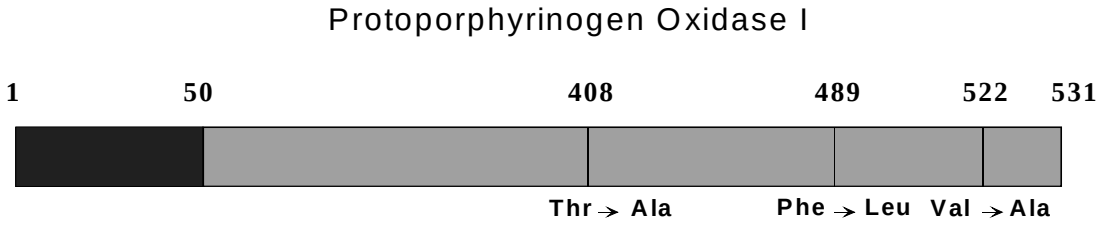


Figure 41: A Schematic view of the PPOX I protein sequence. A putative transit peptide from amino acid 1 to 50 is shown in black. The mutations leading to herbicide resistance and their positions on the protein sequence are indicated.

4.9.2 Growth of bacterial cell culture in presence of acifluorfen

The *E. coli hemG* mutant transformed with plasmids containing either mutated or nonmutated *PPOX I* sequences was grown in liquid LB medium without acifluorfen and in presence of 20 or 100 µM of herbicide. The optical density (OD) of the bacterial cell culture was measured at different time intervals. Fig.42. presents data of OD values measured after 24 h of growth in

presence of acifluorfen. Growth of the *hemG* mutant transformed with nonmutated PPOX I was almost completely inhibited with 20 and 100 µM of acifluorfen. The *hemG* mutant containing the mutated PPOX I cDNA #65 exhibited almost the same growth rate with the two applied concentrations of acifluorfen as in absence of the herbicide. The *hemG* mutant transformed with a plasmid containing the mutated PPOX I sequence #38 was only slightly inhibited at the selected concentrations of the herbicide, while growth of *hemG* containing the mutated PPOX I #51 sequence was more sensitive to the herbicide. Its growth was inhibited by 50% in the presence of 100 µM of acifluorfen.

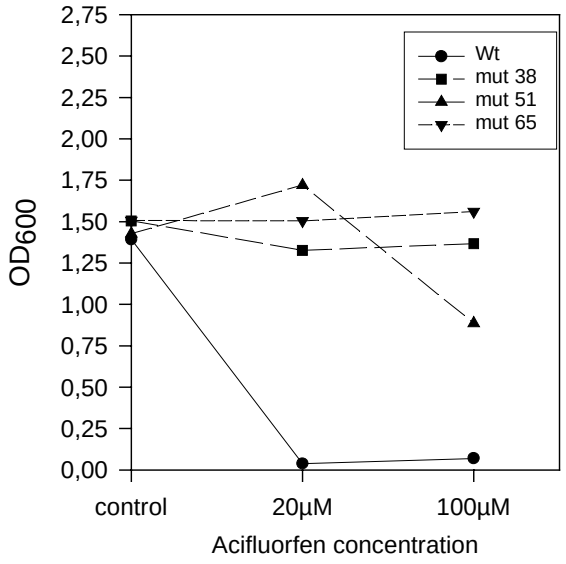


Figure 42: Growth of bacterial cell culture in the presence of acifluorfen. Plasmids containing mutated cDNAs sequences encoding PPOX I were transformed into a *E. coli hemG* mutant and grown in medium containing acifluorfen as described above. Growth of bacterial cells was measured by spectrophotometer as optical density (OD) of bacterial cell culture at 600 nm.

4.9.3 Generation of transgenic plants containing the mutated cDNA sequences encoding PPOX I

The cDNA sequences coding for mutated *N. tabacum* PPOX I were inserted in sense orientation into the binary vector Bin AR behind the CaMV 35S promoter. Valeria Voronetskaya is continuing this work. Three obtained constructs were introduced into *Arabidopsis thaliana* by vacuum infiltration of plants with the solution of *A. tumefaciens.* Transgenic plants were analyzed for expression of PPOX I mRNA and protein and for resistance against peroxidizing herbicides (Valeria Voronetskaya, unpublished data).

5 Discussion

5.1 Cloning and characterization of two isoforms of tobacco PPOX

5.1.1 Cloning and sequencing of tobacco PPOX

Two different cDNA sequences have been identified by complementation of the heme auxotrophic *E. coli hemG* mutant lacking protoporphyrinogen IX oxidase activity. They were designated *PPOX I* and *PPOX II*. Both deduced peptide sequences contain only 27.2% invariant amino acid residues. The antiserum raised against each of the two isoenzymes did not cross-react with the other (Fig. 8). The two enzymes share only a few identical domains, which are also conserved in other bacterial or eukaryotic protoporphyrinogen IX oxidases (Fig. 6). The significance of each invariant amino acid residue for structural or functional roles remains to be elucidated.

Genes encoding for protoporphyrinogen IX oxidase have been identified first from *E. coli* (Sasarman et al., 1993) and *Bacillus subtilis* (Hansson and Hederstedt, 1992) and were designated *hemG* and *hemY*. They represent two distinct protoporphyrinogen oxidizing systems, the oxygen dependent of the HemY-type and the bacterial multi-component system. Both genes did not share any sequence similarity. Generation of protoporphyrinogen oxidase deficient mutants has provided a useful way for the isolation of *PPOX* genes from different organisms by functional complementation of the mutation. The gene for yeast protoporphyrinogen IX oxidase was identified by functional complementation of a *hem14-1* yeast mutant which is deficient in enzyme activity and resembles the HemY protein (Camadro and Labbe, 1996). Interestingly, the *E. coli hemG* mutant could be complemented with human, mouse and *Arabidopsis* cDNA sequences encoding the HemY like protein (Nishimura et al., 1995b; Taketani et al., 1995; Dailey et al., 1995; Narita et al., 1996).

Alignment of PPOX I and II proteins with known sequences revealed significant similarity to protoporphyrinogen IX oxidases, which were identified by *E. coli hemG* complementation. The tobacco PPOX I and II show sequence identity to the *A. thaliana* PPOX of 71.2% / 24.6% (Narita et al., 1996), *B. subtilis* HemY of 28.1% / 25.1% (Hansson and Hederstedt, 1992), mouse PPOX of 24.6% / 24.6% (Dailey et al., 1995), human PPOX of 23.5% / 21.4% (Nishimura et al., 1995b) and *S. cerivisae* PPOX of 19.3% / 19.8% (Camadro and Labbe,

1996). Sequence comparison indicated a close relation of the *Arabidopsis* protein to protoporphyrinogen IX oxidase I.

5.1.2 Translocation of PPOX isoenzymes to plastids and mitochondria

Early enzymes of tetrapyrrole synthesis are exclusively detected in chloroplasts. Protoporphyrinogen IX oxidase is the first enzyme of the pathway whose activity was determined in plastids as well as in mitochondria (Jacobs and Jacobs, 1987). *In vitro* translated protoporphyrinogen IX oxidase I was selectively directed to plastids. It accumulated in membrane and stroma fractions (Fig. 7). However, Western blot analysis of plastidic fractions revealed that the immuno responsive protein was only detected in the membrane fraction of green plastids (Fig. 8). This is consistent with data on the protoporphyrinogen IX oxidase activity that were entirely found in thylakoid membranes and envelopes of spinach chloroplast (Matringe et al., 1992b). The occurrence of the isoenzyme in the stromal fraction might represent an intermediate state of the import or integration process. This has also been observed for other plastid proteins, such as light-harvesting chlorophyll binding proteins and Rieske iron-sulfur protein (Madueno et al., 1993; Yuan et al., 1993). In these cases soluble proteinaceous stromal factors have been proposed to promote integration of the processed protein into the thylakoid membrane. The protoporphyrinogen IX oxidase deduced from the *Arabidopsis* cDNA sequence shares a high similarity with the tobacco isoenzyme I. In contrast to the assumption by Narita et al. (1996), we have shown through our import experiment that PPOX I is not localized in mitochondria but in chloroplasts.

The second isoenzyme is specifically recognized by the mitochondrial import machinery. It was targeted to isolated mitochondria and protected there from proteolysis. The information for mitochondrial import is usually located at the N-terminus of the enzyme. The imported mitochondrial enzyme did not show an obvious reduction in size. The enzymes from human and mouse (Nishimura et al., 1995b; Dailey et al., 1995) share with tobacco isoform II at the N-terminus a rather short stretch of amino acid residues in front of the flavin binding site. The homologous human protein imported *in vitro* into mitochondria also maintained the molecular size (Nishimura et al., 1995b). Thus suggesting similar translocation mechanisms for the mammalian and plant mitochondrial enzyme. Compared to other protoporphyrinogen IX oxidases, the yeast enzyme contains an N-terminal extension of 13 amino acid residues which directs the protein to mitochondria and is visibly cleaved off (Camadro et al., 1994). A small

transit peptide could be cleaved off from tobacco mitochondrial enzyme, although no clear-cut transit sequence is detectable. Alternatively, this enzyme is inserted into the membranes without any modification, assuming that a targeting signal different from a cleavable peptide extension allows trafficking to the inner membrane. A number of proteins which are localized in the inner membrane space, the outer and inner mitochondrial membrane and do not contain N-terminal targeting signals were previously reported (Zimmermann et al., 1979). Apocytochrome c is also initially transferred into the intermembrane space without any processing step. Covalent attachment of heme stabilizes the protein at its target site (Stuart et al., 1990). At the moment, it can be hypothesized that the substrate protoporphyrinogen IX effects routing of protoporphyrinogen IX oxidase to its target site. Along this line, accumulation of the plastidal protoporphyrinogen IX oxidase in the stroma fraction and interruption of the transfer to the thylakoids could also be explained by the lack of protoporphyrinogen IX.

5.1.3 Possible mechanisms of Protogen IX transport from chloroplasts to the mitochondria

The mitochondrial heme synthesis requires a transfer system of protoporphyrinogen IX from chloroplasts that protects the substrate from photooxidation. Herbicidal inhibition of PPOX and feeding of 5-aminolevulinate caused accumulation of protoporphyrin(ogen) IX in the cytoplasm, thus suggesting a directed export of protoporphyrinogens from plastids to mitochondria (Jacobs and Jacobs, 1993). Two pathways of porphyrin transport are possible: Porphyrins could be actively transported across the cytoplasm. Alternatively, protoporphyrinogen IX is directly channeled from enzyme to enzyme both attached to the membranes of chloroplasts and mitochondria. Additional porphyrin carrier proteins have to be postulated in both cases. The activity of protoporphyrinogen IX oxidase in the plastidal envelopes could be interpreted with a plastidal enzyme simultaneously involved in protoporphyrinogen IX transfer and tetrapyrrole synthesis. However, this idea requires a mechanism that modulates the activity of the plastidic isoenzyme between promotion of substrate channeling and catalytic conversion to protoporphyrin (Matringe et al., 1992b).

5.1.4 In vivo expression studies

The mitochondrial isoform of PPOX is exclusively associated with the capacity for heme synthesis. The plastidic enzyme activity functions in formation of heme required for apoproteins of the cytochrome b/f complex and predominantly of chlorophyll. Compartmentalized isoenzymes with different functions were expected to be differentially regulated in response to cellular and environmental requirements. The developmental and clock controlled expression of genes encoding both isoforms did not exhibited significant differences between both subfamilies (Fig. 10 and 11). Immune-reactive protoporphyrinogen IX oxidase I and II followed almost the same pattern as their steady state RNA content (data not shown). We think that protoporphyrinogen IX oxidase expression is also posttranslationally regulated and might have regulatory significance for the distribution of protoporphyrin between both organelles.

The characterization of the two isoenzymes located in the two different cellular compartments will expedite both, studies on the interaction between mitochondrial and plastidal pathway including the regulatory mechanism of substrate distribution; and the identification of additional porphyrin transport carrier.

5.2 Different mechanisms of herbicide resistance

Protoporphyrinogen oxidase is the target of DPEs type herbicides with photodynamic mode of action (Matringe et al., 1989; Witkowski and Halling., 1989). Several strategies have been developed for obtaining resistant plants towards the peroxidizing herbicides directed against PPOX. Asami and Yoshida (1999) classified mechanisms of herbicide resistance as shown in Fig. 43. One way to obtain herbicide resistance can be an alteration of the herbicide binding site in the catalytic cleft of the enzyme preventing stable binding of specific herbicides (Fig. 43A). Overproduction of a herbicide-sensitive target enzyme is another way to introduce herbicide resistance into plant cells (Fig. 43B). Transfer of genes encoding herbicide resistant target enzyme (Fig. 43C) could be an alternative way leading to herbicide resistant crop. The resistance of plant species to herbicides can also be developed by other strategies, such as reduced uptake, sequestration of the herbicides, a rapid metabolic destruction of the herbicides, or of Protogen IX and Proto IX (Fig. 43D).

We used two different approaches to obtain herbicide resistance. In the first approach

transgenic tobacco plants overexpressing either PPOX I or PPOX II were generated. In the second approach a cDNA sequence encoding PPOX I was mutated in the *E. coli* expression system followed by selection of bacterial clones for herbicide resistance.

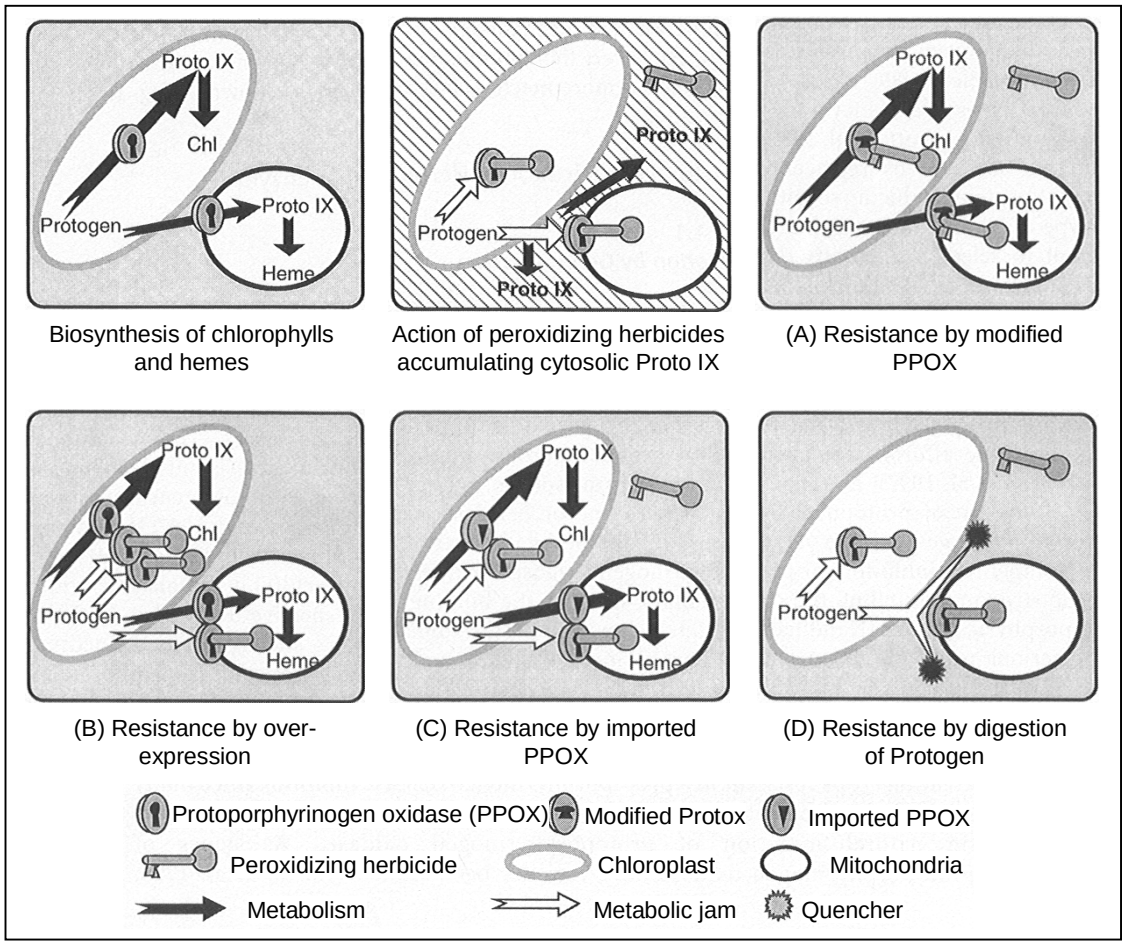


Figure 43: Protoporphyrin IX biosynthesis and its inhibition, with four possible mechanisms for obtaining resistance in plants under peroxidizing herbicide stress (Asami and Yoshida, 1999).

5.2.1 Overexpression of plastidic PPOX leads to resistance to the DPE herbicide acifluorfen

We examined the molecular basis of PPOX I over-expression in tobacco plants leading to resistance against the peroxidizing PPOX-inhibitor acifluorfen. A stable transformed gene construct consisting of the 35S CaMV-promoter linked to the *Arabidopsis thaliana* cDNA sequence encoding the plastidic PPOX isoform resulted in increased expression (Fig. 19) and activity (Fig. 20) in tobacco transformants. The three transgenic lines analyzed had at least a

5-6 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 only prevents accumulation of toxic amounts of protoporphyrin(ogen) IX. The experiments with the PPOX I over-expressing lines demonstrated that excess PPOX I activity did not significantly enhance the synthesis of protoporphyrin IX and did not increase the chlorophyll pool (Table 3). But excess amounts of PPOX compensate the herbicidal effects up to a certain inhibitor concentration at which residual PPOX activity still guarantees a normal metabolic flow in the pathway. It prevents leakage of photosensitizing protoporphyrin(ogen) IX into the cytoplasm and its accumulation in the cytosolic membranes as it is shown in leaf disk experiments (Fig. 17A and B) and after acifluorfen spraying (Fig. 23). Therefore, the tolerance of transgenic plants against the treatment of peroxidizing herbicides, which is normally inhibitory to PPOX in wild type 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 seems to be conceivable that 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 the lack of heme in mitochondria. If this correct, then the plastidic heme synthesizing branch could provide heme which is interchangeable among the different cellular compartments as previously discussed (Watanabe et al., 1998).

Overproduction of a herbicide-sensitive target enzyme is an established idea to introduce herbicide resistance into plant cells, as shown by a sethoxydim-resistant cell line of maize which exhibited a 90-fold increase in resistance due to 2.6-fold increase of acetyl coenzyme A carboxylase activity (Parker et al., 1989), or by the L-phosphinothricin resistant cell line of alfalfa with a 100-fold increase in resistance due to seven fold overproduction of glutamin synthase (Donn et al., 1984).

Other resistance mechanisms against PPOX-inhibition published also can be cited as reference to our approach. Choi et al. (1998) demonstrated diphenyl ether herbicide resistance of transgenic tobacco plants expressing the *Bacillus subtilis* HemY by means of an approximate 50% reduced electrolyte leakage of the transgenic plants in comparison to the wild type. It is, however, not clear if accumulating bacterial protein contributes to the PPOX activity in the plants and if the herbicide tolerance can be explained by reduced accumulation of protoporphyrin IX. A mutant tobacco cell culture line revealed in response to the application of the peroxidizing herbicide S23142 a 10-times higher transcript level encoding the mitochondrial PPOX II than the control culture, whereas the *PPOX I*-RNA level was not changed (Watanabe et al., 1998). The change in *PPOX II*-RNA content corresponds to a two-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 in comparison to a 5-times or transiently 20-times higher accumulation of protoporphyrin IX in wild type culture during dark or light incubation, respectively (Ichinose et al., 1995). A photomixotrophic tobacco cell culture was selected by stepwise increase in concentrations of the herbicide ET 62311 (Horikoshi and Hirooka, 1999; Horikoshi et al., 1999). Analysis of the mutants to elucidate the mechanism of resistance revealed a point mutation Ala 231 Met in the PPOX I cDNA which could be the basis for the resistance of the mutated cell culture. The *Chlamydomonas* cell line *RS-3* showed significant PPOX resistance against inhibition by peroxidizing herbicides resulting in a more than 10-times reduced formation of Mg-porphyrins in comparison to 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 different experimental systems that have been used to demonstrate the resistance mechanisms against the peroxidizing effects of the herbicides, a 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 the Material 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 on leaves of these lines in comparison to the wild type (Fig. 22). The light dosage (= light intensity x exposure time) and the time of application play a major role in the efficiency of the herbicide 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 environmental factors the dosage effects of herbicide action depends on the physiological state of the plants. Plants show a natural variation in susceptibility to peroxidizing herbicides (Sherman et al., 1991). The resistance mechanisms are very complex and not completely understood. They can include enzymatic resistance, increased degradation of the herbicide or the accumulating porphyrins, and improved natural adaptive capacity of the antioxidative pathway for detoxification of reactive oxygen species generated during herbicide action (Knörzer and Böger, 1999). We are currently investigating in transgenic tobacco plants expressing *Arabidopsis* PPOX I whether other resistance mechanisms support the effects of PPOX overexpression in transgenic tobacco. Future exploitation of these plant properties is required to engineer higher resistance against peroxidizing herbicides.

5.2.2 Overexpression of PPOX II in tobacco results only in slight resistance against acifluorfen

We also generated and analyzed transgenic tobacco plants that overexpressed the mitochondrial isoform of PPOX. A full-length cDNA sequence encoding PPOX II from *N. tabacum* (Lermontova et al., 1997) was inserted in sense orientation into the binary vector Bin AR and introduced into the tobacco genome. Most of the tobacco plants containing PPOX II transgenes showed similar growth rates and green pigmentation as the wild type. Among circa 50 transformants only one transgenic line S36 showed a chlorotic phenotype under greenhouse and especially under high light conditions. This phenotype disappeared with the age of the plant. Chlorophyll content in HL-grown transformants was reduced by up to 50 % of the wild type. In contrast, LL- grown transgenic plants had a wild type- like phenotype and the chlorophyll content was only slightly reduced in comparison to the wild type control. Protoporphyrinogen oxidase II is involved in heme synthesis in mitochondria. It can be assumed that overexpression of PPOX II could result in changes of the heme content in

transgenic plants. We were not able to measure heme content in tobacco plants due to experimental difficulties, but it has been demonstrated that heme inhibits ALA synthesis in intact plastids (Chereskin and Castelfranco, 1982). ALA formed in chloroplast is the common precursor of chlorophyll and heme synthesizing in chloroplast and mitochondria. Heme which is synthesized in mitochondria may therefore also be involved in feedback regulation of ALA synthesis. Strong reduction of ALA synthesizing capacity relative to the control was observed in the leaves of transgenic line S36 under high light conditions, while LL-grown transformants had the same ALA synthesizing capacity as the wild type. We can speculate that under high light conditions increased heme content leads to inhibition of ALA synthesis with consequent reduction of chlorophyll content.

Northern and Western blot analysis revealed an increase in the levels of mRNA and protein in PPOX II sense plants relative to the wild type. Expression of mRNA for PPOX I is not effected in PPOX II overexpressing plants (Fig. 39).

As described above, the resistance against peroxidizing herbicides can be achieved by the generation of tobacco plants overexpressing the plastidal isoform of PPOX. Previously Matringe et al. (1989) have demonstrated that PPOX-inhibiting herbicides such as S23142 and acifluorfen inhibit PPOX activity in both chloroplasts and mitochondria *in vitro*. Enzymological studies with the recombinant PPOX II, overproduced in *E. coli,* confirmed that this isoform, as well as PPOX I, is sensitive to the peroxidizing herbicides. (Lermontova et al., 1997). It was predicted that the overexpression of the mitochondrial isoform also could lead to a reduced sensitivity of plants to peroxidizing herbicides. To test the herbicide resistance of PPOX II overexpressing plants, we performed nearly the same experiments as it was described above for PPOX I sense plants.

Germination of seeds from PPOX II transformants on MS medium containing acifluorfen showed that transgenic seeds are sensitive to the herbicide like the wild type (data not shown). After spraying with 10 µM of acifluorfen, PPOX II sense plants, similar to the wild type, developed necrotic lesions within two days. Only leaf discs of LL-and HL-grown PPOX II overexpressing plants after incubation with 500 nM and 1 µM of acifluorfen accumulated ca. 50 % less of protoporphyrin IX than the control (Fig. 40). Therefore, it can be concluded that the concentration of applied herbicides and the way of their application are very important factors for the investigation of herbicide action in plants and for the comparative analysis of the herbicide sensitivity of the wild type and transgenic plants.

Comparison of the data obtained from analysis of PPOX II sense plants with the data for PPOX I sense plants suggested that overexpression of PPOX II leads to a lower resistance of transformants against peroxidizing herbicides. This could be due to several reasons. Expression of the homologous gene very often leads to cosuppression or to low overexpression of the protein encoded by transgene. Comparison of transgenic tobacco plants carrying the PPOX I transgene from *Arabidopsis* and tobacco plants carrying the tobacco PPOX II transgene confirm this observation. In the first case, the protein level of PPOX I was increased up to 6 fold, in the second case the level of PPOX II protein increased about 2 fold in transgenic plants in comparison to the wild type. Another explanation for this difference may be the different impacts of PPOX I and PPOX II in tetrapyrrole biosynthesis. PPOX II contributes to the biosynthesis of mitochondrial heme, while PPOX I is the common enzyme for the plastidal heme and chlorophyll biosynthetic pathways. As was discussed above, inhibition of heme synthesis in mitochondria via inhibition of PPOX II could be overcome in PPOX I overexpressing plants by a compensatory exchange of heme between plastids and mitochondria. It is not known if increased amount of mitochondrial PPOX could compensate inhibition of heme and chlorophyll biosynthesis in plastids. Previously Watanabe et al. (1998) have suggested that resistance of a photomixotrophic tobacco cell culture, mentioned in section 5.2.1, was due to an increasing activity of protoporphyrinogen oxidase and 10 fold increase in the level of mitochondrial PPOX mRNA. They proposed that in mutant cells the excessive Protogen, accumulated due to the inhibition of PPOX I, is metabolized in mitochondria because of higher levels of mitochondrial PPOX. The growth of photomixotrophic tobacco cells depends on both photosynthesis and catabolism of sugar, therefore inhibition of chlorophyll biosynthesis in these cells can not be as critical as in plants. These tissue culture cells are hardly comparable with tobacco plants.

To establish conclusively the role of mitochondrial PPOX in biosynthesis of tetrapyrroles and to elucidate the mechanism of PPOX II inhibition by peroxidizing herbicide it would be useful to overexpress the heterologus gene encoding PPOX II in tobacco. It will make these plants comparable with PPOX I overexpressing plants. Comparative analysis of PPOX I and II overexpressing plants may help to understand the mechanism of the interaction between the two isoforms and the mechanism of herbicide action.

Theoretically, the overexpression of both isoforms of PPOX must lead to a higher tolerance against peroxidizing herbicide (Fig. 43B) than overexpression of either PPOX I or PPOX II.

Therefore, the generation of transgenic plants overexpressing two isoforms of PPOX could lead to a significant increase in herbicide resistance.

5.2.3 Mutagenesis of cDNA encoding PPOX is another way to obtain herbicide resistance

To alter the cDNA sequence encoding PPOX I in order to prevent stable herbicide binding, the plasmid pBS KS containing tobacco *PPOX I* was transformed into the random mutagenesis strain XL1-Red. As mentioned before (section 1.7) PPOX originating from microorganisms is only weakly inhibited by the known tetrapyrrole-dependent photodynamic herbicides. Therefore, a tobacco PPOX I carrying the random mutation leading to herbicide resistance cannot be selected in the presence of bacterial, herbicide tolerant PPOX. To solve this problem, the mutated plasmids were retransformed into the PPOX-deficient *hemG* mutant and herbicide resistance of bacterial colonies was tested on increasing concentrations of acifluorfen. Four *PPOX I*-cDNAs selected from resistant bacterial clones showed point mutations in the coding region leading to the substitution of different amino acids. Two clones (# 36 and 38) were identical; they are mutated at amino acid 408 from ACA (Thr) to GCA (Ala). Clone #51 is characterized by the substitution of Phe by Leu at position 489 (TTT-CTT), whereas in clone #65 the GTA codon for Val was converted to GCA coding for Ala at position 522 (Fig. 41). We compared the growth of *E. coli hemG* mutants transformed with plasmids containing either mutated or nonmutated PPOX I sequences in liquid LB medium without acifluorfen and in presence of 20 or 100 µM of the herbicide. Growth of the *hemG* mutant transformed with nonmutated *PPOX I* was almost completely inhibited with 20 and 100 µM of acifluorfen (Fig. 42). The *hemG* mutant containing the mutated *PPOX I* cDNA #65 exhibited almost the same growth rate at the two applied concentrations of acifluorfen as in absence of the herbicide. The *hemG* mutant transformed with a plasmid containing the mutated PPOX I sequence #38 was only slightly inhibited at the selected amounts of the herbicide while growth of a *hemG* strain containing mutated PPOX I #51 was more sensitive to the herbicide and was inhibited by 100 µM of acifluorfen almost by 50 %.

Several reports exist in the literature describing mutations of PPOX I leading to herbicide resistance. Sequencing of *PPOX I* from a herbicide resistant photomixotrophic tobacco cell culture (section 5.2.1), revealed a point mutation (Ala 231 Met), which could cause the resistance (Horikoshi et al., 1999). A single point mutation (Val 389 Met) of PPOX I of *Chlamydomonas reinhardtii* conferred also herbicide resistance to the *RS-3* mutant strain

(Randolf-Anderson et al., 1998). Ward and Wolrath mentioned three different mutations in the PPOX I sequence from *Arabidopsis* (Ala 220 Tyr; Gly 221 Ser; Tyr 426 Cys) and one mutation in PPOX I from maize (Ala 166 Tyr) in the patent which was submitted in 1995. Sequence alignment revealed that the maize mutation corresponds to one of the mutations in *Arabidopsis* and is also identical to the mutation (Ala 231 Met) in the photomixotrophic tobacco cell culture described above. The mutations described here for tobacco PPOX I are different from the ones previously published. They are also located in those amino acid regions, which are conserved among all known plastidal isoforms of PPOX. Until now there is no information available on the herbicide binding domain of PPOX. It could be assumed that all peptide domains, carrying mutated amino acids could be involved in binding of the porphyrin and herbicide. Structural analysis of FeCh co-crystallized with N-methylmesoporphyrin revealed that several distinct domains on the N- and C-terminal ends are involved in porphyrin binding (Lecerof et al., 2000).

5.3 Deregulation of tetrapyrrole biosynthesis by expression of antisense mRNA for plastidal PPOX in tobacco plants

A direct approach to determine the significance of an enzyme for the control of the metabolic flux in a pathway is to progressively decrease in planta the amount of the target enzyme and to assay the extent of catalytic deficiency. We generated transgenic tobacco plants carrying a gene for plastidal protoporphyrinogen oxidase in antisense orientation under the control of the CaMV 35S promoter in the binary vector BinAR. Under greenhouse conditions with an average light intensity of circa 300 µmol photons m^{-2} s^{-1} the transformants were generally characterized by a growth rate slower than or similar to that of control plants. Three transgenic lines, which showed necrotic lesions, were selected for more detailed investigations. Molecular analysis of these selected transformants revealed decreases in PPOX transcript levels and in the amount of PPOX I protein (Fig. 27) resulting in a 2 to 10 fold accumulation of Proto IX (Fig. 29) in the transgenic plants in comparison to the wild type. This could be an explanation for the necrotic phenotype of PPOX I antisense plants based on the common knowledge about the photoreactivity of Proto IX.

The light dependence of tetrapyrrole-induced cellular damage has been described for the action of DPEs herbicides and ALA feeding (Rebeiz et al., 1984, 1988). To study the effect of the light dosage on PPOX I antisense plants, wild type and progenies of the T1 generation of

transgenic line AS19 were grown under different light conditions. The obtained data revealed that the extent of necrotic damage on transgenic plants was inversely dependent on the light intensity. Transformants grown in the greenhouse showed slightly reduced growth rates and had only a few necrotic lesions (Fig. 25A). Transgenic plants transferred from the greenhouse to low light (70 µmol m^{-2} s^{-1}) developed necrotic lesions within 48 hours (Fig. 25C, D). The strongest necrosis appeared on the middle leaves (from leaf 4 to 8) of six-week-old transgenic plants (Fig. 26C) In contrast to that, transformants which were exposed to high light (550 µmol m^{-2} s^{-1}), grew even better than in the greenhouse and had normal pigmentation without necrotic spots (Fig. 25B, D). Growth of the selected transgenic plants under light intensities up to 1000 µmol m^{-2} s^{-1} did not result in the formation of necrotic lesions. It was assumed that under different light conditions transgenic plants could accumulate different amounts of Proto IX, which would be responsible for the extent of necrotic damage. Therefore, the content of Proto IX was measured in control and transgenic plants, which were transferred from the greenhouse to low and high light conditions (Fig. 30). Levels of Proto IX in leaves 1-4, 5-6 were not significantly different between transformants exposed either to low light or high light intensities. However, lower amounts of Proto IX were accumulated in leaves 7-8 of LL-illuminated plants than in the respective leaves of HL-illuminated transformants. The decreased amount of Proto IX in leaf 7-8 of LL-grown transformants could be explained with its degradation due to the higher degree of necrotic lesions on these leaves (Fig. 25C, D). In PPOX I antisense plants the levels of PPOX I transcript and protein were reduced to the same extent relatively to the wild type under all selected growth conditions (Fig. 28). Therefore, different phenotypical changes under low light and high light intensities in PPOX I antisense plants are not due to the different amounts of accumulating Proto(gen) IX and also not due to the different expression of PPOX I mRNA and protein. This finding encouraged me to search for another mechanism which might be involved in the different response to low light and high light intensities in PPOXI antisense plants.

It is known that tetrapyrrole intermediates are highly photoreactive; they can easily be excited and then transfer energy or electrons to O_2. Reactive oxygen species (ROS) are produced upon exposure to light and oxygen. ROS in plants are normally detoxified by enzymatic and chemical antioxidants present in all compartments, especially in photosynthetic organelles (Foyer et al., 1994). Therefore, the contents of low molecular weight antioxidants under different growth conditions in wild type and transgenic plants expressing antisense mRNA for

PPOX I were analyzed (Fig. 32). Our data showed that control and transgenic plants 4 days after transfer to low light conditions have strongly decreased levels of total and reduced ascorbate and glutathione, respectively, and also declined levels of tocopherol. Transfer of transgenic plants, adapted for one week to low light conditions, into the growth chamber with high light intensity led to an increase of the levels of antioxidants including tocopherol. In contrast to that, in high light-adapted plants the levels of antioxidants were decreased after transfer to the low light conditions (data not shown). This could be explained by changes in the level of photosynthesis, which decreased 6-fold after transfer of tobacco plants from the greenhouse to low light conditions (data not shown). The photosynthetic light reaction provides the ascorbate-glutathione cycle (the main detoxification pathway of ROS) with reductants via NADPH.

Previous studies have showed that low molecular weight antioxidants could have several functions in oxidative stress. They can detoxify reactive oxygen species which are formed during oxidative stress (Foyer, 1993). The antioxidants can also be important in keeping tetrapyrrolic intermediates in their reduced form (Jacobs et al., 1996; Mock et al., 1998). Since we can not distinguish between reduced and oxidized forms of Proto(gen) in our experiments, it is difficult to conclude how antioxidants are involved in the response against accumulating Proto(gen) IX in PPOX I antisense plants. Two different mechanisms could be proposed. If in PPOX I antisense plants Protogen IX is oxidized to Proto IX under both growth conditions, the lower phototoxicity of Proto IX under high light conditions could be due to the higher capacity of plants to detoxify the formed ROS in contrast to low light grown plants, which have strongly decreased amounts of radical scavengers like tocopherol, ascorbate and glutathione. The second mechanism is based on the assumption that LL-illuminated plants have a lower capacity than HL–illuminated plants to keep Protogen IX in its reduced state due to decreased amounts of antioxidants. This suggestion can be supported with previously obtained data, which demonstrated that oxidation of Uro(gen) to Uro can be inhibited by ascorbate *in vitro* (Jacobs et al., 1996).

The fact that Proto IX accumulation causes a “threshold” effect on cells could be useful in understanding the role of antioxidants in response to Proto(gen) IX accumulation under low and high light conditions. Iinuma et al. (1994) have demonstrated that accumulation of Proto IX to a certain concentration did not lead to oxidative cell damage, while the cells with further increase of this amount exhibited a consistent phototoxic response. These observations could

be explained by an “overloading” of the detoxification systems, as in the case of transformants grown under low light conditions. In contrast to that, HL-grown plants containing the same amounts of Proto(gen) IX as LL-grown plants could be prevented from photooxidative damage by increased amounts of antioxidants.

Plants expressing the antisense transgenes for PPOX I could be compared with plants treated with peroxidizing herbicides. Both inhibition systems lead to a decrease of PPOX activity either by inhibition or reduced expression of the enzyme, resulting in the accumulation of excessive amounts of Proto(gen) IX which finally causes necrotic leaf lesions. Finckh and Kunert (1985) investigated the protecting role of ascorbate and tocopherol against herbicide-induced lipid peroxidation in higher plants. Tested plant species showing substantial amounts of both ascorbate and tocopherol and a ratio of ascorbate to tocopherol concentration between 10 and 15:1 were highly protected against phytotoxic action of the herbicides. It was suggested that the extent of peroxidative cell damage after herbicide treatment was determined by the amount of ascorbate and tocopherol. Low concentrations of ascorbate and tocopherol in plant cells potentiated the effect of an accumulating protoporphyrin IX on the peroxidative process. These data are in agreement with our conclusion about the role of antioxidants in plant response to the accumulation of Proto(gen) IX.

Previous studies of Kruse et al. (1995b) and Mock and Grimm (1997) on transgenic plants showed that porphyrin accumulation was the result of the inhibition of UROD or CPO activity due to the expression of antisense mRNAs encoding the corresponding enzyme. In contrast to PPOX I antisense plants, reduced activity of either CPO or UROD increased the level of porphyrinogen substrates up to 500-fold in transformants relative to control plants. Analysis of light-dependent necrosis on UROD antisense plants (Mock and Grimm, 1997) revealed the opposite tendency upon different light intensities in comparison to PPOX I antisense plants. UROD antisense plants were grown for 10 weeks under a 6-h light/ 18-h dark regime with 300µmol photons $m^{-2}s^{-1}$. Plants were then grown for additional 4 weeks under these conditions or under 16-h light/ 8-h dark cycles at 300 or 90µmol m^{-2} s^{-1}. Under each light regime UROD antisense plants grew slower than wild-type plants and showed reduced leaf area and plant size. Plants became photodynamically susceptible during a long photoperiod at high light, as indicated by the necrotic lesions on most of the transgenic plants (Mock and Grimm, 1997). Necrotic damage was drastically reduced when plants were exposed to low-light intensity (90µmol m^{-2} s^{-1} for 16 h) or short light periods (300µmol $m^{-2}s^{-1}$ for 6 h).

The mechanisms which can explain the difference between PPOX I, UROD and CPO antisense plants in response to low and high light intensities have to be elucidated. The nature, properties and subcellular localization of porphyrin intermediates may help to answer this question. No significant difference was obtained in the formation of singlet oxygen after UV-A irradiation of Proto, Copro and hematoporphyrin in acetone solution (Arakane et al., 1996). However, comparing the amounts of porphyrin intermediates that accumulate in UROD and CPO antisense plants (up to 500 fold relatively to the wild type) with the amounts of Proto IX in PPOX I antisense plants (2-10 fold) suggested that Proto IX is much more toxic than Uro and Copro. An explanation for the different toxicity of porphyrin intermediates *in vivo* can be their different subcellular localization. Matringe et al. (1992) have demonstrated that PPOX is an integral protein of both the thylakoid and the envelope membranes of spinach chloroplasts while UROD and CPO are localized in the chloroplast stroma. This influences also the subcellular localization of the corresponding tetrapyrrole intermediates. Porphyrin intermediates have different lipophilicity which determines their membrane binding capacity. Lee and co-workers (1993) demonstrated that Proto IX is the most lipophilic of the early porphyrin intermediates. As mentioned above (section 1.6; 1.8), accumulation of porphyrin intermediates leads to lipid peroxidation followed by membrane destruction. Therefore, association of Proto IX with chloroplast membranes could potentiate its phototoxicity in comparison to Uro and Copro. When comparing the efficacy of Uro, Copro and Proto for vascular damage, only lipophilic Proto showed a phototoxic effect on endothelial cells *in vitro* (Strauss et al., 1995).

The localization of excessive amounts of porphyrins does not necessarily correspond to the sites where porphyrins accumulate under normal physiological conditions. It was shown that inhibition of PPOX by DPEs herbicides leads to the accumulation of its substrate, Protogen IX, which leaks out of the plastid and is oxidized in the cytoplasm to Proto IX (Matringe and Scalla, 1988; Sandmann et al., 1990; Jacobs et al., 1991; Lee and Duke, 1994). Jacobs and Jacobs (1993) investigated the formation of porphyrin intermediates by isolated barley plastids in darkness fed with porphyrin precursor ALA and in the presence or absence of DPEs herbicides. In the absence of herbicides about 50 % of the Proto IX formed was found in the extraplastidic medium. In contrast to that, Urogen and magnesium protoporphyrin were located mainly within the plastid. If the incubation was carried out in the presence of herbicide, Proto IX formation by the plastids was completely abolished, but large amounts of

Protogen accumulated in the extraplastidic medium. The authors assumed that Proto IX and Protogen IX found outside the plastid did not arise from plastid lysis, since the percentage of plastid lysis, measured via stromal marker enzymes, was far less than the percentage of these porphyrins in the extraplastidic fraction. From this work it has been concluded (Jacobs and Jacobs, 1993) that Proto(gen) export took place under conditions of a massive excess of porphyrin production within the plastids caused by higher levels of ALA.

In PPOX I antisense plants excessive amounts of Protogen IX could also be accumulated in the cytoplasm where it is more toxic than in chloroplasts, since in chloroplasts it is prevented from oxidation by the highly reducing environment (Jacobs and Jacobs, 1994). The accumulation of Proto(gen) IX outside of the plastids could be explained by the existence of a transport machinery for the transfer of Protogen IX into mitochondria needed for the synthesis of mitochondrial heme. It is still unclear if this transport machinery is specific for Protogen IX or if it is used for the transfer of other porphyrins too, especially when they are accumulating in excessive amounts. It is also not clear where excessive amounts of Uro(gen) and Copro(gen) were accumulated in UROD and CPO antisense plants, respectively.

Comparison of the results presented above with previously published data indicates that deregulation of tetrapyrrole biosynthesis on the levels of UROD, CPO and PPOX I led to the accumulation of the corresponding substrates. Excessive amounts of accumulating porphyrins in all cases resulted in necrotic damage of transgenic plants. However, these transgenic plants differently respond to the changes of light conditions. The mechanism which is responsible for this difference remain still unclear. As mentioned above, it is difficult to distinguish between reduced and oxidized forms of tetrapyrroles. Therefore, the kinetics of porphyrinogen accumulation and their oxidation to the corresponding porphyrins, which are phototoxic, were not studied before. The establishment of methods, which will allow determining separately the reduced and oxidized forms of porphyrins, could be the next step which may help to solve numerous questions that arose from the studies on transgenic plants with deregulated biosynthesis of tetrapyrroles. To understand the difference between UROD, CPO and PPOX I antisense plants it also would be useful to show the subcellular localization of the accumulating porphyrin intermediates.

6 Abstract

1. Protoporphyrinogen IX oxidase (PPOX) is the last enzyme in the common pathway of heme and chlorophyll biosynthesis. Two different full length tobacco cDNA sequences were cloned by complementation of the protoporpyhrin IX (Proto IX) accumulating *E. coli hemG* mutant rescuing it from heme auxotrophy. These cDNA sequences were designated *PPOX I* and *PPOX II*, respectively. *PPOX I* cDNA sequence encoded a protein of 548 amino acid residues protein with a putative transit sequence of 50 amino acid residues, the *PPOX II* cDNA a protein of 504 amino acid residues. Both deduced protein sequences share 27,2 % identical amino acid residues. *In vitro* translated PPOX could be translocated to plastids. The PPOX II was targeted to mitochondria. The mature plastidal and the mitochondrial isoenzyme were overexpressed in *E. coli*. Bacterial extracts containing the recombinant mitochondrial enzyme exhibit high PPOX activity relative to control strains, while the plastidal enzyme could only be expressed as an inactive peptide.

2. Protoporphyrinogen oxidase is the molecular target of diphenyl ether type (DPEs) herbicides. Their inhibitory mode of action is explained by rapid oxidation of accumulating protoporphyrinogen IX (Protogen IX) to the photosensitizing Proto IX, which subsequently generates reactive oxygen species (ROS) during oxidative breakdown. Several strategies have been developed to obtain plants resistant toward such peroxidizing herbicides. In presented work two different approaches were used to obtain herbicide resistance.

a) In the first approach, transgenic tobacco plants overexpressing either plastidal or mitochondrial isoform of PPOX were generated. The transformants were screened for low Proto IX accumulation upon treatment with the DPE herbicide acifluorfen.

Transgenic plants, containing the sense transgenes for *Arabidopsis* PPOX I, showed lower susceptibility against acifluorfen than the wild type in experiments including leaf disc incubation and foliar spraying with herbicide. The resistance towards acifluorfen was conferred by 5-6 fold increase in the level of the plastidic isoform of PPOX. The *in vitro* activity of this enzyme extracted from plastids of selected transgenic lines was at least 5-times higher than the control activity. Herbicide treatment which is normally inhibitory to PPOX did not significantly impair the catalytic reaction in transgenic plants and, therefore, did not cause photodynamic damage in leaves. Thus overproduction of the plastidal isoform of

protoporphyrinogen oxidase neutralizes the herbicidal action, prevents the accumulation of the substrate protoporphyrinogen IX and, consequently, abolishes the light-dependent phototoxicity of acifluorfen.

Insertion of the *N. tabacum* PPOX II transgene into the genome of tobacco plants led to a circa 2 fold increase of the levels of PPOX II mRNA and protein in transformants in comparison to the wild type. Transgenic plants overexpressing PPOX II showed only slight resistance against acifluorfen, in contrast to the PPOX I overexpressing plants. This finding could be explained either by low overexpression of PPOX II or by the different roles of the two isoforms of PPOX in tetrapyrrole biosynthesis.

b) In the second approach, the tobacco *PPOX I* cDNA sequence was randomly mutagenized in the *E. coli* XL-Red mutator strain. The mutated plasmids were retransformed into *E. coli hemG* mutant and resistant bacterial colonies were selected on increasing concentrations of the PPOX inhibitor acifluorfen. Three *PPOX I* cDNAs selected from resistant bacterial clones showed point mutations in the PPOX I coding region leading to the substitution of a different amino acid. All three mutations are located in the cDNA sequence for highly conserved amino acid residues.

3. To study the effect of PPOX I deficiency, transgenic plants carrying a gene for PPOX I in antisense orientation were generated. Selected transgenic lines were characterized by decreased levels of PPOX I mRNA and protein leading to the accumulation of Proto(gen) IX, the substrate of PPOX. Antisense inhibition of PPOX resulted in reduced growth rate and necrotic damage of the leaves. The formation of necrotic leaf lesions inversely depends on the light intensities. Exposure of transgenic plants, which were grown before in the greenhouse, to low light conditions (70 µmol m^{-2} s^{-1}) led to necrotic damage of the leaves. In contrast to that, the same transgenic lines after exposure to high light were characterized by only slight necrotic damage. The Proto(gen) IX content and the expression levels of PPOX I mRNA and protein were similar under low and high light conditions. However, the light effect was strongly correlated with the levels of low molecular weight antioxidants (ascorbate, glutathione and tocopherol).

Taken together, the applied combination of molecular biological and physiological approaches extents our knowledge about the role of protoporphyrinogen IX oxidase in chlorophyll biosynthesis and in other metabolic processes of plants.

7 Zusammenfassung

1. Protoporphyrinogen-IX-Oxidase (PPOX) ist das letzte gemeinsame Enzym im verzweigten Stoffwechselweg der Häm- und Chlorophyllbiosynthese. Zwei verschiedene Vollängen-cDNA-Klone aus Tabak (*Nicotiana tabacum*) konnten wegen ihrer Eigenschaft isoliert werden, die Häm-Auxotrophie der Protoporphyrin-IX (Proto IX)-akkumulierenden *E. coli hemG*-Mutante zu komplementieren. Die cDNA-Sequenzen wurden als *PPOX I* bzw. als *PPOX II* bezeichnet. Durch *PPOX I* wird ein Protein mit 548 Aminosäureresten inkl. einer vermuteten Transitpeptid-Sequenz von 50 Resten kodiert, während *PPOX II* für ein Protein mit 504 Aminosäureresten kodiert. Beide abgeleiteten Proteinsequenzen weisen 27,2% identische Aminosäuren auf. *In vitro*-Translationsprodukte von *PPOX I* wurden in Plastiden importiert, wohingegen *PPOX II*-Produkte in Mitochondrien-Präparationen nachgewiesen werden konnten. Es gelang, beide maturen Isoenzyme in *E. coli* zu exprimieren. Bakterienextrakte, die das rekombinante mitochondriale Enzym exprimieren, weisen eine hohe PPOX-Aktivität im Vergleich zum Kontrollstamm auf, während das plastidäre Isoenzym nur als inaktives Polypeptid gebildet wurde.

2. PPOX ist das molekulare Target von Herbiziden des Diphenylether (DPE)-Typs. Deren hemmende Wirkungsweise läßt sich durch die Akkumulation und rasche Oxidation von Protoporphyrinogen (Protogen) IX zum photosensibilisierenden Proto IX erklären, was die nachfolgende Generierung von zellschädigenden reaktiven Sauerstoffspezies (ROS) zur Folge hat. Verschiedene Strategien sind entwickelt worden, um Pflanzen zu erzeugen, die gegenüber solchen peroxidierenden Herbiziden resistent sind. Ausgewählte Ansätze wurden in dieser Arbeit verfolgt:

a) Transgene Tabakpflanzen konnten generiert werden, die entweder die plastidäre oder die mitochondriale Isoform von PPOX überexprimieren. Die Transformanten wurden daraufhin untersucht, ob sie eine geringe Akkumulation von Proto IX nach der Behandlung mit dem DPE-Typ Herbizid Acifluorfen aufwiesen.

Tabakpflanzen, die das *PPOX I*-Transgen aus *Arabidopsis thaliana* in S*ense*-Orientierung exprimieren, sind durch eine im Vergleich zum Wildtyp (WT) geringere Anfälligkeit gegenüber Acifluorfen gekennzeichnet, was sowohl die Behandlung von Blattscheiben als auch das Besprühen der Blätter ganzer Pflanzen betrifft. Die vergrößerte Resistenz gegenüber

Acifluorfen wird offensichtlich durch eine fünf- bis sechsfach erhöhte Menge der plastidären Isoform von PPOX vermittelt, die mit einer in gleichem Maße erhöhten extrahierbaren *in vitro*-Enzymaktivität im Vergleich mit WT-Pflanzen einhergeht. Eine Herbizidbehandlung, die normalerweise die PPOX hemmt, hat keinen signifikanten Einfluß auf die Katalyse in den transgenen Pflanzen und führt deshalb nicht zu photodynamischen Schäden an den Blättern. So führt die Überexpression der plastidären Isoform von PPOX dazu, daß es zu keiner Anhäufung von Proto IX kommt, wodurch die lichtabhängige Phytotoxizität von Acifluorfen aufgehoben wird.

Wurde PPOX II aus Nicotiana tabacum in Tabak überexprimiert, konnte eine ca. zweifache Zunahme der entsprechenden Transkript- und Proteinmenge im Vergleich zu WT-Pflanzen festgestellt werden. Im Vergleich mit den PPOX I-überexprimierenden Pflanzen war die Resistenz gegenüber Acifluorfen aber eher gering. Dieses Ergebnis könnte durch das niedrige Expressionsniveau des Transgens oder aber durch mögliche unterschiedliche Funktionen der beiden Isoformen von PPOX im Tetrapyrrol-Metabolismus erklärt werden.

b) Im zweiten Ansatz, Resistenz zu erzeugen, wurde die *PPOX I*-cDNA aus Tabak im *E. coli* Mutator-Stamm XL-Red nach dem Zufallsprinzip mutagenisiert. Die mutierten Plasmide wurden in den *hemG*-Stamm von *E.coli* re-transformiert und die Bakterienkolonien anschließend auf Medien mit steigenden Konzentrationen von Acifluorfen selektiert. Drei cDNAs für *PPOX I*, die aus resistenten Kolonien isoliert werden konnten, wiesen Punktmutationen in einem stark konservierten Abschnitt des kodierenden Bereichs auf, welche zum Austausch von jeweils einer Aminosäure führten.

3. Um die Auswirkungen von *PPOX I*-Defizienz zu untersuchen, wurden transgene Tabakpflanzen erzeugt, die das *PPOX I*-Gen in Antisense-Orientierung enthalten. Ausgewählte transgene Linien wiesen erniedrigte Gehalte von *PPOX I*-mRNA und –Protein auf, was zur Akkumulation von Proto(gen) IX führte. Sichtbare Auswirkungen der Antisense-Inhibierung von *PPOX I* waren verlangsamtes Wachstum der Pflanzen und Nekrose-Schäden an den Blättern. Das Ausmaß der Blattläsionen verhielt sich invers zur verabreichten Lichtintensität: An den Blättern von transgenen Pflanzen, die zuvor im Gewächshaus angezogen worden waren, entwickelten sich rasch Nekrosen, nachdem die Pflanzen anschließend Schwachlichtbedingungen ausgesetzt wurden (70 µmol m^{-2} s^{-1}). Im Gegensatz dazu wiesen die gleichen Transformanten unter dem Einfluß von höheren Lichtintensitäten nur

sehr schwache Nekrosen auf. Der Gehalt an Proto(gen) IX und das Expressionsniveau für *PPOX I*-Transkript und –Protein unterschieden sich kaum zwischen den beiden unterschiedlichen Lichtbedingungen, was eine komplexere Ursache für den Lichteffekt vermuten läßt. Der Status des antioxidativen Schutzsystems der transgene Pflanzen wurde unter den verschiedenen Bedingungen analysiert. Es wurde beobachtet, daß der Transfer von Pflanzen aus Gewächshaus- in Schwachlichtbedingungen innerhalb weniger Tage in einer massiven Erniedrigung der Gehalte an niedermolekularen Antioxidantien sowohl in WT- als auch in den transgenen Pflanzen resultierte.

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Acknowledgements

This work was done at the Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben.

First of all I would like to express my gratitude to Dr. Bernhard Grimm, head of the Chlorophyll Biosynthesis group, for giving me the opportunity to work in his research group, for his careful supervision, helpful and stimulating discussions. I am also grateful to him for his help during writing and correcting this thesis.

I would like to thank Dr. E. Kruse for the organellar import experiment and for the introduction into molecular biological techniques. I am thankful to other colleagues from our group, V. Voronetskaya, A. Alawady and S. Mishra, for the nice and stimulating atmosphere in the group, for scientific debates, for their help during experimental work and in preparation of this thesis. I am particularly grateful to U. Keetman for reading and correction of some sections in the thesis and also for his help in computer presentation of this work.

I would like to thank Mrs. P. Linow, Mrs. E. Fraust and Mrs. A. Püschel for their excellent technical assistance. I am grateful to Mrs. S. König for DNA sequencing, Mrs. H. Ernst and Mrs. B. Schäfer for the photographic work. I would also like to thank Mrs. U. Tiemann and Mrs. A. Kilian for the fast and qualified help with the computer presentation of graphics.

I am especially indebted to Dr. D. Enneking for proofreading of this thesis.

I am also thankful to Dr. H-P. Mock, Dr. M. Melzer and Dr. M. Peisker for the collaboration during this work.

Finally, I thank D. Demidov for helpful discussions and interesting suggestions and for sharing new experimental methods with me.

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Erklärung

Hiermit erkläre ich, daß ich meine Arbeit selbständig und ohne fremde Hilfe verfaßt habe, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die benutzten Werke kenntlich gemacht habe. Diese Arbeit wurde noch nicht als Bewerbung für die Promotion an dieser oder anderen Universität benutzt.