Cloning a Gene Coding for Norflurazon Resistance in Cyanobacteria

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The herbicide norflurazon inhibits carotene biosynthesis in photosynthetic organisms by blocking the enzyme phytoene dehydrogenase (= phytoene desaturase). We have isolated norflurazon-resistant mutants of the cyanobacterium *Synechococcus* PCC 7942. The herbicide-resistance gene from the mutant NFZ4 has been cloned by genetic complementation of the resistance trait in wild type cells. The experiment described here illustrates the usefulness of employing cyanobacteria to clone herbicide-resistance genes in a quick and simple way.

Introduction

Studies of herbicide-resistant plants have provided an insight on the mode of action of herbicides and on the biochemical and physiological properties of their target proteins. The development of new techniques for DNA-mediated transformation of plants have paved the way to the possibility for genetic engineering of herbicide resistance in crop plants. However, a prerequisite of such studies is the availability of a herbicide-resistant biotype from which it is possible to clone the resistance conferring gene.

A number of bleaching herbicides such as norflurazon, fluridone and fluorochloridone block carotenogenesis by inhibiting phytoene dehydrogenase (= phytoene desaturase) which converts phytoene into phytofluene and ζ-carotene. Treatment of plants with these herbicides result in a decrease of carotenoids and chlorophylls, and a concurrent accumulation of phytoene [1, 2]. Studying carotenogenesis in a cell-free system using daffodil chromoplasts or photosynthetic membranes from the cyanobacterium *Synechocystis,* showed that phytoene accumulation originates from a direct interference of norflurazon with the catalytic activity of phytoene dehydrogenase and not by inhibition of the biosynthesis of the enzyme [3, 4]. It has been demonstrated recently that norflurazon inhibits phytoene dehydrogenase in a non-competitive manner that involves a reversible binding to the enzyme [5].

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Molecular description of carotenogenesis in general, and of phytoene dehydrogenase in particular has been lacking. Several of the genes involved in carotenogenesis have been cloned from *Rhodobacter* [6–8], among them the gene *crt*I which encodes phytoene dehydrogenase. However, due to a large phylogenetic gap, this gene did not hybridize with DNA from higher plants (J. Hearst, personal communication).

Cyanobacteria (blue-green algae) provide an excellent system for cloning herbicide-resistance conferring genes. Their photosynthetic apparatus and metabolism are very similar to those of plants, yet their prokaryotic nature allows sophisticated genetic manipulations. In addition, herbicide-resistant mutants can be easily isolated [9]. Cloning a resistance-conferring gene from cyanobacteria and analysis of the molecular basis of the resistance are important first steps towards the study of the gene also in higher plants.

We describe here the cloning of the gene that confers norflurazon resistance in the cyanobacterium *Synechococcus* PCC 7942. Our working hypothesis was that, similar to the case of atrazine [10] and sulfonylurea [11] resistance, a point mutation in the target protein, in our case phytoene dehydrogenase, can confer herbicide resistance, while still allowing the enzyme to function.

Materials and Methods

Strains and growth conditions

Cell cultures of *Synechococcus* PCC 7942 were grown in BG 11 medium at 35 °C as described [12]. Norflurazon-resistant mutant strains were grown

as above with 4 μM norflurazon added to the growth medium. For selection of kanamycin-resistant transformants, cells were plated on solid BG 11 medium containing 1.5% agar and 10 μg/ml kanamycin.

Molecular cloning

Methods for extracting DNA from *Synechococcus* PCC 7942 and for transformation of cyanobacterial cells were according to Williams and Szalay [12]. Restriction enzyme digestion, Southern hybridization and cloning followed conventional protocols [13] except where noted below. The vector pBR 329 k was constructed as follows: a 1.2 kb *Pst*I fragment containing the aminoglycoside 3′-phosphotransferase gene from Tn 903 [14] was inserted into the *Pst*I site of pBR 329 (Fig. 1). This was necessary since ampicillin resistance is a poor selectable marker in cyanobacteria. Deletions were constructed using the Promega Erase-a-base kit. The *E. coli* strain MV 1190 was used as a host for the plasmid vectors pBR 329 K and pUC 118. Restriction and modifying enzymes were purchased from United States Biochemical Co. (Cleveland, OH).

Biochemical analysis

Procedures for carotenoid determination in *Synechococcus* PCC 7942, as well as *in vivo* and *in vitro* carotenogenesis assays have been previously described [15, 16].

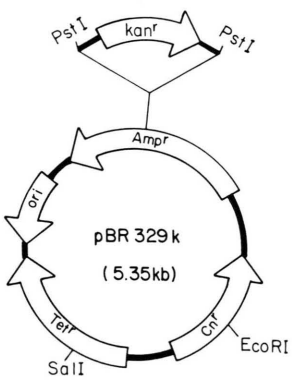

Fig. 1. Structure of plasmid pBR 329 k. See text for details.

Results

Cloning the norflurazon resistance gene

Several norflurazon-resistant mutant strains have been isolated following mutagenesis [15]. Biochemical characterization showed the resistance to be manifested at the level of phytoene dehydrogenation [15, 16]. DNA was extracted from cells of mutant NFZ4 and digested with the restriction enzyme *Eco*RI. The cleaved DNA was transfected into cells of the wild type (wt) strain. Homologous recombination, involving a double crossing-over event between the transfected DNA and the cyanobacterial chromosome, could result in a particular exchange that replaces the sequence in the chromosome with the herbicide resistant mutation [17]. Norflurazon-resistant colonies have appeared following selection on plates containing 1.0 μM norflurazon at the estimated frequency of 10^{-5}. The recovery of the norflurazon-resistant transformants indicates that the resistance in NFZ4 is a genetic trait that is most probably encoded by a single gene.

In order to clone this gene, a genomic DNA library of mutant NFZ4 was constructed in the *Eco*RI site of the *E. coli* plasmid pBR 329 k. The library was amplified in *E. coli* and was transfected in the form of closed circular plasmids into wild type cells of *Synechococcus* PCC 7942. Transformants were selected for growth on medium containing kanamycin and norflurazon. The double-resistance phenotype acquired by wt cells is a result of a stable transformation of the cells with both the kanamycin and norflurazon-resistance genes. Since pBR 329 k is unable to replicate autonomously in cyanobacteria, the only way that these two genes could be maintained in the transformant was by integration into the cyanobacterial genome by a single crossing-over event between the cyanobacterial DNA insert in the recombinant pBR 329 k and its homologous sequence in the cyanobacterial chromosome. As a result, the entire plasmid was integrated into the genome, creating a merodiploid for the segment it carried (Fig. 2).

DNA was extracted from one of the partial diploid strains and cut with the restriction enzyme *Sal*I. The *Sal*I resulting fragments were then self-ligated and used to transfect *E. coli* cells. The transformants, which were selected for growth on kanamycin-containing LB medium, contained a

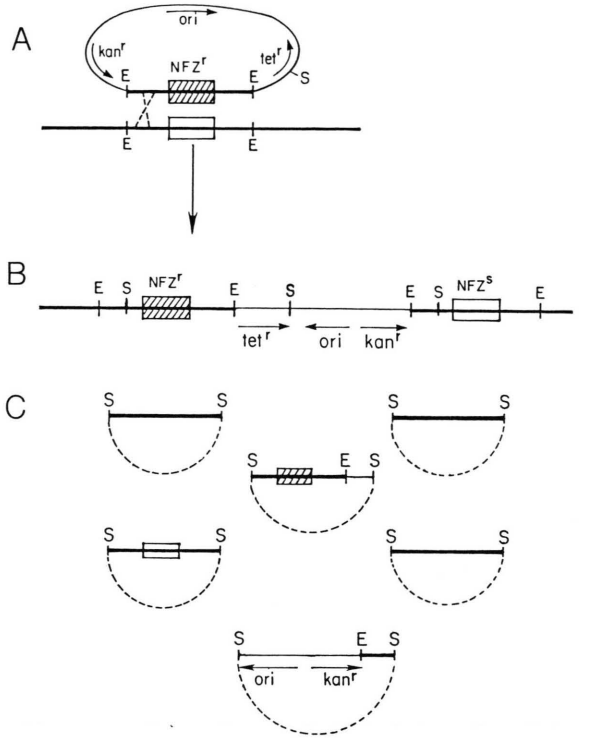

Fig. 2. Strategy used to clone the norflurazon-resistance (NFZ^r) gene. A genomic library from herbicide-resistant mutant NFZ4 in the *Eco*RI site of pBR 329k was transfected to wild type cells. A single crossover event between the transfected plasmid and the cyanobacterial chromosome (A) lead to its integration into the cyanobacterial genome next to the homologous sequence of the insert (B). A kanamycin-resistant (kan^r) and NFZ^r transformant was selected. "Self ligation" of the *Sal*I fragments of the transformant DNA, followed by transformation to *E. coli*, "rescued" the plasmid along with a portion of the original insert. (E-*Eco*RI; S-*Sal*I; ori-origin of DNA replication in *E. coli*).

portion of the pBR 329k vector along with part of the original *Eco*RI fragment (Fig. 2). This plasmid was designated pPD6.

The *Sal*I–*Eco*RI 800bp insert in pPD6 was used as a probe to screen the original NFZ4 genomic library for the plasmid clone containing the complete norflurazon-resistance conferring insert. This plasmid, pPD2, contained a 10 kb *Eco*RI insert that was able to transfer norflurazon resistance when used to transfect wild type *Synechococcus* PCC7942 cells. The transformed strain was designated PD2.

Localization of the norflurazon-resistance mutation

A restriction map of the 10 kb insert of pPD2 was constructed (Fig. 3). In order to localize the mutation that confers norflurazon resistance, each of the restriction fragments shown in Fig. 3 was checked for its ability to transfer norflurazon resistance *via* DNA transformation. The *Sal*I–*Bam*HI 2.8 kb fragment was found positive and was further analyzed to localize the mutation (Fig. 3).

Analysis of phytoene dehydrogenase from PD2

Similar to NFZ4, norflurazon resistance in PD2 was found to be manifested, both *in vivo* and *in*

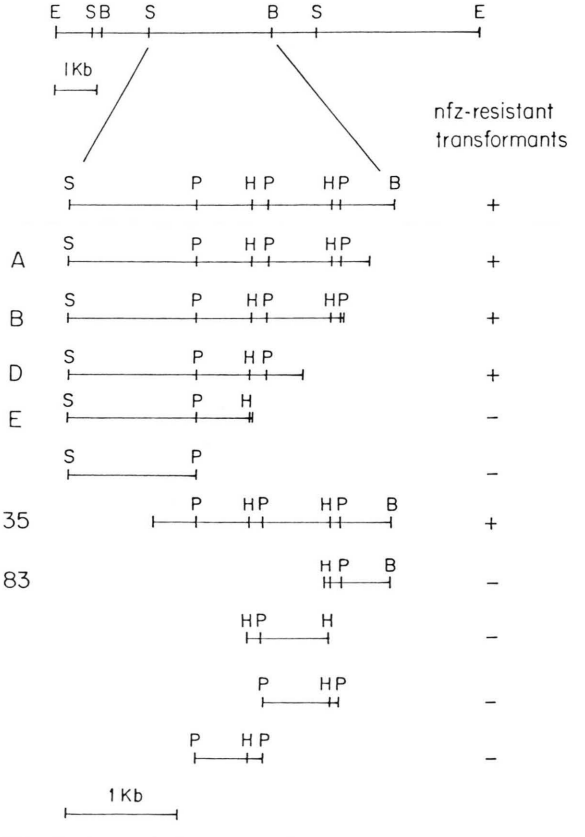

Fig. 3. A restriction map of the 10 kb *Eco*RI fragment cloned from the genome of *Synechococcus* PCC7942 in the plasmid pPD2. Mapping the mutation of norflurazon resistance (nfz^r) on the *Bam*HI–*Sal*I DNA fragment was done by deletions that were constructed and transfected to wild type cells of *Synechococcus* PCC7942. Transformation to nfz^r (+) was tested. (E-*Eco*RI; H-*Hind*III; P-*Pst*I; S-*Sal*I; different deletions are marked A, B, D, E, 35 and 83).

vitro, at the level of phytoene dehydrogenase in NFZ4 (Table I).

Table I. Factors of resistance and *in vitro* activity in *Synechococcus* PCC 7942 wild type and resistant strain PD2.

Strain	*in vivo* I_{50}	RF	*in vitro* k_i	RF	Conversion rate [%]
WT	0.11	–	0.09	–	76
PD2	1.22	12.2	1.37	15.2	37

RF: Resistance factor is the ratio of mutant I_{50} or k_i *versus* wild type values.
The conversion rate was determined as radioactivity from ^{14}C-geranylgeranyl pyrophosphate incorporated into ζ-+β-carotene relative to the radioactivity in phytoene + ζ-+β-carotene.

Measurement of the efficiency of the phytoene dehydrogenation reaction in the cell-free system revealed a drop of 41% in PD2 relative to wild type (Table I). Phenotypically, this decrease in phytoene dehydrogenase activity is manifested also in carotenoid composition (Fig. 4). In wild type cells, β-carotene makes up slightly over one quarter of all carotenoids, with zeaxanthin being the most abundant (62%). In PD2, there is an 18% drop in β-carotene content, but a large drop of 67% in the amount of xanthophylls. Consequently, β-carotene in PD2 makes up close to half (48%) of all carotenoids.

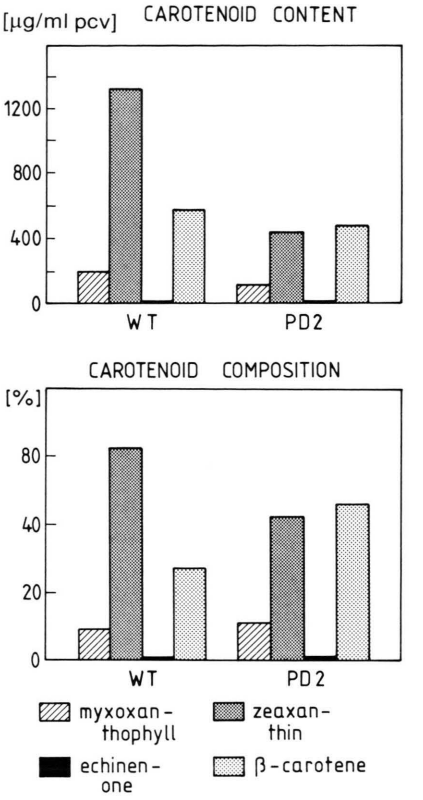

Fig. 4. Carotenoid and xanthophyll content and composition in *Synechococcus* PCC7942 wild type and PD2 strains. In contrast to WT, PD2 accumulated 15-*cis* phytoene up to 20% of total colored carotenoids.

Discussion

We describe here the cloning of a DNA fragment from a norflurazon-resistant mutant of *Synechocossus* PCC7942 that confers norflurazon resistance upon transfection to wild type cyanobacteria. Several lines of evidence indicate that this fragment may encode the gene for the enzyme phytoene dehydrogenase. As has previously been shown, the resistance to norflurazon in the original mutant strain NFZ4 is manifested both *in vivo* and *in vitro* at the level of phytoene dehydrogenase [15]. A similar pattern of resistance is found also for PD2, the strain genetically engineered to herbicide resistance with the gene cloned from NFZ4.

Furthermore, norflurazon resistance is accompanied by a drop in the activity of phytoene dehydrogenase. The enzyme in PD2 is 41% less efficient than that of the wild type in converting phytoene to ζ-+β-carotene. This can be explained if the mutation that confers resistance to nurflurazon occurred in the gene for phytoene dehydrogenase, and this alteration also interferes with the enzymatic activity. Since PD2 is completely isogenic with the wild type strain, except for the mutation for herbicide resistance, it is probable that the mutation has occurred in the gene encoding phytoene dehydrogenase.

The reduced efficiency of phytoene dehydrogenase results also in an altered pattern of carotenoid composition. While the amount of β-carotene is only slightly lowered in PD2, there is a significant reduction in the amount of xanthophylls. The xanthophylls are biosynthetic products of β-carotene. Apparently, a basic concentration of β-carotene is necessary for cell growth, while xanthophyll concentration is less critical. It seems that a mecha-

nism exists in *Synechococcus* PCC 7942 that limits xanthophyll synthesis under conditions of limited β-carotene supply.

Cyanobacterial cells can be transformed to norflurazon resistance by two mechanisms: 1. Integration of an additional fragment, containing the herbicide-resistance mutation, as a result of a single crossing-over event between a plasmid and the chromosome; 2. By a gene replacement due to a double crossing-over event between a DNA fragment and its homologous sequence in the chromosome. Only in the first case is it possible to reisolate the plasmid by the plasmid rescue technique. When linear DNA is transfected to cyanobacteria, transformation must occur *via* the double cross-over mechanism. This was the case when deletions of the *Sal*I–*Bam*HI fragment were used (Fig. 3). In contrast to DNA fragment D, fragment E failed to transfer NFZ^r. The region that was deleted from fragment D to give fragment E defines the location of the mutation. However, the internal *Hind*III–*Hind*III fragment, that contains that entire region, did not confere resistance when transfected to wt cells. This result is anticipated assuming that the double cross-over mechanism necessitates a several hundred base pairs region surrounding the mutation on each side in the transforming fragment [17]. When the mutation is located too close to the end of the transforming DNA fragment, it can no longer be transferred to the chromosome by crossing-over of DNA molecules. The deletion analysis, together with the inability of the internal *Pst*I and *Hind*III fragments to transform wt cells to norflurazon-resistance places the mutation in the region of the first *Hind*III–*Pst*I junction.

While the biochemical data indicates that the norflurazon-resistance gene encodes phytoene dehydrogenase, the possibility cannot be ruled out that norflurazon acts through an as yet unidentified protein associated with the carotenogenesis complex. Molecular biological studies, including the sequencing of the gene, will allow an accurate identification.

[1] P. G. Bartels and C. McCullough, Biochem. Biophys. Res. Commun. **48,** 16 (1972).
[2] G. Sandmann and P. Böger, in: Target Sites of Herbicides of Herbicide Action (G. Sandmann and P. Böger, eds.), pp. 25–44, CRC Press, Boca Raton, Fl. 1989.
[3] P. Beyer, K. Kreuz, and H. Kleinig, Planta **150,** 435 (1980).
[4] I. E. Clarke, G. Sandmann, P. M. Bramley, and P. Böger, FEBS Lett. **140,** 203 (1982).
[5] G. Sandmann, H. Linden, and P. Böger, Z. Naturforsch. **44c,** 787 (1989).
[6] J. M. Pemberton and C. M. Harding, Curr. Microbiol. **14,** 25–29 (1986).
[7] G. Giuliano, D. Pollock, and P. A. Scolnik, J. Biol. Chem. **261** (28), 12925–12929 (1986).
[8] G. A. Armstrong, M. Alberti, F. Leach, and J. E. Hearst, Mol. Gen. Genet. **216,** 254–268 (1989).
[9] J. Hirschberg, N. Ohad, I. Pecker, and A. Rahat, Z. Naturforsch. **42c,** 102–112 (1987).
[10] J. Hirschberg, A. Ben Yehuda, I. Pecker, and N. Ohad, in: Plant Molecular Biology (D. von Wettstein and N. H. Chua, eds.), pp. 357–366, Plenum Publ., New York 1987.
[11] K. Y. Lee, J. Townsed, J. Tepperman, M. M. Black *et al.*, EMBO J. **7,** 1241–1248 (1988).
[12] J. G. K. Williams and A. A. Szalay, Gene **24,** 37 (1983).
[13] T. Maniatis, E. F. Fritsch, and J. Sambrook, Molecular Cloning a Laboratory Manual, Cold Spring Harbor Laboratory 1982.
[14] A. Oka *et al.*, J. Mol. Biol. **147,** 217 (1981).
[15] H. Linden, G. Sandmann, D. Chamovitz, J. Hirschberg, and P. Böger, Pestic. Biochem. Physiol. **36,** 46–51 (1990).
[16] G. Sandmann, Methods Enzymol. **167,** 329 (1988).
[17] S. S. Golden, Methods Enzymol. **167,** 714 (1988).