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Chloroplast-coded atrazine resistance in *Solanum nigrum: psbA* loci from susceptible and resistant biotypes are isogenic except for a single codon change

Pierre Goloubinoff and Marvin Edelman
Department of Plant Genetics, Weizmann Institute of Science, Rehovot 76100, Israel

Richard B. Hallick*
Department of Chemistry, University of Colorado, Boulder, CO 80309, USA

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ABSTRACT

The 32-kDa photosystem II protein of the chloroplast is thought to be a target molecule for the herbicide atrazine. The psbA gene coding for this protein was cloned from *Solanum nigrum* atrazine-susceptible ('S') and atrazine-resistant ('R') biotypes. The 'S' and 'R' genes are identical in nucleotide sequence except for an A to G transition, predicting a Ser to Gly change at codon 264. The same predicted amino acid change in psbA was previously shown for an *Amaranthus hybridus* 'S' and 'R' biotypes which had, in addition, two silent nucleotide changes between the genes (Hirschberg, J. and McIntosh, L., Science 222, 1346-1349, 1983). Occurrence of the identical, non-silent change in psbA in different 'S' and 'R' weed biotype pairs suggests a functional, herbicide-related role for this codon position.

INTRODUCTION

Atrazine (2-chloro-4-(2-propylamino)-6-ethylamino-*s*-triazine) is a commonly used herbicide in crop fields and along roads and railways. It acts by blocking electron transport at the reducing side of photosystem II (1). Atrazine-resistant biotypes have been reported in many weed species (2,3). The resistance trait was shown to be maternally inherited in several instances (4), including *Solanum nigrum* (5). Based on studies *in vivo* and with isolated chloroplasts atrazine and other herbicides are believed to interact with a chloroplast DNA coded, photosystem II protein having an apparent M_r of 32-kDa (6,7). Synthesis of this protein is not markedly affected in atrazine-resistant biotypes (8); however, binding of azidoatrazine to the 32-kDa protein is greatly reduced in resistant plants as compared to susceptible ones (9).

Hirschberg and McIntosh (10) reported three nucleotide differences between the psbA gene, coding for the 32-kDa protein, from an atrazine-susceptible ('S') and an atrazine-resistant ('R') plant of *Amaranthus hybridus*. One of these differences would result in an amino acid change from Ser to Gly at position 227 (equivalent to position 264 in the numbering scheme of Zurawski et al., ref:11). The other two occurred in silent, third-base

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positions. Therefore the two *A. hybridus* biotypes are not isogenic. Thus, the question arises whether the amino acid difference between the two decoded sequences is causally related to atrazine herbicide resistance in weeds, or is merely a random neutral mutation, expressing the divergence between the two particular plants which were investigated.

Demonstration of the same amino acid difference in the decoded psbA sequences of another 'S' and 'R' weed biotype pair would be important evidence for a functional, herbicide-related significance to this mutational event. Accordingly, psbA genes from atrazine-susceptible and atrazine-resistant biotypes of the weed, *S. nigrum* were cloned and sequenced. We show that the psbA nucleotide sequences for this biotype pair are identical except at one position. This single difference predicts a Ser to Gly change between *S. nigrum* 'S' and 'R' biotypes in the same residue as was reported for *A. hybridus*.

MATERIALS AND METHODS

Materials

Phage M13 mp8 and mp9 replicative forms, and restriction enzymes *Eco*RI, *Hin*dIII, *Pst*I, *Sau*3A1, *Xho*II, and *Bam*HI were obtained from New England Biolabs. (α-^{32}P)-dATP and (^{35}S)-dATPαS were from New England Nuclear. *E. coli* HB101 (12) and *E. coli* JM 101 (13) were used as hosts for plasmid and phage vectors, respectively. *E. coli* DNA-polymerase I large-fragment and T4 DNA ligase were obtained from Boehringer Mannheim, while deoxy- and dideoxy-nucleotide triphosphates were from PL Inc.

Isolation of psbA Clones from *S. nigrum*

Atrazine-susceptible and atrazine-resistant *S. nigrum* plants were grown from seeds collected in France and provided by J. Gasquez, INRA, Dijon. Chloroplast DNA, extracted from leaves of 'S' and 'R' *S. nigrum* plants (14,15), was kindly provided by J. Gressel and N. Cohen. A 5 kb *Bam*HI fragment carrying the psbA gene sequence and flanking regions was isolated from 'S' and 'R' DNAs. The fragment was identified in each case by hybridization to SP247/5, a cloned probe containing an internal psbA sequence from *Spirodela oligorrhiza* (16). The 5 kb *Bam*HI fragments from the 'S' and 'R' biotypes were then cloned in pBR322 to yield plasmids pSNS48 and pSNR6, respectively.

Subcloning in mp8 and mp9 Phage Vectors

Plasmids pSNS48 and pSNR6 were digested with *Xho*II and cloned in the *Bam*HI site of mp8, yielding mpS and mpR, respectively. mpS and mpR were each found to contain a 1202 bp fragment spanning the psbA gene from position -140 to +1062. Replicative forms of mpS and mpR were used for *Sau*3A1 subcloning in mp8 and mp9 phage vectors. Four *Sau*3A1 clones were derived from mpS and one

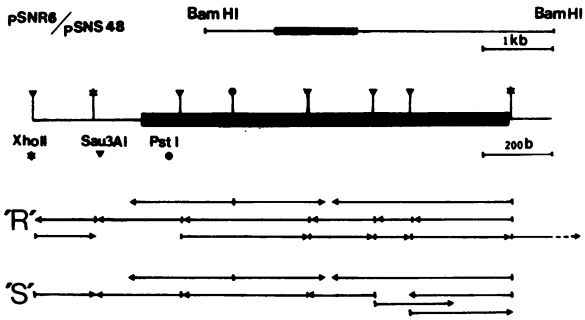


Figure 1: Sequencing strategy for *S. nigrum* 'R' and 'S' psbA genes. Several *Sau*3Al and the *Xho*II fragments were subcloned from the initial pSNR6 and the pSNS48 plasmids into M13 mp8 or mp9 phage vector. The *Pst*I and the remaining *Sau*3Al fragments were derived from the *Xho*II subclones. The 3'-flanking region (dashed arrow) was sequenced only for the 'R' gene.

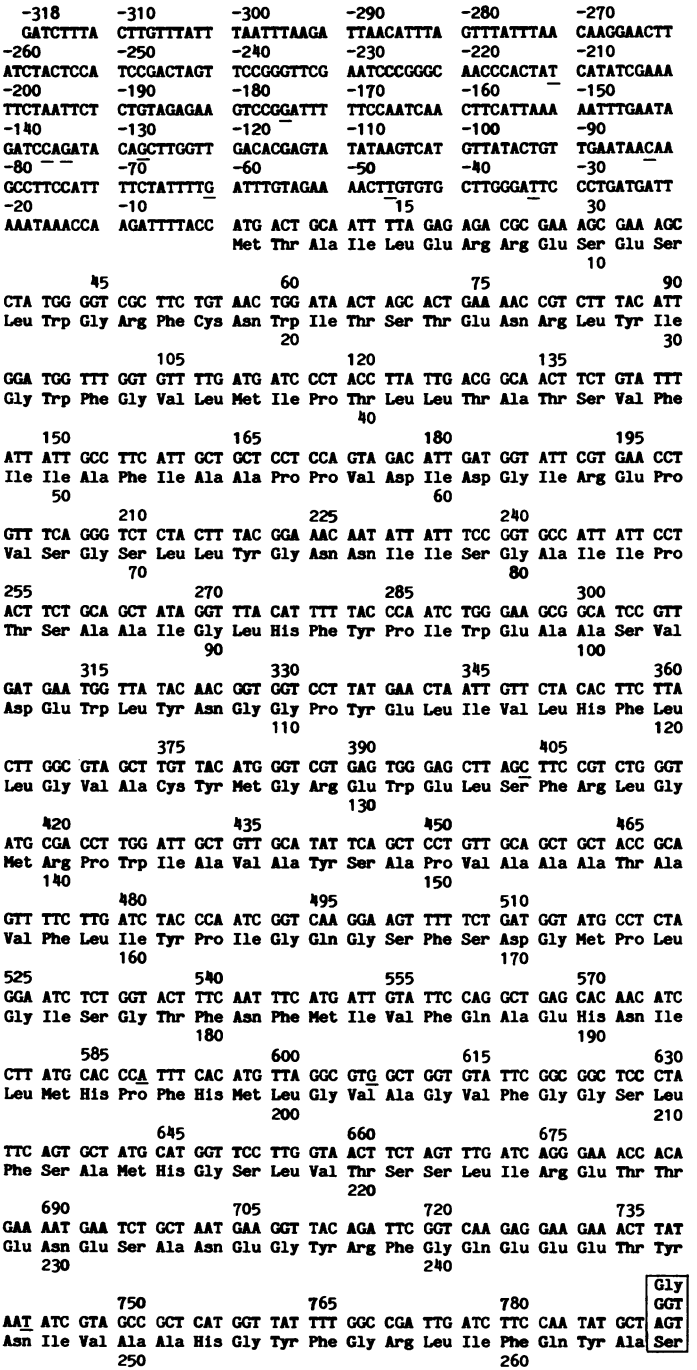
from mpR. All of the remaining *Sau*3Al clones were derived by direct subcloning of pSNS48 and pSNR6 in mp8 and mp9. *Pst*I subclones were derived by excising an 816 bp fragment from mpS and mpR, and recloning it in the opposite direction in mp8. Single-stranded mpS and mpR were also used directly for sequencing from position 1062 through position 530.

DNA Sequence Analysis

Subcloned M13 recombinant phages were used to prepare single-stranded templates for sequence analysis by the dideoxy chain termination method (17). Either (α-^{32}P)-dATP or (^{35}S)-dATPαS (18) were used as radiolabels. DNA was electrophoresed at constant voltage of 1300 V in 6% polyacrylamide, 8 M urea slab gels (0.2-0.4 X 170 X 350 mm) bound to one of the glass electrophoresis plates (19). The gel plates were backed by 4 mm aluminum plates to reduce temperature gradients. After electrophoresis the gels were fixed in 10% acetic acid, dried on the glass plate and autoradiographed on X-ray film (AGFA curix RP2).

RESULTS AND DISCUSSION

The strategy used in sequencing the 'R' and 'S' *S. nigrum* psbA genes is shown in Fig. 1. For both genes, the entire coding region was determined at least twice using independent clones. All of the *Sau*3Al sites within the coding region, as well as the single *Pst*I site, were read by means of overlapping clones. The region spanning nucleotide 773 to 885 was sequenced in four independent clones for the 'S' gene and three independent clones for the 'R' gene.


-318 -310 -300 -290 -280 -270
GATCTTTA CTTGTTTATT TAATTTAAGA TTAACATTTA GTTTATTTAA CAAGGAACTT
-260 -250 -240 -230 -220 -210
ATCTACTCCA TCCGACTAGT TCCGGGTTCG AATCCCGGGC AACCCACTAT CATATCGAAA
-200 -190 -180 -170 -160 -150
TTCTAATTCT CTGTAGAGAA GTCCGGATTT TTCCAATCAA CTTCATTAAA AATTTGAATA
-140 -130 -120 -110 -100 -90
GATCCAGATA CAGCTTGGTT GACACGAGTA TATAAGTCAT GTTATACTGT TGAATAACAA
-80 -70 -60 -50 -40 -30
GCCTTCCATT TTCTATTTTG ATTTGTAGAA AACTTGTGTG CTTGGGATTC CCTGATGATT
-20 -10 -15 30
AAATAAACCA AGATTTTACC ATG ACT GCA ATT TTA GAG AGA CGC GAA AGC GAA AGC
Met Thr Ala Ile Leu Glu Arg Arg Glu Ser Glu Ser
10
45 60 75 90
CTA TGG GGT CGC TTC TGT AAC TGG ATA ACT AGC ACT GAA AAC CGT CTT TAC ATT
Leu Trp Gly Arg Phe Cys Asn Trp Ile Thr Ser Thr Glu Asn Arg Leu Tyr Ile
20 30
105 120 135
GGA TGG TTT GGT GTT TTG ATG ATC CCT ACC TTA TTG ACG GCA ACT TCT GTA TTT
Gly Trp Phe Gly Val Leu Met Ile Pro Thr Leu Leu Thr Ala Thr Ser Val Phe
40
150 165 180 195
ATT ATT GCC TTC ATT GCT GCT CCT CCA GTA GAC ATT GAT GGT ATT CGT GAA CCT
Ile Ile Ala Phe Ile Ala Ala Pro Pro Val Asp Ile Asp Gly Ile Arg Glu Pro
50 60
210 225 240
GTT TCA GGG TCT CTA CTT TAC GGA AAC AAT ATT ATT TCC GGT GCC ATT ATT CCT
Val Ser Gly Ser Leu Leu Tyr Gly Asn Asn Ile Ile Ser Gly Ala Ile Ile Pro
70 80
255 270 285 300
ACT TCT GCA GCT ATA GGT TTA CAT TTT TAC CCA ATC TGG GAA GCG GCA TCC GTT
Thr Ser Ala Ala Ile Gly Leu His Phe Tyr Pro Ile Trp Glu Ala Ala Ser Val
90 100
315 330 345 360
GAT GAA TGG TTA TAC AAC GGT GGT CCT TAT GAA CTA ATT GTT CTA CAC TTC TTA
Asp Glu Trp Leu Tyr Asn Gly Gly Pro Tyr Glu Leu Ile Val Leu His Phe Leu
110 120
375 390 405
CTT GGC GTA GCT TGT TAC ATG GGT CGT GAG TGG GAG CTT AGC TTC CGT CTG GGT
Leu Gly Val Ala Cys Tyr Met Gly Arg Glu Trp Glu Leu Ser Phe Arg Leu Gly
130
420 435 450 465
ATG CGA CCT TGG ATT GCT GTT GCA TAT TCA GCT CCT GTT GCA GCT GCT ACC GCA
Met Arg Pro Trp Ile Ala Val Ala Tyr Ser Ala Pro Val Ala Ala Ala Thr Ala
140 150
480 495 510
GTT TTC TTG ATC TAC CCA ATC GGT CAA GGA AGT TTT TCT GAT GGT ATG CCT CTA
Val Phe Leu Ile Tyr Pro Ile Gly Gln Gly Ser Phe Ser Asp Gly Met Pro Leu
160 170
525 540 555 570
GGA ATC TCT GGT ACT TTC AAT TTC ATG ATT GTA TTC CAG GCT GAG CAC AAC ATC
Gly Ile Ser Gly Thr Phe Asn Phe Met Ile Val Phe Gln Ala Glu His Asn Ile
180 190
585 600 615 630
CTT ATG CAC CCA TTT CAC ATG TTA GGC GTG GCT GGT GTA TTC GGC GGC TCC CTA
Leu Met His Pro Phe His Met Leu Gly Val Ala Gly Val Phe Gly Gly Ser Leu
200 210
645 660 675
TTC AGT GCT ATG CAT GGT TCC TTG GTA ACT TCT AGT TTG ATC AGG GAA ACC ACA
Phe Ser Ala Met His Gly Ser Leu Val Thr Ser Ser Leu Ile Arg Glu Thr Thr
220
690 705 720 735
GAA AAT GAA TCT GCT AAT GAA GGT TAC AGA TTC GGT CAA GAG GAA GAA ACT TAT
Glu Asn Glu Ser Ala Asn Glu Gly Tyr Arg Phe Gly Gln Glu Glu Glu Thr Tyr
230 240
Gly
750 765 780 GGT
AAT ATC GTA GCC GCT CAT GGT TAT TTT GGC CGA TTG ATC TTC CAA TAT GCT AGT
Asn Ile Val Ala Ala His Gly Tyr Phe Gly Arg Leu Ile Phe Gln Tyr Ala Ser
250 260

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795                 810                 825                 840
TTC AAC AAC TCT CGT TCG TTA CAC TTC TTC CTA GCT GCT TGG CCT GTA GTA GGT
Phe Asn Asn Ser Arg Ser Leu His Phe Phe Leu Ala Ala Trp Pro Val Val Gly
                    270                                     280
        855                 870                 885                 900
ATC TGG TTT ACC GCT TTA GGT ATT AGC ACT ATG GCT TTC AAC CTA AAT GGT TTC
Ile Trp Phe Thr Ala Leu Gly Ile Ser Thr Met Ala Phe Asn Leu Asn Gly Phe
                        290                                         300
                915                 930                 945
AAT TTC AAC CAA TCT GTA GTT GAC AGT CAG GGT CGT GTA ATT AAC ACT TGG GCT
Asn Phe Asn Gln Ser Val Val Asp Ser Gln Gly Arg Val Ile Asn Thr Trp Ala
                                    310
    960                 975                 990                1005
GAT ATC ATC AAC CGT GCT AAC CTT GGT ATG GAA GTT ATG CAT GAA CGT AAT GCT
Asp Ile Ile Asn Arg Ala Asn Leu Gly Met Glu Val Met His Glu Arg Asn Ala
    320                                 330
           1020                1035                1050
CAT AAC TTC CCT CTA GAC CTA GCT GCT ATC GAA GCT CCA TCT ACA AAT GGA TAA
His Asn Phe Pro Leu Asp Leu Ala Ala Ile Glu Ala Pro Ser Thr Asn Gly  *
               340                                 350
      1070        1080        1090        1100        1110        1120
  GATCCCAG  CCTAGTCTAT  AGGAGGTTTT  GAAAAGAAAG  GAGCAATAAC  CATTTTCTTG
      1130        1140        1150        1160        1170        1180
TTCTATCAAG  AGGGTGCTAT  TGCTCCTTTC  TTTTTTTCTT  TTTCTTTATT  AATTTCCTAG
1183
 TAT
```

Figure 2: The nucleotide and the decoded amino acid sequences of psbA from atrazine-susceptible and atrazine-resistant *S. nigrum*. The sole difference between the 'S' and 'R' sequences is shown in the boxed region. A single A to G base transition accounts for the Ser to Gly change. The nucleotide divergences from *N. debneyi* (11) are underlined. Nucleotides downstream of the gene which are overlined are not present in *N. debneyi*.

The complete nucleotide sequences of the atrazine-susceptible and atrazine-resistant *S. nigrum* psbA genes, plus flanking regions is given in Fig. 2. The decoded amino acid sequences are also shown. Among the six higher plant psbA sequences previously reported (10,11,20-22), those from *Nicotiana debneyi* and *N. tabacum* (which are members of the Solanaceae) most closely resemble the sequence in Figure 2. The nucleotide differences between the *S. nigrum* and *N. debneyi* sequences are underlined. From position -227 to -1, nine differences between the two genes were found (~4% divergence); however, the region proposed to be related to the *E. coli* promoter sequence (11) is entirely conserved. The same region (-80 to -120) which is also highly conserved in *Sinapis alba* is required for accurate *in vitro* transcription in a homologous transcription system (23). Among the 1062 coding nucleotides of the gene, ten differences (~0.9% divergence) were recorded; however, all these occur in third-base wobble positions within the open reading frame. Thus, the decoded atrazine-susceptible *N. debneyi* and *S. nigrum* polypeptides have identical amino acid sequences. Among the 121 nucleotides downstream of the gene, four divergent events (~3% divergence) were counted; however, the stable stem-and-loop structure of the 3'-flanking sequence (11) is conserved.

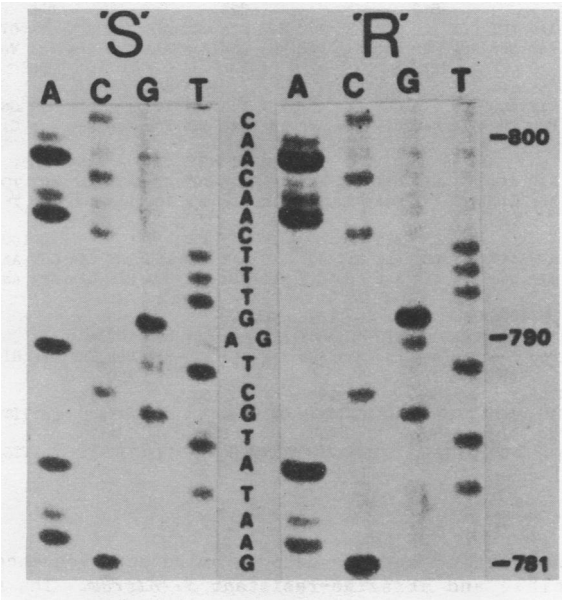

Figure 3: Autoradiogram of DNA-sequencing gels (dideoxy method) showing the A to G transition between psbA from *S. nigrum* 'S' and 'R' biotypes. Nucleotide positions are indicated on the right.

The sole difference between *S. nigrum* atrazine-susceptible and atrazine-resistant biotypes over the 1384 nucleotides for which they were compared is shown in Figure 3. A single A to G base transition at nucleotide 790 of the psbA gene is clearly discerned. This corresponds to an amino acid change from Ser to Gly at position 264, as indicated in the boxed region in Figure 2. The sequence data presented for psbA of *S. nigrum* 'S' and 'R' biotypes, together with that reported for *A. hybridus* (10), strongly imply a relationship between the amino acid change at this position and atrazine-resistance in weed plants.

Partial psbA sequences of another independent *S. nigrum* 'S' and 'R' biotype pair have recently been determined (24). The 270 nucleotides resolved in this case are identical to a segment of the complete gene sequence presented for the 'S' and 'R' biotype in Figure 2. Erickson et al. (25) have likewise recently sequenced part of psbA from a mutant of the green alga *Chlamydomonas reinhardii* which is resistant to atrazine and diruron (3-(3,4-dichlorophenyl)-1,1-dimethylurea). The only difference in the coding regions of the gene from the mutant the wild-type, was at codon 264, where Ala replaced Ser. A Ser to Ala change also occurs at the equivalent position in the decoded psbA sequence from a diuron-resistant mutant of *Euglena gracilis* (26; Johanning-

meier and Hallick, manuscript in preparation).

In summary, while Ser is conserved at position 264 in all decoded psbA sequences reported for atrazine-susceptible organisms (10,11,20-28), it is replaced in five herbicide-resistant mutants. We can therefore assume that Ser at position 264 is not less stringently conserved than the other amino acids of the decoded gene. At the same time, the high frequency of mutation at this codon emphasizes a key (although not exclusive (29)) role for position 264 in this type of herbicide resistance. Perhaps a relationship exists between this position on the gene and the overlapping-binding-region for triazine and urea herbicides found on the 32kDa protein (30).

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*R.B.H. was a visiting fellow of the Dobrin Center for Nutrition and Plant Research, The Weizmann Institute of Science, Rehovot, Israel. Current Address, Department of Biochemistry, Biological Sciences West, University of Arizona, Tucson, AZ 85721.

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