A New psbA Mutation Yielding an Amino-Acid Exchange at the Lumen-Exposed Site of the D1 Protein

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In eight metribuzin-resistant photoautotrophic cell cultures of *Chenopodium rubrum* (Thiemann and Barz, 1994 a, b) sequence analyses of a part of the psbA gene coding for the photosystem-II D1 protein had revealed different double and triple mutations within the herbicide binding niche of the protein (Schwenger-Erger *et al.,* 1993). Two pairs of the examined cell lines carried identical mutations within this part of the protein, although their growth performance and their herbicide resistance patterns were different, both at the cellular and the thylakoid level. Starting from the known part of the psbA gene we have amplified the remaining psbA sequences using inverse polymerase chain reaction. Thus the complete sequence of the coding part of the gene was elucidated. After sequence analyses we found an additional amino acid exchange at the position 184 (ile → asn) of the D1 protein in cell line L1. Metabolic consequences of this mutation are discussed. Partial sequence analyses of the psbD gene of the herbicide resistant cell culture lines revealed no mutation within that part of the D2 protein, which is in direct contact with the D1 protein.

Introduction

In previous studies eight metribuzin-resistant photoautotrophic cell cultures of *Chenopodium rubrum* were established via multiple step selection procedure and characterized for their photosynthetic and growth performance (Thiemann and Barz, 1994a, b). Metribuzin inhibits the photosynthetic electron transport by displacing the secondary electron acceptor Q_B from its binding niche at the D1 protein of photosystem II (PSII). Since most of the commonly observed PS II herbicide resistances are based upon an altered D1 protein of photosystem II and because [^{14}C]-metribuzin-binding to isolated thylakoids had been shown to be reduced within the resistant cell lines as compared to the wildtype cells (Thiemann and Barz, 1993), sequence analyses of a part of the psbA gene were carried out. The DNA part investigated codes for the amino acids 209–291 containing the Q_B- and herbicide binding niche of the D1 protein. This region is highly conserved and shows 72% identity in all PSII-containing species characterized thus far (Svensson *et al.,* 1993). Our results revealed different double and triple mutations of the D1 protein in the photoautotrophic resistant cell lines (Thiemann and Barz, 1994a; Schwenger-Erger *et al.,* 1993).

Two pairs of cell lines, L1 and L8 as well as L4 and L7 carried the same mutations in the examined part of the psbA gene, although their growth behaviour and – more important – their herbicide resistance patterns were quite different, both at the level of cells and of isolated thylakoids (Thiemann and Barz, 1994a, b; Schwenger-Erger *et al.,* 1993). Whereas the amino acid 219 val was replaced by ile in the D1 protein of all four mbz-resistant cell lines the amino acid residue 251 ala was replaced by thr within the cell lines L4 and L7 and by val in case of the cell lines L1 and L8. The main mutation site within the psbA gene of higher plants leading to resistance against triazine herbicides, was determined within the region between the transmembrane helices IV and V of the D1 protein at position 264, where serine is replaced by glycine (Hirschberg and McIntosh, 1983; Reith and Strauss, 1987; Barros and Dyer, 1988) or threonine (Sato *et al.,* 1989). In algae mutations in positions 219, 251, 255, 256 and 275 are also known,

Abbreviations: Mbz, metribuzin (4-amino-6-*t*-butyl-4,5-dihydro-3-methylthio-1,2,4-triazin-5-one); QA, QB, primary and secondary electron acceptor of photosystem II; kDa, kilodalton; PS II, photosystem II.

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which confer resistance to several classes of herbicides (Erickson *et al.*, 1985; Johanningmeier *et al.*, 1987; Wildner *et al.*, 1990). From these results we concluded that further mutations either within the remaining part of the psbA gene, in the genes for other neighbouring proteins interacting with the herbicide binding site or in the lipid environment of the D1 protein might exist in one or several of these four cell lines.

Investigations on the lipid composition of membranes of cell lines L4, L7 and wild type cells revealed some characteristic differences between the wildtype cells on one hand and the higher resistant cell line L4 and the lower resistant line L7 on the other. These differences were detected in the thylakoid lipids and to a higher degree even within the cellular lipids (C. Schwenger-Erger, N. Weber, W. Barz, unpubl. results).

In addition to the lipid analyses we continued our efforts to find further mutations within the hitherto not investigated part of the psbA gene. In this report we describe our results to amplify these remaining sequences of the chloroplast encoded psbA gene. The chloroplasts contain a DNA of appr. 120–160 kb, on average, which encodes for organellar tRNAs, rRNAs and about 60–90 plastidic proteins (Ohyama *et al.*, 1986; Shinozaki *et al.*, 1986; Hiratsuka *et al.*, 1989; Wolfe *et al.*, 1992). In consideration of the relatively small plastidic genome we choose the method of inverse polymerase chain reaction (Triglia *et al.*, 1988; Ochman *et al.*, 1988; Silver and Keerikatte, 1989), because we assumed that the probability to obtain a satisfactory yield of a circular template containing the remaining psbA gene sequences, generated via restriction of cpDNA and subsequent ligation, would be sufficiently high. Sequence analyses of the finally amplified cpDNA fragments indeed showed the presence of a new mutation site.

Material and Methods

Cell cultures

The photoautotrophic *Chenopodium rubrum* cell cultures were established as described by Thiemann and Barz (Thiemann and Barz, 1994a). Cultivation and harvest of the cells were performed as described earlier (Schwenger-Erger *et al.*, 1993).

DNA preparation

DNA enriched in cpDNA was isolated from lyophilized thylakoids prepared according to Nelson *et al.* (Nelson *et al.*, 1970). Analogous to the method described by Doyle and Doyle (Doyle and Doyle, 1990) 50 mg of lyophilized thylakoids were resuspended in prewarmed extraction buffer (2% (w/v) cetyltrimethylammoniumbromide, 1.4 M NaCl, 0.2% (v/v) β-mercaptoethanol 20 mM EDTA, 100 mM Tris-(hydroxymethyl)-aminomethan-HCl, pH 8.0) and incubated for 45 min at 63 °C. After protein extraction with chloroform: isoamylalcohol (24:1, v/v) the DNA was precipitated with 70% isopropanol for 2 hours and then pelleted by centrifugation (5 min, 10,000×*g*). The pellet was washed with 1 ml of washing buffer (76% ethanol, 100 mM ammoniumacetate), dried and resuspended in 20 µl aqua bidest.

Amplification of a psbA gene fragment using inverse polymerase chain reaction (IPCR)

Restriction and ligation of cpDNA

cpDNA was restricted with Eco RI (5 µg cpDNA in 18.5 µl aqua dest., 1.5 µl "one forall" buffer (Pharmacia), 5 U Eco RI) at 37 °C for about 4 hours. After inactivation of the restriction enzyme (10 min, 65 °C) T4-polynucleotide-ligase-mix (Pharmacia) was added to obtain a sufficient amount of circular fragments of cpDNA, which arise as a by-product of the ligase reaction and which may serve as a template for the amplification of border sequences starting from an internal gene segment.

Polymerase chain reaction

PCR was carried out with two oligonucleotides constructed from the known part of the psbA gene. Oligo 1(5'-ccgccgaatacaccagctac-3') is homologous to bases 604 to 623 of the coding strand of the gene, oligo 2 (5'-actatggctttcaacttaaacgg-3') is homologous to bases 874 to 896 of the non-coding strand.

The reaction mixture contained 8.25 µl aqua bidest, 5 µl reaction buffer (Pharmacia),1 µl primer solution (oligo 1 + oligo 2, 100 pmol each), 0.5 µl dNTP solution (dATP, dCTP, dGTP, dTTP, 2 mmol, respectively), 5 µl restricted and ligated

cpDNA and 0.25 µl Taq-polymerase (5 U/µl, Pharmacia).

The PCR was carried out in a Perkin Elmer cycler 2400 and the conditions were kept as follows: after a primary denaturation step (5 min, 90 °C) 35 cycles were performed with denaturation for 15 sec at 90 °C, annealing for 15 sec at 50 °C and elongation for 30 sec at 72 °C. A terminal elongation phase was run for 5 min at 72 °C.

Identification and isolation of the PCR fragment

The PCR reaction products were separated via horizontal agarose gel electrophoresis (1% agarose in 0.5 × TBE buffer; running buffer: 0.5 × TBE buffer; electrophoretic conditions: 100 mV, 2 h; ethidiumbromide-stained). To prove whether the obtained PCR-products contain psbA gene sequences the agarose gels were blotted according to the protocol of Chomczynski (Chomczynski, 1992) and nitrocellulose filters were hybridized with a homologous psbA gene probe directed at the 3'-terminus of the psbA gene.

Bands at 2,700 bp were eluted from the agarose gels using the jetsorb extraction kit (Genomed).

Transformation

Transformation of PCR products and selection of transformants were carried out with the pMOSBlue T-vector kit (Amersham) according to the instructions of the manufacturer.

Sequencing

DNA sequencing was performed according to the chain-termination-method of Sanger (Sanger *et al.*, 1977) using ^{35}S-dATP as the labelling compound together with the T7-sequencing kit (Pharmacia). At least two transformants of two different IPCR experiments were sequenced for each cell line.

Amplification of a psbD gene fragment

PCR was carried out with two oligonucleotides constructed from the known sequence of the psbD gene of spinach (Holschuh *et al.*, 1984). Oligo psbD1(5'-gctgcttggcctgtagtagg-3') is homologous to bases 828 to 847 of the coding strand of the gene, oligo psbD2 (5'-ccaaggaaccatgcatagcac-3') is homologous to bases 635 to 656 of the non-coding strand.

The reaction mixture contained 8.25 µl aqua bidest, 5 µl reaction buffer (Pharmacia),1 µl primer solution (oligo 1 + oligo 2, 100 pmol each), 0.5 µl dNTP solution (dATP, dCTP, dGTP, dTTP, 2 mmol, respectively), 50 ng cpDNA in 5 µl aqua bidest. and 0.25 µl Taq-polymerase (5 U/µl, Pharmacia).

The PCR was performed as follows: after a primary denaturation step (5 min, 90 °C) 35 cycles were performed with denaturation for 15 sec at 90 °C, annealing for 15 sec at 54 °C and elongation for 30 sec at 72 °C. A terminal elongation phase was run for 5 min at 72 °C.

Isolation and transformation of the obtained 212bp fragments was performed as described above. Identification of the fragments was carried out by sequencing and subsequent sequence alignments.

Results and Discussion

Using the method of "inverse polymerase chain reaction" we have now amplified those parts of the psbA gene which had not been investigated in our earlier studies (Schwenger-Erger *et al.*, 1993). Restriction of plastidic DNA, subsequent (self)ligation of the obtained fragments and finally an amplification step using two oligonucleotides constructed from the known part of the gene (see Materials and Methods) were successfully performed. Eco RI was selected as restriction enzyme for the primary DNA-degradation, because no cleavage site for this enzyme exists within the psbA gene of *Spinacia oleracea* and *Nicotiana debneyi* (Zurawski *et al.*, 1982). Fig. 1 shows the separated PCR reaction products obtained from the cpDNA of the resistant cell lines L1, L4, L7 and L8 of *Chenopodium rubrum*, which contain psbA gene sequences upstream of bp 623 and downstream of bp 874. The main product was found at about 2,700 bp for each cell line and Southern analyses (hybridization with a probe directed at the 3'-terminus of the psbA gene) verified that this band contains psbA gene sequences (data not shown). In some PCR runs we found small portions of by-products in the low molecular weight range, which failed to hybridize with the Southern probe. The same 2,700 bp PCR fragment was obtained with

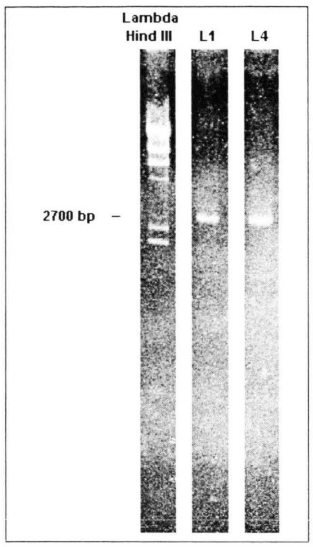

Fig. 1. Polaroid photo of the inverse polymerase chain reaction products of L1, L4 obtained with plastidic DNA-fragments of *Chenopodium rubrum* cell lines L1, L4, L7 and L8 separated by horizontal agarose gel electrophoresis (1% agarose in 0.5 × TBE buffer; running buffer: 0.5 × TBE buffer; running conditions: 100 mV, 2h; ethidiumbromide-stained).

plastidary DNA of wildtype cells. It should be emphasized that at least in case of a relatively small genome the method of inverse polymerase chain reaction is quite suitable to amplify borders of a gene from which only internal sequence information is available.

Sequence analyses of the cloned PCR products, together with the previously sequenced part of the gene (Schwenger-Erger *et al.*, 1993), resulted in the whole coding sequence of the psbA gene for the wildtype cells (accession number: Y 14732) and the four resistant cell lines L1, L4, L7 and L8 of *Chenopodium rubrum*. At present psbA gene sequences are available from approximately 20 higher plant species (Svensson *et al.*, 1991). At the level of the nucleotide sequence the *Chenopodium rubrum* psbA gene shows 98% homology to the spinach sequence (Zurawski *et al.*, 1982) and at the level of the amino acid sequence the D1 protein of *Chenopodium rubrum* shows 100% homology to the D1 protein of *Spinacia oleracea*, *Nicotiana debneyi* (Zurawski *et al.*, 1982), *Nicotiana tabacum* (Sugita and Sugiura, 1984), *Solanum nigrum* (Goloubinoff *et al.*, 1984), *Nicotiana plumbaginifolia* (Pay *et al.*, 1988) and *Gossypium hirsutum* (Ulmasov *et al.*, 1990).

In case of the *Chenopodium rubrum* cell line L1 we found an additional mutation at the amino acid position 184 of the D1 protein, where ile is replaced by asn (Table I). However, in the cell lines L4, L7 and L8 no additional mutations were detected in the D1 protein. Table I summarizes the results of our sequence analyses of the psbA gene of the four metribuzin-resistant cell lines L1, L4, L7 and L8 in comparison to the wildtype cells.

Since the amino acid 184 is located within the lumenal helix arranged parallel to the thylakoid membrane it is not in direct contact with the region of herbicide and Q_B-binding (Trebst, 1987). Parts of the lumen-exposed sequence connecting the helices III and IV of the D1 protein are thought to be involved in forming the manganese cluster or stabilizing O_2-production and especially asn 170 is thought to be directly involved in manganese coordination (Nixon and Diner, 1992). At present a direct contribution of amino acid 184 to these phenomena cannot be decided. In view of our data future work should be devoted to this aspect.

Sequence analyses of the psbD gene between bp 635 and 828, coding for the region between the helices D and E of the D2 protein, revealed identical sequences for the resistant and susceptible cultivars of *Chenopodium rubrum* (data not shown). Therefore, an influence of an altered D2 protein on the herbicide binding and thereby on

Table I. Mutation sites of the psbA gene of the four metribuzin-resistant cell lines L1, L4, L7 and L8 of *Chenopodium rubrum* in comparison to the wildtype (WT) (compare Schwenger-Erger *et al.*, 1993).

Cell Line	Codon 184	Codon 219	Codon 251
WT	ATT lie	GTA val	GCT Ala
L1	AAT asn	ATA ile	GTT val
L4		ATA ile	ACT thr
L7		ATA ile	ACT thr
L8		ATA ile	GTT Val

the resistance patterns of the cell lines could be excluded.

Finally, the above mentioned differences in herbicide resistance and growth performance (Thiemann and Barz, 1994a, b; Schwenger-Erger *et al.*, 1993) between the four selected cell lines can most likely not be explained by action of the D1 protein.

In view of our data on the D1 and D2 proteins we tend to consider the observed differences in the lipid composition (C. Schwenger-Erger, N. Weber, W. Barz, unpubl. results) as the main cause for different properties of the herbicide resistant cell lines L1, L4, L7 and L8 of *Chenopodium rubrum*.

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