Transformation of Tobacco with a Mutated Cyanobacterial Phytoene Desaturase Gene Confers Resistance to Bleaching Herbicides

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Carotenoids are constituents of the photosynthetic apparatus and essential for plant survival because of their involvement in protection of chlorophylls against photooxidation. Certain classes of herbicides are interfering with carotenoid biosynthesis leading to pigment destruction and a bleached plant phenotype. One important target site for bleaching herbicides is the enzyme phytoene desaturase catalysing the desaturation of phytoene in ζ-carotene. This enzymatic reaction can be inhibited by norflurazon or fluridone. We have transformed tobacco with a mutated cyanobacterial phytoene desaturase gene (*pds*) derived from the *Synechococcus* PCC 7942 mutant NFZ4. Characterization of the resulting transformants revealed an up to 58 fold higher norflurazon resistance in comparison to wild type controls. The tolerance for fluridone was also increased 3 fold in the transgenics. Furthermore, the transformed tobacco maintained a higher level of D1 protein of photosystem II indicating a lower susceptibility to photooxidative damage in the presence of norflurazon. In contrast, the genetic manipulation did not confer herbicide resistance against ζ-carotene desaturase inhibitors.

Introduction

Carotenoids are vital for all organisms with oxygenic photosynthesis. Located in the antenna and core complexes of photosystem I and II carotenoids fullfill important functions in stabilization of pigment-protein complexes and light harvesting (Plumley and Schmidt, 1995; Lee and Thornber, 1995; Ros *et al.*, 1998). Moreover, they are indispensible for photoprotection due to their ability to dissipate excess light energy as harmless heat and their potential to quench free radicals and singlet oxygen (Krinsky, 1979; Niyogi, 1999). The first comitted step in carotenoid biosynthesis is the formation of phytoene from two molecules of geranylgeranyl diphosphate by the enzyme phytoene synthase (Psy). The colourless phytoene undergoes a series of desaturation steps (Sandmann, 1994; Beyer *et al.*, 1994). Four of them lead to the production of the red lycopene. In higher plants, algae and cyanobacteria two different enzymes are responsible for the conversion of phytoene into lycopene, namely phytoene desaturase (Pds) and ζ-carotene desaturase (Zds). Phytoene desaturase carries out the first two-step desaturation to yield ζ-carotene which is the substrate for the second desaturation sequence catalyzed by ζ-carotene desaturase (Fig. 1). Recently, it has been shown that carotenoid desaturation and the formation of all-*trans* lycopene in higher plants requires the presence of a distinct carotenoid isomerase which has been cloned in two laboratories using mutants of *Arabidopsis* or tomato, respectively (Park *et al.*, 2002; Isaacson *et al.*, 2002). Subsequent cyclizations of lycopene give rise to carotenes which can then be converted to xanthophylls by the introduction of various oxy-, keto- and epoxy-groups.

Inhibition of coloured carotenoids causes a dramatic reduction in pigment content known as bleaching which finally results in plant death because of missing photoprotection. One of the key

Abbreviations: SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; DW, dry weight; SD, standard deviation; rpm, rounds per minute.

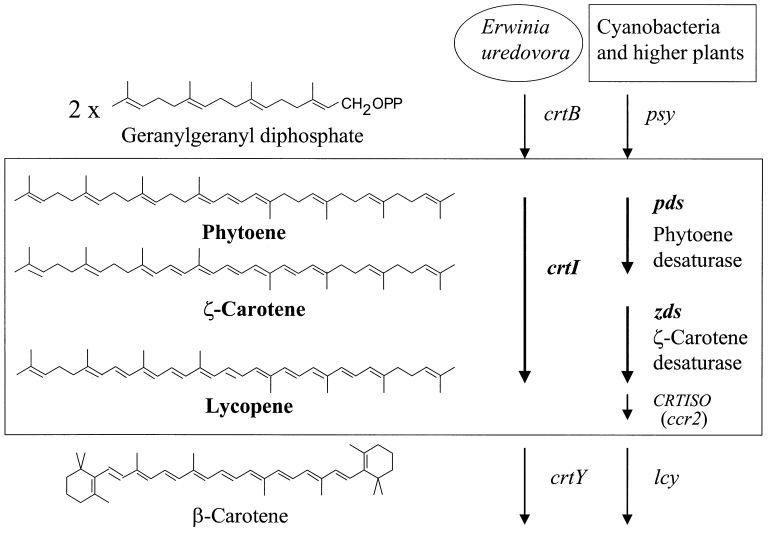

Fig. 1. Comparative scheme of the carotenoid biosynthetic pathway in bacteria and higher plants. *psy*: phytoene synthase gene, *pds*: phytoene desaturase (plant-type) gene, *crtI*: phytoene desaturase gene, *lcy*: lycopene cyclase gene, *zds*: ζ-carotene desaturase gene, *crtB*: bacterial phytoene synthase gene, *crtY*: bacterial lycopene cyclase gene, *CRTISO (ccr2):* carotenoid isomerase gene.

targets for bleaching herbicides is the enzyme phytoene desaturase (Böger and Sandmann, 1998a). With respect to their sequence and biochemical properties one can distinguish between plant-type phytoene desaturases (Pds) found in higher plants, algae and cyanobacteria and the well conserved desaturases of the *crtI* type (García-Asua *et al.*, 1998). The *crtI*-type desaturases of bacteria and fungi carry out three or four desaturation steps and are not susceptible to herbicides targeting Pds whereas the plant type phytoene desaturases are inhibited. For several bleaching herbicides a reversible binding to the substrate phytoene and a non-competitive inhibition could be demonstrated by investigation of enzyme kinetics. Using selective screening procedures several norflurazon-resistant mutants of *Synechococcus* PCC 7942 could be isolated. Some of these mutants exhibited a higher tolerance towards bleaching herbicides and had a lower catalytic activity (Chamovitz *et al.*, 1990; Linden *et al.*, 1990). A point mutation in the phytoene desaturase gene causing an amino acid substitution at the protein level was shown to be responsible for herbicide resistance in mutant NFZ 4 (Chamovitz *et al.*, 1991). We have expressed the cyanobacterial phytoene desaturase gene derived from the *Synechococcus* NFZ 4 mutant in a constitutive manner in tobacco plants. In this study the resulting transgenic tobacco was tested for its resistance to bleaching herbicides targeting the plant-type phytoene desaturase as well as for its sensitivity to photodamage upon norflurazon treatment.

Material and Methods

Chemicals and enzymes

If not stated otherwise, chemicals were purchased from Fluka (Buchs, Germany), Merck (Darmstadt, Germany), Riedel deHaen (Seelze, Germany), Roth (Karlsruhe, Germany), Serva (Heidelberg, Germany) and Sigma (Deisenhofen, Germany). Enzymes for DNA manipulations were from Life Technologies Inc. (Gaithersburg, USA), New England Biolabs (Schwalbach, Germany), Qiagen (Hilden, Germany) and Roche Diagnostics (Mannheim, Germany).

Herbicides

Norflurazon (4-chloro-5-methylamino-2-(α,α,α-trifluoro-*m*-tolyl)pyridazin-3(2*H*)-one) was provided from Sandoz (Basel, Switzerland), Fluridone (1-methyl-3-phenyl-5-(α,α,α-trifluoro-*m*-tolyl)-4-pyridone) from Eli Lilly (Indianapolis, USA) and LS 80707 (ethyl-*cis*-5-methyl-6-ethyl-2-phenyl-5,6-dihydropyran-4-one-3-carboxylate) from Rhone-

Poulenc (Lyon, France). All herbicides were dissolved in pure methanol.

DNA manipulations

DNA manipulations followed the methods and techniques described in Sambrook *et al.* (1989).

Plant material and transformation

Tobacco (*Nicotiana tabacum* L. var. Samsun) was used as experimental plant material. A mutated phytoene desaturase gene isolated from the NFZ4 mutant of *Synechococcus* PCC7942 (Chamovitz *et al.*, 1991; 1993) was subcloned in the intermediate vector pBS and a *Sph* I site was introduced at the 5‘ end by site directed mutagenesis according to Sayers and Eckstein (1989). The 1.6 kb fragment encoding the cyanobacterial phytoene desaturase was reisolated by restriction digest with *Sph* I and *Kpn* I and inserted in the *Sph* I/*Kpn* I digested vector pTRA3XN, a pUC 118 derivative. This ligation led to an in-frame fusion of the bacterial gene with a transit sequence for chloroplast import, namely the small subunit of the ribulose bisphosphate carboxylase (SSU) of pea. To build up a construct suitable for plant transformation, the 1.8 kb fragment consisting of the transit sequence and the coding region for phytoene desaturase was gel isolated after *Xba* I/*Eco* R I digestion, blunted with Klenow enzyme and ligated in the *Sma* I site of the binary vector pGPTV-Kan-P35S CaMV, a pBIN derivative (Bevan, 1984; Becker *et al.*, 1992). The expression of the foreign gene in the plant transformation vector was under constitutive promoter control (Odell *et al.*, 1985). The binary vector was introduced into *Agrobacterium tumefaciens* LBA 4404. Tobacco was transformed by agroinfection following the leaf dics method of Horsch *et al.* (1985). Regeneration was performed in the presence of the antibiotic kanamycin as selection marker. Positive transformants were screened for the presence of the foreign transgene by PCR using specific primers for the cyanobacterial *pds* gene.

Plant growth and inhibitor treatments

Seeds of non-transformed tobacco and *pds* transgenics were surface-sterilized. For inhibitor treatments 40–60 seeds were allowed to germinate in sterile 250 ml Erlenmeyer flasks containing 10 ml liquid Murashige and Skoog basal (Murashige and Skoog, 1962). To each flask 5 µl of methanol with inhibitor was added. The controls had the equivalent volume of pure methanol. The Erlenmeyer flasks were then incubated in a shaker at 100 rpm in a thermostated culture room at a constant temperature of 23 °C. A light/dark cycle of 14/10 h was applied. Samples were illuminated with white light of 65 µmol m^{-2} s^{-1} provided by a combination of fluorescent tubes (Osram L-FLUORA and Osram Universal White). After an incubation period of 6 days the plantlets were harvested. In case where heterozygous plantlets were to be analyzed, the resulting seedlings of germination of seeds from line Pds1 (T1) were grouped according to the segregation into their bleaching phenotype. Azygous bleached plantlets were removed and the hemizygous and homozygous plants kept separately for analysis.

Growth of adult plants

Small sterile plantlets were first put in autoclaved glass containers (Weck, Wehr, Germany) and after two to three weeks transfered to soil in the green house until they reached the 6–8 leaves stage. In order to create comparable conditions between transgenics and controls the plants were then incubated in a growth chamber (Heraeus Vötsch, Hannover, Germany) at 23 °C and a humidity of 65%. A light/dark cycle of 14/10 h was used and white light intensity was 50 µmol m^{-2} s^{-1}.

Pigment extraction and analysis

For pigment analysis inhibitor treated plantlets and corresponding wild type controls were harvested, rinsed with water which was then removed and the fresh weight determined by weighing. The samples were subsequently shock-frozen and freeze-dried. Freeze-dried material was ground and then repeatedly extracted with 80% (v/v) acetone until the cell debris was white. The pigment extracts were combined and subjected to spectroscopical pigment determination. Pigment content was estimated using the formula of Lichtenthaler and Wellburn (1983). In case of very low pigment concentrations the amount of pigment (especially chlorophylls) was rechecked by HPLC analysis ac-

cording to Gilmore and Yamamoto (1991). Pigments were identified by their retention times, comparison of spectral data with literature values and co-chromatography with authentic standards (cp. Goodwin and Britton, 1988). I_{50} values were estimated from inhibition curves for carotenoid formation or chlorophyll content or obtained from Dixon plots by linear regression analysis. The values were derived from three to 14 independent determinations with a minimum of 40 seedlings. The factor of resistance (FR) in the transgenics represents the ratio of I_{50} of transgenics versus the I_{50} of the wild type.

RNA isolation and expression analysis

Total RNA was isolated from seedlings (and adult plants) as described in Kuntz *et al.* (1992). First strand cDNA was obtained by reverse transcription of 2 μg total RNA using the reverse transcriptase from Life Technologies and the oligo dT primer. The reaction was performed at 37 °C for 2 h as recommended by the supplier. After dilution 80 ng of the resulting cDNA was used as template for a PCR amplification with primers pdCya1 (5′ccgctccgacgaagacatcttgg) and pdCya2 (5′cgagcatcagacagacggtgc) specific for the cyanobacterial phytoene desaturase. Typically a 50 μl reaction contained: 1 μl template (80 ng of cDNA), 5 μl reaction buffer (10×), 3 μl 25 mM $MgCl_2$, 20 pmol of each primer and 0.5 μl Taq-polymerase (Gibco BRL, Life Technologies, Gaithersburg, MD, USA). PCR was carried out in a thermocycler PTC-100 (MJ Research). PCR conditions were as follows: 4 min denaturation at 94 °C, then 30 cycles (1 min denaturation at 94 °C, 0.5 min annealing at 56 °C and 1.5 min extension at 72 °C) and a final extension step of 5 min at 72 °C.

Western blot analysis

Total proteins were extracted from 100–150 mg of 7 days old seedlings after inhibitor treatment. For this purpose the seedlings were frozen and ground in a mortar. The above mentioned amount of frozen powder was directly transfered into a 2 ml Eppendorf tube containing 200 μl extraction buffer (50 mM N-Tris-(hydroxymethyl)aminomethane/HCl pH 8, 4 mM $MgCl_2$, 2% (w/v) SDS, 100 μM PMSF and 100 mM DTT), vortexed and incubated at 100 °C for 10 min. Subsequently, the samples were centrifuged for 10 min and the supernatant transfered in a new 1.5 ml tube. Proteins were precipitated with 4 volumes of ice-cold acetone for at least 1 h. The precipitated proteins were pelleted by centrifugation for 10 min and the organic supernatant discarded. To remove salts the protein pellet was washed twice with 70% (v/v) ethanol and air-dried. Proteins were redisolved in 50 μl of 1:100 diluted extraction buffer.

Protein concentration was determined following the procedure of Bradford (1976).

30 μg of total protein was separated under denaturing conditions on a 10% (w/v) polyacrylamide gel according to Lämmli (1970). Western blotting was carried out in principal as described by Towbin *et al.* (1979). The proteins were therefore blotted onto a PVDF membrane (Immobilon P, Millipore, Eschborn, Germany) using a wet blot apparatus (TE 22, Hoefer Scientific Instruments, San Franscisco, USA). Subsequently, the membrane was probed with a D1 antibody against the D1 protein of *Bumilleriopsis filiformis* (Herrmann *et al.*, 1985). The immunosignal was revealed by chemoluminescence using the ECL Western blotting detection reagents of Amersham Pharmacia Biotech (Buckinghamshire, UK) after exposure to film (Hyperfilm ECL, Amersham, Braunschweig, Germany).

Results

Transgene expression and inheritance

The expression of the cyanobacterial *pds* transgene was investigated by RT-PCR from total RNA of the transformed seedlings. RNA of the non-transformed wild type served as control. Fig. 2 shows the resulting PCR products of transformant Pds1 and controls separated on a 1.2 (w/v)% agarose gel. Only the reverse transcription of RNA of the transgenic plant yielded the correct product of 700 bp after amplification with primers specific for the cyanobacterial *pds* gene. No RT-PCR product could be obtained using RNA of the wild type or the transgenic line ET4–208 overexpressing the *crtI*-type phytoene desaturase of *Erwinia uredovora*. Table I demonstrates the segregation pattern for kanamycin resistance used as selectable marker in the T2 generation of transformant Pds1 obtained after self-fertilization of plants from the T1 generation. The 3:1 ratio of kanamycin resist-

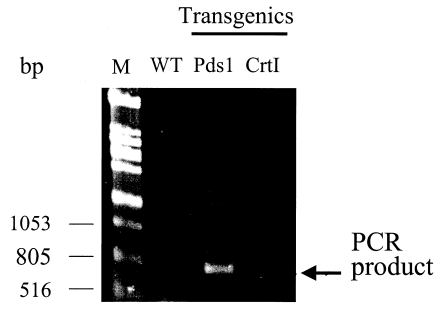

Fig. 2. Expression of the cyanobacterial transgene in tobacco as investigated by RT-PCR. 80 ng of total RNA derived from tobacco leaves of the wild type as well as transformants harboring the cyanobacterial Pds (Pds1) or the *crtI*-type phytoene desaturase (CrtI) from *Erwinia uredovora* were reverse transcribed. The resulting cDNA was subjected to PCR using primers specific for the mutated cyanobacterial transgene and the derived PCR products separated on a 1% (w/v) agarose gel as depicted.

ant to sensitive seedlings indicates a single insertion of the transgene. All subsequent experiments were carried out with heterozygous as well as homozygous plantlets of line Pds1.

Pigment content and composition in wild type and transgenic pds plants

To evaluate the impact of the additional *pds* gene on pigment content and composition pigments were extracted from transformants and corresponding wild type controls. No significant alteration in the amount of total pigments could be detected (Fig. 3). The carotenoid pattern in adult plants was only slightly modified demonstrating a small increase in the relative amount of violaxanthin (Table II). The carotenoid distribution of seedlings did not vary significantly between non-transformed controls and transgenics.

Table I. Segregation pattern of the T1 seeds of Pds transgenic line 1 on selection with kanamycin.

Seedlings	Kan Res	Kan res	Kan sens
Number	163	307	166
%	25.6	48.3	26.1

Seeds were germinated on Murashige and Skoog basal media containing 100 μg/ml kanamycin as selective marker. Kan: kanamycin, Res: resistant (homozygous), res: resistant (hemizygous), sens: sensitive.

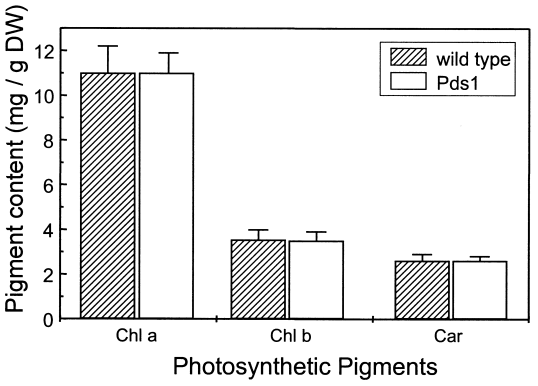

Fig. 3. Pigment content of the wild type and phytoene desaturase transformants of *Nicotiana tabacum* L. Total pigments were extracted from freeze-dried seedlings with 80% (v/v) acetone and quantified spectroscopically according to Lichtenthaler and Wellburn (1983). WT: non-transformed tobacco, Pds1: transgenic line overexpressing the overexpressing the mutated cyanobacterial phytoene desaturase of mutant NFZ 4 of *Synechococcus* PCC 7942, n= 19 for the wild type and n = 15 for the *pds* transgenics (± SD).

Table II. Carotenoid composition in the wild type and different phytoene desaturase transformants of *Nicotiana tabacum* L.

Carotenoid distribution (%)	Wild type	ET4−208	Pds1
Neoxanthin	8.5 ± 0.6	8.8 ± 0.6	10.1 ± 0.7
Violaxanthin	10.6 ± 0.04	15.6 ± 1.6	12.3 ± 0.4
Lutein	36.4 ± 0.7	32.2 ± 0.6	37.6 ± 0.1
β-Carotene	41.5 ± 0.01	43.4 ± 2.8	40.1 ± 1.1

Total pigments were extracted from freeze-dried material with 80% acetone and quantified spectroscopically according to Lichtenthaler and Wellburn (1983). After extraction of total pigments, the pigments were separated on HPLC using a Spherisorb 5 μ ODS1 column and a gradient program as described in Gilmore and Yamamoto (1991). Peak areas from the chromatogram corresponding to the individual carotenoids were integrated and the carotenoid distribution expressed in %. WT: non-transformed tobacco, Pds1: transgenic line overexpressing the overexpressing the mutated cyanobacterial phytoene desaturase of mutant NFZ 4 of *Synechococcus* PCC 7942, ET4-208: transformant overexpressing the *crtI*-type phytoene desaturase of *Erwinia uredovora.* chl: chlorophyll, car: carotenoids. $n = 4$.

Inhibitor treatments and determination of I_{50} values

Resistance to the phytoene desaturase inhibitors norflurazon and fluridone were investigated *in vivo.* For this purpose seedlings were germi-

nated in the presence of various herbicide concentrations in liquid Murashige and Skoog media as described in Materials and Methods. As expected, the amount of photosynthetic pigments decreased with increasing inhibitor concentrations. The inhibitory effect was quantified by determination of the I_{50} values for pigment synthesis. The non-transformed wild type plants showed a high sensitivity towards the bleaching herbicide norflurazon resulting in a sharp decrease in the amount of photosynthetic pigments with elevated inhibitor concentrations (Fig. 4). The I_{50} value for inhibition of the synthesis of coloured carotenoids was found to be approximately 0.052 μM. In contrast, hemizygous seedlings of the Pds1 transformant harboring the mutated cyanobacterial gene were much more resistant exhibiting I_{50} values of around 0.65 μM. For the homozygous seedlings an even higher I_{50} value of ca. 3 μM could be determined (Table III). Similar results were found when the analysis was based on chlorophyll content. In case of the Pds inhibitor fluridone a 50% inhibition of coloured carotenoids (and also a corresponding decrease in chlorophyll content) was shown to occur at an inhibitor concentration of approx. 6.6 nM in the wild type. A slightly higher tolerance towards fluridone was observed in the transgenics overexpressing the mutated cyanobacterial *pds*. An I_{50} value of approx. 19.7 nM was estimated for the homozygous progeny. No difference between the response of wild type and transgenics was observed with respect to the ζ-carotene desaturase inhibitor LS 80707 (data not shown).

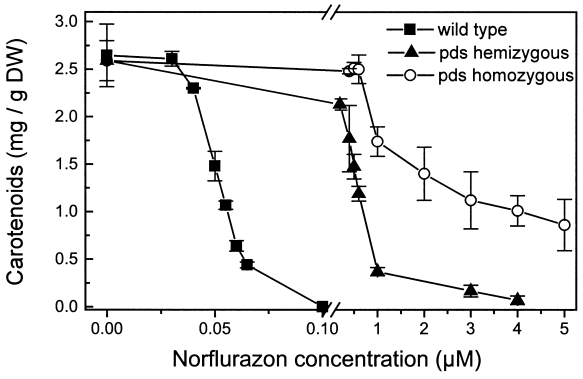

Fig. 4. Impact of different concentrations of the bleaching herbicide norflurazon on carotenoid formation in wild type and Pds transformants. Carotenoid content of wild type controls and transformants of line Pds1 were determined in dependence of increasing herbicide concentrations after extraction of total pigments with acetone and spectroscopic estimation of carotenoid content according to Lichtenthaler and Wellburn (1983). n = 3–14 independent determinations, SD = standard deviation).

Table III. I_{50} values for the inhibition of coloured carotenoids by various Pds inhibitors in different tobacco transgenics transformed with an additional phytoene desaturase.

Wild type and Transgenic lines	I_{50} value Norflurazon [M]	Fluridone [M]
Wild type	5.2×10^{-8}	6.6×10^{-9}
Hemizygous *pds* transformant	6.5×10^{-7}	9.7×10^{-9}
Homozygous *pds* transformant	3×10^{-6}	2×10^{-8}
Homozygous *crtI* transformant	$\geq 10^{-4}$	1.2×10^{-5}

Pds1: Transformant with an additional phytoene desaturase gene from mutant NFZ4 of *Synechococcus* PCC 7942; ET4-208: Transformant with an additional *crtI*-type phytoene desaturase from *Erwinia uredovora*, WT: Wild type control. I_{50} values of ET4-208 from Misawa *et al.* (1993).

Influence of different norflurazon concentrations on D1 protein level in wild type and transgenics

Photosystem II (PS II) is susceptible to light stress and known to become inhibited by high light exposure. This phenomenon is called photoinhibition (Aro *et al.*, 1993). As an integral part of PS II, the D1 protein can be degraded by light stress and is subjected to repair mechanisms. Accordingly, the amount of the D1 protein is a suitable parameter for photodamage. We have investigated the D1 protein level upon treatment with different concentrations of the bleaching herbicide norflurazon in wild type and transgenic tobacco plants. Fig. 5 shows the level of D1 protein in dependence of the norflurazon concentration. Whereas the amount of D1 protein diminishes drastically with increased inhibitor concentration and is hardly visible after treatment with 0.2 μM, this herbicide concentration did not provoke a pronounced decrease in the D1 protein level of the transformant with mutated cyanobacterial Pds.

Discussion

Carotenoid biosynthesis is one of the major targets for bleaching herbicides (Sandmann and Böger, 1994b). In particular, attention has focussed on the desaturation of phytoene by the ac-

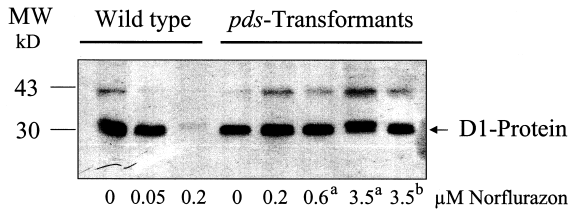

Fig. 5. Western blot analysis of the D1 protein level in Pds transformant 1 and wild type upon norflurazon treatment. 30 µg of total protein were separated on a 10% (w/v) polyacrylamide gel and subsequently blotted onto a PVDF membrane (Millipore). The membrane was probed with a polyclonal antibody against the D1 protein of *Bumilleriopsis filiformis* and the steady-state D1 protein level detected by chemoluminescence using ECL as described in Material and Methods. a: hemizygous, b: homozygous transformants.

tion of phytoene desaturases. Inhibition of this enzymatic reaction led to a decrease in coloured carotenoids and – as a secondary effect – also to a drop in chlorophyll content finally resulting in plant death due to unsufficient photoprotection. However, chemical mutagenesis and subsequent selection in the presence of bleaching herbicides resulted in the isolation of several cyanobacterial mutants resistant to norflurazon. For mutant NFZ 4 of *Synechococcus* PCC 7942 the underlying molecular basis for the enhanced tolerance has been unravelled. A point mutation in the *pds* gene was identified leading to an amino acid substitution of valine to glycine which obviously confered resistance against the bleaching herbicide norflurazon in this strain (Chamovitz *et al.*, 1991). In order to evaluate the impact of this gene for herbicide resistance in higher plants we have transformed tobacco plants with the mutated phytoene desaturase gene of NFZ 4. Furthermore, we have analyzed pigment content and composition in wild type and transgenics to check for alterations in carotenoid synthesis and accumulation.

Determination of total pigment content did not show any significant differences between the transformed tobacco overexpressing the cyanobacterial transgene and the corresponding wild type controls (Table II). Therefore, it is unlikely that phytoene desaturase is rate-limiting for carotenoid formation in higher plants as it was proposed for cyanobacteria (Chamovitz *et al.*, 1993). This assumption is corroborated by the recent finding that overexpression of the endogenous phytoene desaturase gene in tobacco did not cause any metabolic effects (Busch *et al.*, 2002). In addition, the carotenoid pattern of our tobacco transformants was investigated by HPLC. Only small changes in carotenoid distribution were found in accordance with previous analysis using the NFZ 4 mutant of *Synechococcus* PCC 7942. The tobacco transformants containing the cyanobacterial PDS had a slightly elevated relative amount of violaxanthin (Table II). A much higher increase in violaxanthin was detected in transgenic tobacco plants which were transformed with the *crtI*-type phytoene desaturase gene of *Erwinia uredovora* (Misawa *et al.*, 1993; 1994). The same phenomenon was observed in transgenic *crtI* tomato fruits where the genetic manipulation led to alterations in the expression level of endogenous carotenoid genes (Römer *et al.*, 2000). An enhancement of β-carotene and its derivatives was also detected in rice endosperm after transformation with the *crtI* gene in combination with the *Narcissus* phytoene synthase (Ye *et al.*, 2000).

Inhibitor experiments using the bleaching herbicide norflurazon demonstrated the aquisition of a remarkable resistance in the transgenics versus the non-transformed controls (Fig. 3, Table III). The I_{50} value for the synthesis of coloured carotenoids was about 0.052 µM in the wild type and 3 µM for the homozygous transgenic line Pds1 which corresponds to a resistance factor of approx. 58 in the transformants. Moreover, comparison of the resistance factors in the tobacco transgenics revealed dramatically different values for the inhibitors norflurazon and fluridone. Whereas transformation with the mutated cyanobacterial gene rendered the transgenics highly resistant (up to 58 fold) to norflurazon, the tolerance for fluridone was only increased 3 fold. This finding further substantiates the impact of the mutation in the *pds* transgene on norflurazon resistance since enhanced tolerance based on elevated protein levels should have had similar effects on both inhibitors. In contrast, the 3 fold increase in resistance to fluridone seems to be the consequence of protein overaccumulation. These results are in agreement with data obtained from investigations of the cyanobacterial mutant NFZ 4 in which also different resistance factors have been found for norflurazon and fluridone (Linden *et al.*,1990, Chamovitz *et al.*, 1993) possibly caused by distinct inhibitor binding

sites. As in the cyanobacterial mutant NFZ 4 the calculated norflurazon resistance factor of 58 in the tobacco transformants is too high to be attributed just to an increase in the total amount of phytoene desaturase enzyme due to the presence of the additional transgene with identical biochemical function but is rather the result of the mutation in the introduced gene product. This conclusion is further supported by previous determinations of Ki values for the cyanobacterial NFZ 4 mutant which was the source of the transgene for tobacco transformation (Linden *et al.*, 1990). As expected the genetic manipulation did not confer herbicide resistance to the Zds inhibitor LS80707 in accordance with studies on cyanobacteria. In contrast, transformation with the structurally and functionally distinct phytoene desaturase of *Erwinia uredovora* caused resistance to Pds and Zds inhibitors (Misawa *et al.*, 1993; 1994). In various studies the impact of carotenoid inhibitors has been previously examined on the transcriptional level showing either an up-regulation of mRNA levels or unchanged amounts of transcripts of carotenoid biosynthetic genes depending on plant species and conditions (Al-Babili *et al.* , 1999; Corona *et al.*, 1996; Guiliano *et al.,* 1993; Simkin *et al*., 2000; Wetzel and Rodermel, 1998). However, these analyses lay beyond the scope of the present investigation and will be a matter of further studies.

The enhanced tolerance to norflurazon resulted in the maintainance of higher levels of photosynthetic pigments and D1 protein in the Pds tobacco transgenics at a norflurazon concentration which led to severe bleaching in the wild type (Fig. 5). Since our experimental approach is only detecting the steady state level of the D1 protein, no conclusion can be made about the turnover of this protein.

In summary, we were able to show that transformation with the mutated cyanobacterial phytoene desaturase gene of mutant NFZ 4 from *Synechococcus* PCC 7942 could successfully confer resistance towards Pds inhibitors to higher plants. The transgenic tobacco plants exhibited a higher norflurazon resistance level (up to 58 fold) than the wild type making the transformants also less susceptible to photodamage in the presence of bleaching herbicides.

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