A Rapid Method for Partial mRNA and DNA Sequence Analysis of the Photosystem II *psb*A Gene

Dirk Naber, Udo Johanningmeier, and Jack J. S. van Rensen

Laboratory of Plant Physiological Research, Agricultural University, Gen. Foulkesweg 72, 6703 BW Wageningen, The Netherlands (D. N. and J. J. S. v. R.) and Lehrstuhl Biochemie der Pflanzen, Ruhr-Universität, Postfach 102148, D-4630 Bochum 1, Bundesrepublik Deutschland

Z. Naturforsch. **45c,** 418–422 (1990); received November 3, 1989

Triazine Resistance, Sequence Analysis, Polymerase Chain Reaction, *Chenopodium album*

Single amino acid substitutions in the D1 protein of photosystem II may cause resistance to various herbicides. In all organisms studied these substitutions are located in or between helices IV and V of the protein. The increasing number of herbicide-resistant organisms necessitates development of a rapid methodology to characterize deviations from the wildtype sequence. Here, two procedures are described to identify mutations in the *psb*A gene, which is coding for D1. These procedures involve the isolation and amplification of DNA and RNA and subsequent sequencing reactions without the need to clone the *psb*A gene. A triazine-resistant and a -susceptible biotype of *Chenopodium album* were used as model species. An A to G transition, giving rise to a serine to glycine mutation at position 264 in the D1 protein, is found in the resistant plant.

Introduction

From many higher plants, triazine-resistant biotypes have been found. In algae, cyanobacteria and photosynthetic bacteria mutants resistant to various herbicides have been induced. The resistance is caused by a decreased binding affinity of the herbicide to the target protein, the D1 (32 kDa, herbicide binding) protein of photosystem II (PS II). This prevents the herbicide from displacing the secondary quinone electron acceptor Q_B from its binding site, thus assuring an uninterrupted electron transport from PS II to the quinone pool in the presence of the herbicide. In several weed species, triazine- or DCMU-resistance was found to be related to a single amino acid substitution at position 264 in D1 [1–6]. The part of the protein containing the substituted amino acid residue is located towards the outside of the thylakoid membrane between transmembrane helices IV and V [7]. It provides the binding sites for the PS II electron acceptor Q_B, as well as for many different classes of PS II herbicides.

The D1 protein is encoded by the *psb*A gene. This gene is highly conserved in plants, algae and cyanobacteria. It is located on the circular chloroplast genome [8, 9]. This molecule exists in up to several hundreds of identical copies per chloroplast, comprising about 1% of total plant DNA. The complete chloroplast DNA sequences were determined in several higher plants [10, 11], whereas *psb*A sequences are available from many organisms (see *e.g.* [1–6, 12–15]). The D1 protein is one of the most abundant chloroplast proteins, with a high rate of turnover in the light [9, 16]. Like its product, *psb*A mRNA is present in large amounts, especially in plants grown under high-light intensity [17].

The analysis of herbicide-resistant mutants has become an important tool for the understanding of herbicide-protein interactions. The increasing number of genetically transformed organisms requires a fast method to determine mutations. Two such methods are described here using triazine-resistant and -susceptible *Chenopodium album* as a model plant. The first method described involves direct DNA sequencing of isolated total plant DNA, using the Sanger dideoxy chain termination method [18]. The second method can be used for mRNA sequence analysis. This procedure has already been applied to *Chlamydomonas* and

Abbreviations: CTAB, cetyl-trimethyl-ammonium-bromide; D1, PS II reaction center protein carrying acceptor Q_B; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PCR, polymerase chain reaction; PS II, photosystem II; Q_B, secondary quinone electron acceptor of PS II.

Reprint requests to Dr. J. J. S. van Rensen.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/90/0500–0418 $ 01.30/0

*Euglena psb*A mRNAs [23]. The method proved to work in higher plants as well.

In both procedures, the polymerase chain reaction (PCR) can be used to amplify the *psb*A DNA or make sufficient amounts of cDNA (*via* a single reverse transcriptase reaction) from mRNA. In this case, only very low amounts of plant material are required.

Materials and Methods

Plant material

Leaves were collected from wildtype and a triazine-resistant biotype of common lambsquarters (*Chenopodium album* L.). Isolated thylakoids from the resistant biotype showed a 1000-fold decrease in sensitivity to triazine compounds as compared to the wildtype. The measured pI_{50} of the mutant is 4–4.5, that of the wildtype 7.5. Cross-resistances of this mutant were described before [19].

Nucleic acid isolation

Plant DNA and RNA were isolated simultaneously by a modification of the cetyl-trimethyl-ammonium-bromide (CTAB) nucleic acid precipitation method as described by Taylor and Powell [20].

Leaf material (10 g) was frozen in liquid nitrogen and ground in a cooled mortar. Immediately 2% (w/v) of β-mercaptoethanol and 10 ml boiling extraction buffer (2% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA and 100 mM Tris-HCl, pH 8) were added. After cooling down to 50 °C, the mixture was extracted with chloroform/iso-amylalcohol (24/1). The aqueous phase was collected. Then 2 ml 10% (w/v) CTAB/0.7 M NaCl was added and the extraction repeated. The upper phase was precipitated with 10 ml precipitation buffer (1% (w/v) CTAB, 10 mM EDTA, 50 mM Tris-HCl, pH 8 and, freshly added 1% β-mercaptoethanol). After 30 min the precipitated nucleic acids were collected by centrifugation. The pellet was resuspended in 20 ml solution containing 1 M CsCl, 50 mM NaCl, 5 mM EDTA and 50 mM Tris-HCl, pH 8 and carefully loaded on a cushion of 5.7 M CsCl, 50 mM NaCl, 5 mM EDTA and 50 mM Tris-HCl, pH 8. After centrifugation for 15 h at 107,000 × *g* RNA was pelleted, while DNA banded around the interface. The nucleic acids were further purified by 2 ethanol precipitations and resuspended in 1 mM EDTA, 10 mM Tris-HCl, pH 8. Gel electrophoresis, blotting and hybridization were performed according to standard procedures [21].

DNA amplification

For DNA sequence analyses the PCR technique [22] was used to sequence DNA from a very small amount (less then 1 µg) of total DNA. Two oligonucleotides, synthesized on an Applied Biosystems model 381A DNA synthesizer, of 23 and 20 nucleotides length were used (5′ $CC^{A}/_{G}TTTA^{A}/_{G}GTTGAAAGCCATAGT$ and 5′ $GT^{A}/_{T}GCTGGTGTATTCGG^{T}/_{C}GG$). The first one hybridizes to bases 874–896 of the RNA-like strand, the other to bases 604–623 of the coding strand [12]. Sequences were chosen to match most known *psb*A genes from higher plants and *Chlamydomonas*. Amplification of the 293-base fragment was performed in 100 µl buffer containing 20 nmol of each primer, 0.1 mM of each of the 4 deoxynucleotides, 0.05% (v/v) Nonidet P40, 0.05% (v/v) Tween-20, 3 mM $MgCl_2$ and 10 mM Tris-HCl, pH 8.0. About 2–3 units of Taq-polymerase (BRL) were used for each amplification. The number of amplification cycles was 40, with temperatures of 40 °C (annealing, 1 min), 55 °C (chain elongation, 3 min) and 92 °C (denaturation, 1 min). After completion of the reactions, samples were allowed to stand at room temperature for 10 min, in order to assure renaturation of single-strand DNA.

Sequence analysis

The RNA sequencing reactions were performed as described [23] using 2 µl AMV reverse transcriptase (15 u/µl). For DNA sequence analysis about 25 µg of total nucleic acid or 0.1 µg of amplified fragment was used as a template for standard sequencing reactions. In both types of reactions 5×10^6 cpm 5′-[^{32}P]-labeled synthetic primer (5′ $GG^{A}/_{T}GT^{G}/_{A}AACCAAATACC$) was used. It is complementary to the mRNA in the region from bases 744 to 761.

Using a third oligonucleotide for sequencing a PCR fragment avoids the need to remove excess of amplification primers, which is necessary when either of these is to be used in the sequencing reactions. Fragments were separated on 8% polyacryl-

amide-urea sequencing gels, electrophoresed at 70 W. After running, gels were exposed to Kodak X-Omat AR X-ray film using an intensifying screen at −80 °C.

Results and Discussion

The binding environment on the D 1 protein for many herbicides is the region between the membrane spanning helices IV and V [7]. This part of the protein, located between amino acid residues 211 and 275, is the region where all mutations causing PS II-herbicide resistance investigated until now are located [6, 24]. For the analysis of a triazine- or DCMU-resistant mutant, it seems justified to assume that the mutation is located within this fragment of D 1. Thus, it seems unnecessary to determine the complete *psb*A DNA sequence. Using one single primer, a stretch of at least 200 nucleotides can easily be read on a single gel.

The *psb*A nucleotide sequence shows large homologies between different species. This allows the use in many photosynthetic organisms of a selective synthetic probe as a universal primer, using either DNA or mRNA as a template for synthesis of a complementary DNA strand in the presence of dideoxy nucleotides [23]. When this probe is 5′-labeled with a [γ-^{32}P]nucleotide, the synthesis can be performed without using any further radioactive labels. The advantage is a signal of constant intensity, independent of the length of the synthesized fragment.

Using 10 g (fresh weight) leaf material, 100–150 µg DNA and 3–4 mg RNA could be isolated. The isolated DNA could be cut completely with restriction endonucleases without additional purification by CsCl-ethidium bromide equilibrium centrifugation. In the RNA preparation several distinct bands of rRNA could be discriminated by agarose gel electrophoresis.

Isolated total DNA from *Chenopodium album* (0.5 µg) was used as template in a PCR amplification experiment, in combination with the 20- and 23-mer primers described above. One-tenth of the reaction mixture was loaded on an 0.8% agarose gel and stained with ethidium bromide (Fig. 1). Except for the desired fragment, no amplification products could be detected. This indicates that the primer combination is specific for the *psb*A sequence.

Using the described 17-mer primer the *psb*A sequence could be determined in the region coding for D 1 amino acid residues 280 down to 220. In Fig. 2 an autoradiograph is shown of the *Chenopodium album psb*A-DNA, containing the mutation giving rise to triazine resistance. The results are summarized in Table I.

In the part of the *Chenopodium psb*A gene shown in Table I, not considering codon 264, 22 nucleotide differences were detected compared to *Chlamydomonas*. This indicates more than 85% homology on the DNA level. Only 8 bases differ

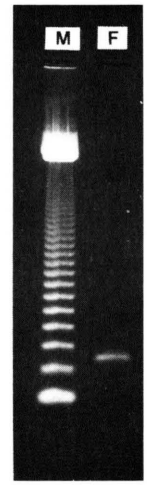

Fig. 1. Agarose gel electrophoresis from a 293-bp amplification product of the *psb*A gene, spanning the region between transmembrane helices IV and V. F = fragment, M = 123-bp DNA ladder.

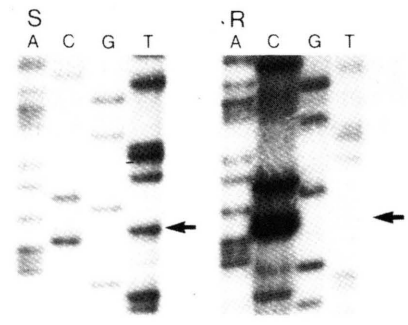

Fig. 2. Autoradiograph of *Chenopodium album* total DNA sequence analysis. Arrows indicate the site of mutation (base 790). S = sensitive, R = resistant biotype. Letters above lanes indicate bases of the synthesized cDNA strand.

Table I. Partial sequence of the *psb*A gene product of *Chenopodium album*, wild type and a triazine-resistant mutant.

5′	230														240										
1	GAA	ACA	ACT	GAA	AAC	GAA	TCA	GCT	AAC	GAA	GGT	TAC	CGT	TTC	GGT	CAA	GAA	GAA	GAA						
2		C	A		T		T		C	A			A	A	T		G								
3		T	A		T		T		T	G			A	A	G		G								
S		T	A		T		T		T	G			A	A	G		G								
R		T	A		T		T		T	G			A	A	G		G								

	250																260								
1	ACT	TAC	AAC	ATT	GTA	GCT	GCT	CAT	GGT	TAC	TTT	GGT	CGT	CTA	ATC	TTC	CAA	TAC	GCT						
2		T	C	C						T	T	T	A	T	G	T		T							
3		T	T	T						T	T	C	A	T	G	T		T							
S		T	T	T						T	C	C	A	T	G	T		T							
R		T	T	T						T	C	C	A	T	G	T		T							

	264					270											280								
1	**TCT**	TTC	AAC	AAC	TCT	CGT	TCA	TTA	CAC	TTC	TTC	TTA	GCT	GCT	TGG	CCG	GTA								
2	**AGT**						T									G									
3	**AGT**						T									T									
S	**AGT**						T									T									
R	**GGT**						T									T									

1 = *Chlamydomonas reinhardtii* [12]; 2 = *Amaranthus hybridus* [3]; 3 = *Chenopodium album* [25]; S = *Chenopodium album*, wildtype; R = *Chenopodium album*, triazine-resistant biotype. The table was constructed from sequencing results obtained with both total DNA and mRNA sequence analyses. Indicated are nucleotides different from those found at the same positions in *Chlamydomonas reinhardtii* [12]. The codon 264 causing a Ser–Gly mutation is indicated in bold lettering.

from the sequence as found in the corresponding part of *Amaranthus hybridus*, and 1 mutation distinguishes our *Chenopodium album* from another line of *Chenopodium album* as described by Bettini *et al.* [25]. These mutations are all silent, *i.e.* none of them leads to a different amino acid sequence of the D1 protein. In the mutant the codon 264 (AGT) of the wildtype, coding for serine, is changed to GGT (glycine). This leads to the incorporation of glycine in the D1 protein. Apparently this changes the binding environment for the triazine herbicides, causing the observed resistance. Presumably the ability to form an H-bond with the herbicide is lost, which results in a weaker binding, and correspondingly faster release from the site. This property seems to be the direct reason of the resistance, rather than a decrease in the rate of binding [6].

The number of triazine-resistant weed species is steadily increasing (*e.g.*, [26]). Almost all triazine-resistant biotypes analyzed until now, including the *Chenopodium album* described here, have an alteration of serine-264 into glycine. In case only a little plant material is available, the PCR technique may become a convenient and fast method to specifically amplify *psb*A-DNA from newly found resistant biotypes, in order to search for mutations in the D1 protein.

Acknowledgement

Automated oligonucleotide synthesis was supported by a grant from the Minister für Wissenschaft und Forschung des Landes Nordrhein-Westfalen.

[1] M. D. C. Barros and T. A. Dyer, Theor. Appl. Genet. **75,** 610–616 (1988).
[2] P. Goloubinoff, M. Edelman, and R. B. Hallick, Nucleic Acids Res. **12,** 9489–9496 (1984).
[3] J. Hirschberg and L. McIntosh, Science **222,** 1346–1349 (1983).
[4] J. Hirschberg, A. Bleecker, D. J. Kyle, L. McIntosh, and C. J. Arntzen, Z. Naturforsch. **39c,** 412–420 (1983).
[5] M. Reith and N. A. Straus, Theor. Appl. Genet. **73,** 357–363 (1987).
[6] D. Naber, Ph.D. Thesis, Agricultural University Wageningen, The Netherlands (1989).
[7] A. Trebst, Z. Naturforsch. **42 c,** 742–750 (1987).
[8] J. R. Bedbrook, G. Link, D. M. Coen, L. Bogorad, and A. Rich, Proc. Natl. Acad. Sci. U.S.A. **75,** 3060–3064 (1978).
[9] A. K. Mattoo, U. Pick, H. Hoffman-Falk, and M. Edelman, Proc. Natl. Acad. Sci. U.S.A. **78,** 1572–1576 (1981).
[10] K. Ohyama, H. Fukuzawa, T. Kohchi, H. Shirai, T. Sano, S. Sano, K. Umesono, Y. Shiki, M. Takeuchi, Z. Chang, S. Aota, H. Inokuchi, and H. Ozek, Nature **322,** 572–574 (1986).
[11] K. Shinozaki, N. Hayashida, and M. Sugiura, Photosynth. Res. **18,** 7–31 (1988).
[12] J. M. Erickson, M. Rahire, and J.-D. Rochaix, EMBO J. **3,** 2753–2762 (1984).
[13] A. Spielmann and E. Stutz, Nucleic Acids Res. **11,** 7157–7167 (1983).
[14] K. Oishi, D. Shapiro, and K. K. Tewari, Mol. Cell Biol. **4,** 2556–2563 (1984).
[15] G. Zurawski, H. J. Bohnert, P. R. Whitfeld, and W. Bottomley, Proc. Natl. Acad. Sci. U.S.A. **79,** 7699–7703 (1982).
[16] M. Wettern, Plant Sci. **43,** 173–177 (1986).
[17] A. Lönneborg, S. R. Kalla, G. Samuelsson, P. Gustafsson, and G. Öquist, FEBS Lett. **240,** 110–114 (1988).
[18] F. Sanger, S. Nicklen, and A. R. Coulsen, Proc. Natl. Acad. Sci. U.S.A. **74,** 5463–5467 (1977).
[19] M. A. K. Jansen, J. H. Hobé, J. C. Wesselius, and J. J. S. van Rensen, Physiol. Vég. **24,** 475–484 (1986).
[20] B. Taylor and A. Powell, Focus **4,** 4–6 (1982).
[21] T. Maniatis, E. F. Fritsch, and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 1982.
[22] R. K. Saiki, D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich, Science **239,** 487–491 (1988).
[23] U. Johanningmeier, U. Bodner, and G. F. Wildner, FEBS Lett. **211,** 221–224 (1987).
[24] J.-D. Rochaix and J. M. Erickson, Trends in Biol. Sci. **13,** 56–59 (1988).
[25] P. Bettini, S. McNally, M. Sevignac, H. Darmency, J. Gasquez, and M. Dron, Plant Physiol. **84,** 1442–1446 (1987).
[26] J. L. P. van Oorschot and H. J. M. Straathof, Annales de l'Association Nationale pour la Protection des Plantes **3,** 267–275 (1988).