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Atrazine resistance in the grass *Poa annua* is due to a single base change in the chloroplast gene for the D1 protein of photosystem II

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Summary. We report here the first molecular characterization of maternally inherited atrazine resistance in a monocot. As has been found in dicots, resistance in the grass *Poa annua* is correlated with a decrease in the ability of herbicides to bind to thylakoids and with an alteration in the gene for the D1 protein of photosystem II which would result in a change from a serine to an alanine residue at position 264 of the protein. Azidoatrazine, a photoaffinity-labelled analogue of atrazine, binds to the putative product of this gene in sensitive but not resistant biotypes of *P. annua*. The wheat gene and protein are shown to resemble those of the atrazine-sensitive *P. annua* biotype.

Key words: Atrazine resistance – D1 protein sequence – wheat – *Poa annua*

Introduction

A plant may be resistant to a herbicide for a number of reasons. Either the protein with which the herbicide reacts may be overproduced (Donn et al. 1984) or it may be altered so that it is no longer affected by the herbicide (Arntzen et al. 1979). Alternatively, the herbicide may be detoxified by the plant (Shimabukuro et al. 1971), or excluded from its site of action (Fuerst et al. 1985). A further mechanism of resistance involves the scavenging of toxic by-products of herbicide action (Harper and Harvey 1978).

Panicoid grasses such as maize, sorghum, sudangrass and sugarcane are atrazine resistant (Jensen et al. 1977) because they contain high levels of glutathione S-transferase in their leaves (Shimabukuro et al. 1971; Mozer et al. 1983). This enzyme conjugates glutathione to the herbicide rendering it non-toxic. Resistance of this type appears to be controlled by a single recessive nuclear gene inherited in a Mendelian fashion (Grogan et al. 1963). Maternally inherited atrazine resistance, however, is found in festucoid grasses such as *Poa annua* (Darmency and Gasquez 1981) and *Bromus tectorum* (Gressel et al. 1982), which suggested that the resistance in these grasses is controlled by an organelle gene rather than a nuclear one.

We show here that in an atrazine-resistant biotype of *P. annua* there is a single base change in the chloroplast gene which codes for the D1 protein of photosystem II (PSII). This change, which would result in an amino acid change in the D1 protein, is correlated with a dramatic decrease in the binding of atrazine to the thylakoids of the resistant *P. annua*. This finding is discussed in relation to recent advances concerning the structure and role in photosynthesis of the D1 protein.

Materials and methods

Leaves of 1 to 8 week old *P. annua* plants and of 10 day old wheat plants (*Triticum aestivum* cv. Mardler) were used as a source of thylakoids. The thylakoids were isolated and their capacity to bind herbicide assayed basically as described by Tischer and Strotmann (1977). This, in essence, consisted of incubating the thylakoids with [^{14}C] atrazine and then measuring the amount of unbound atrazine remaining. The method of Pfister et al. (1981) was used for the photoaffinity labelling of thylakoids. Bound [^{14}C] azidoatrazine (49.4mCi/m mol, Pathfinder Laboratories Inc., St. Louis, Missouri, USA) was covalently linked to protein by irradiating preparations

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with short wave u.v. light. The extracted protein was then fractionated on a 10%–16% polyacrylamide gradient gel according to the method of Laemmli (1970) as modified by Roscoe and Ellis (1982). The gels were then stained and autoradiographed.

For the immunological detection of D1, similarly fractionated thylakoid protein was transferred to nitrocellulose (BA85, Schleicher and Schuell) by the method of Vaessen et al. (1981). After transfer, filters were incubated for 2 h at room temperature in a buffer containing 10 m*M* Tris/HCl (pH 7.5), 0.35 m*M* NaCl, 2% (w/v) milk powder (Marvel) to saturate any remaining protein-binding sites. The filters were then washed twice in distilled water and twice in RIA buffer (10 m*M* Tris/HCl, pH 7.5, 0.15 *M* NaCl, 0.1% deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulphate). After this the filters were incubated overnight at 25 °C in RIA buffer containing the diluted (1:250) antiserum to D1 (obtained from Dr. L. McIntosh) and unreacted serum then removed by two RIA washes. [^{125}I]-labelled protein A was absorbed by bound antiserum during a 2 h incubation at 25 °C in RIA buffer. Subsequently the filters were washed three to four times in RIA buffer and twice in distilled water to remove unreacted protein A before being baked at 65 °C for 30 min and autoradiographed.

The standard methods used to clone and sequence the wheat and *P. annua* genes for the D1 protein will be specified elsewhere (Barros and Dyer, in preparation) in a paper devoted to the characterization of the genes themselves. A comparison of the derived amino acid sequences of these proteins with the sequence of the L protein of the bacterium *Rhodopseudomonas viridis* was carried out using Los Alamos programmes (Kanehisa 1982) operating on a VAX 750 computer.

Results

The amount of atrazine bound by thylakoids isolated from wheat and *P. annua* is shown in Fig. 1. The results, expressed as the amount of atrazine bound by thylakoids containing a specific amount of chlorophyll, are plotted as a function of the amount of atrazine which remains unbound. The data show that there is a very marked difference in the binding of atrazine by thylakoids isolated from wheat and the herbicide-susceptible *P. annua* compared with that bound by the resistant *P. annua* biotype. In the thylakoids of susceptible plants atrazine binding increases with herbicide concentration up to a saturation level, whilst the specific binding is absent in thylakoids from resistant plants. Similar binding patterns have been reported for thylakoids from susceptible and resistant biotypes of *Senecio vulgaris* (Pfister et al. 1979) and *Amaranthus retroflexus* (Oettmeier et al. 1982). Furthermore, the maximum level of binding observed in this study (=2.9 n mol/mg chlorophyll) was similar to that measured for atrazine and other PSII-inhibiting herbicides by Tischer and Strotmann (1977) and Laasch et al. (1982).

From these results it may be calculated that there are 337 and 360 chlorophyll molecules per herbicide binding site in wheat and susceptible *P. annua* respectively. This is similar to the number of chlorphylls per PSII unit measured by others (Tischer and Strotmann 1977; Malkin et al. 1981) suggesting that there is just one binding site and therefore D1 component per reaction centre.

The nature of the protein which binds atrazine was determined by photoaffinity labelling (Fig. 2). This shows that the azidoatrazine could be linked to a thylakoid protein (M_r 33.9 KDa) from the herbicide sensitive plants corresponding in the size to protein D1. However, no thylakoid protein from the resistant bio-

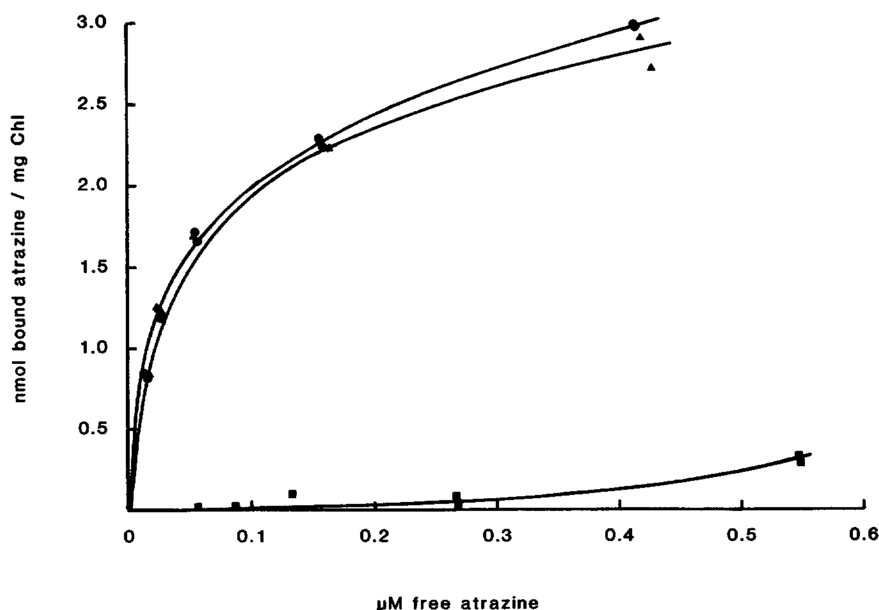

Fig. 1. Atrazine binding by thylakoids from wheat (●) and from a sensitive (▲) and resistant (■) biotype of *P. annua*. *Chl*=chlorophyll

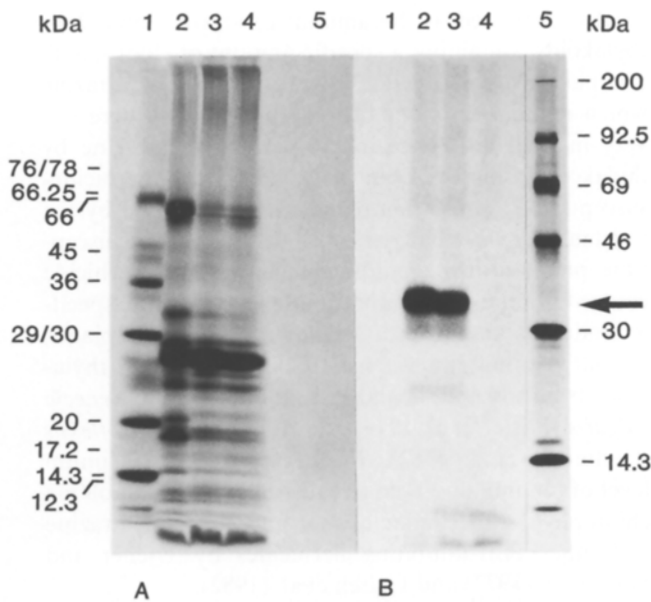

Fig. 2. [^{14}C] azidoatrazine-labelled thylakoid proteins from wheat and *P. annua* fractionated by polyacrylamide gel electrophoresis. *Panel A* shows the proteins stained with Coomassie Blue and *B* shows the fluorograph obtained from the same gel. *Lane 2* contained wheat protein; *lanes 3* and *4* contained protein from atrazine sensitive and atrazine resistant *P. annua* plants, respectively. *Lanes 1* and *5* contained molecular weight standards

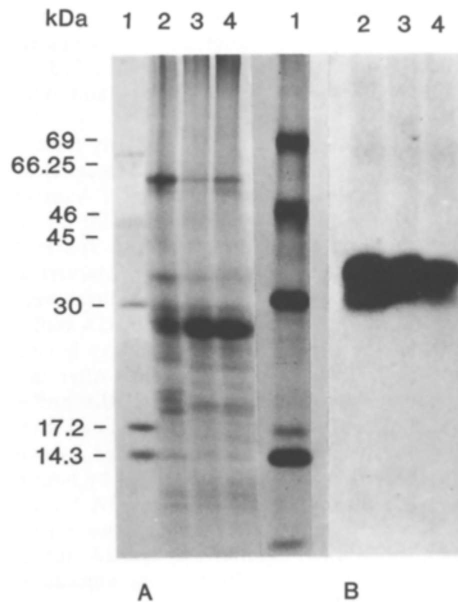

Fig. 3. Immunological detection of D1 after fractionation of thylakoid proteins of wheat and *P. annua* by polyacrylamide gel electrophoresis. *Panel A* shows the proteins stained with Coomassie Blue and *B* shows the fluorograph of the immunoblots. *Lanes 2* contained wheat protein; *lanes 3* and *4* contained protein from atrazine sensitive and atrazine resistant *P. annua* plants, respectively. *Lanes 1* contained molecular weight standards

MTAILERRES	TSLWGRFCNW	ITSTENRLYI	GWFGVLMIPT	LLTATSVFII	AFIAAPPVDI	60
DGIREPVSGS	LLYGNNIISG	AIIPTSAAIG	LHFYPIWEAA	SVDEWLYNGG	PYELIVLHFL	120
LGVACYMGRE	WELSFRLGMR	PWIAVAYSAP	VAAATAVFLI	YPIGQGSFSD	GMPLGISGTF	180
NFMIVFQAEH	NILMHPFHML	GVAGVFGGSL	FSAMHGSLVT	SSLIRETTEN	ESANEGYKFG	240
QEEETYNIVA	AHGYFGRLIF	QYASFNNSRS	LHFFLAAWPV	VGIWFTALGI	STMAFNLNGF	300
NFNQSVVDSQ	GRVINTWADI	INRANLGMEV	MHERNAHNFP	LDLAVLEVPS	ING	353

Fig. 4. Amino acid sequence of D1 in the atrazine-sensitive biotype of *P. annua.* The underlined residue at position 264 is where there is a serine to glycine change in the resistant biotype. Underlined residues at positions 345 and 346 are where there are alanine and valine residues respectively in wheat, the rest of the wheat sequence being identical to that of the *P. annua* sensitive biotype. Single letter amino acid code: A: alanine, C: cysteine, D: aspartate, E: glutamate, F: phenylalanine, G: glycine, H: histidine, I: isoleucine, K: lysine, L: leucine, M: methionine, N: asparagine, P: proline, Q: glutamine, R: tyrosine, S: serine, T: threonine, V: valine, Y: tyrosine, W: tryptophan

type was labelled, confirming the loss of a specific atrazine-binding site in the latter.

To show that this loss in binding site in the resistant *P. annua* plants was not due to the total loss of D1, the presence of this protein in all three types of thylakoid was demonstrated immunologically (Fig. 3). The proteins were fractionated by gel electrophoresis and then D1 detected using an antibody to this protein. The main protein band observed in each preparation corresponded exactly in position to that labelled by azidoatrazine (not shown). As with azidoatrazine labelling, a smaller component was also detected which could have been either a breakdown product or D1 in a different conformation.

A single nucleotide difference was found in the genes coding for D1 in sensitive and resistant *P. annua* plants (Barros and Dyer, in preparation). This is a adenine to guanine change at position 790 in the coding sequence and would result in the replacement of a serine by glycine residue in amino acid 264 in D1 (Fig. 4). No other differences were found either in the coding sequence or in the flanking sequence 140

nucleotides upstream or 122 nucleotides downstream of the gene. The wheat gene is identical with that of the sensitive biotype at position 790 but differs from both the *Poa* genes at several other positions. Most of these nucleotide differences do not change the coding properties of the gene; only a valine to alanine change at position 345 and a leucine to valine change at position 346 results (Fig. 4). The difference between the atrazine sensitive and resistant plants at position 264 is identical to that found in sensitive and resistant biotypes of various species of *Amaranthus* (Hirschberg and McIntosh 1983; McNally et al. 1987), of *Solanum nigrum* (Goloubinoff et al. 1984) and of *Senecio vulgaris* (R. Blyden and J. C. Gray, unpublished results) and similar differences have been found in *Chlamydomonas rheinhardii* (Erickson et al. 1984) and *Anacystis nidulans* (Golden and Haselkorn 1985).

Discussion

Considerable progress is being made in determining the molecular basis of atrazine resistance. It was first established (Bouges-Bocquet 1973; Velthuys and Amesz 1974) that the herbicide inhibits photosynthesis by preventing electron transfer from the primary quinone electron acceptor Q_A to the secondary quinone acceptor Q_B at the reducing side of PSII. The D1 (diffuse band 1) protein (also known as the peak D protein, 32 kDa protein and the Q_B-binding protein) was implicated in the electron transfer processes, with a change in the gene for this protein being correlated with the acquisition of the atrazine-resistant phenotype. This was initially shown in the dicot *Amaranthus hybridus* (Hirschberg and McIntosh 1983) and the unicellular green alga *Chlamydomonas reinhardii* (Erickson et al. 1984) and subsequently in the cyanobacterium *Anacystis nidulans* (Golden and Haselkorn 1985) and in the dicots *Solanum nigrum* (Goloubinoff et al. 1984) and *Senecio vulgaris* (R. Blyden and J. C. Gray, unpublished results). Conversion of atrazine sensitive *A. nidulans* to the resistant form by transformation with the altered *psbA* gene has provided the conclusive proof that the specific alteration found in its sequence was that which changed the phenotype (Golden and Sherman 1984).

The results presented here show that in the atrazine resistant biotype of the monocot grass *P. annua* the same type of resistance mechanism is found (Fig. 4). The close similarity between the *psbA* gene of wheat and that of *P. annua*, which is modified in the atrazine-resistant plants, suggest that if a similar change was made in wheat, atrazine resistance would result. Also, it now seems reasonable to expect that all of the maternally inherited atrazine resistance which occurs in higher plants (e.g. in *Brassica campestris, B. napus, Bromus tectorum, Chenopodium* spp., *Bidens tripartita* and *Polygonum* spp., see Gressel et al. 1982) will be of the same type.

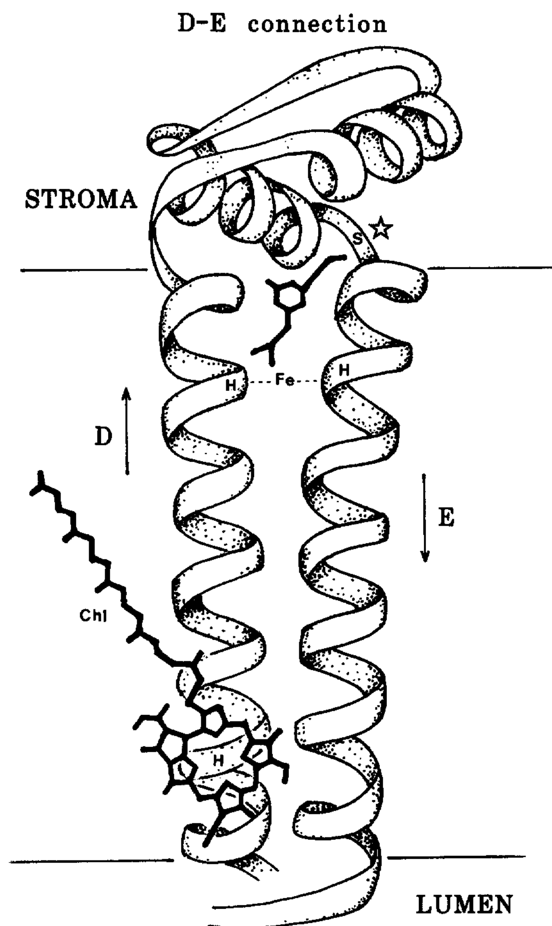

Fig. 5. Stylized representation of the possible conformation of the quinone pocket of D1. The two transmembrane helices D and E pass through the membrane in opposite directions. A histidine residue near the lumen end of helix D is the probable ligand for the Mg^{2+} of one of the "special pair" of chlorophylls at the photocentre and other histidine residues towards the stromal ends of both the D and E helices are ligands of an iron atom. Serine 264 (S*), mutation of which may result in herbicide resistance, is located in the D-E connection near the start of helix E and probably forms a hydrogen bond with the herbicide itself, shown here in the pocket near the stromal surface. (See Fig. 6 for the precise positioning of particular residues)

It is now possible to present a graphic picture of how atrazine and functionally related herbicides may exert their influence on photosynthesis. This is because recently there have been substantial advances in the determination of the structure of PSII reaction centres. These studies suggest that the herbicide binding protein D1 is a integral component of this centre (Nixon et al.

1986; Nanba and Satoh 1987) and that normally it binds a quinone Q_B involved in electron transport and one of a "special pair" of chlorophyll molecules where the primary photosynthetic process take place. It also seems to bind iron which could be involved in the transfer of electrons to Q_B from Q_A.

The model of D1 structure currently receiving most support suggests that it has five rod-like α-helices (A-E) which pierce the thylakoids vertically or possibly at a slight angle (Deisenhofer et al. 1985; Trebst 1986; Sayre et al. 1986). The fourth (D) and fifth (E) helices and D-E connecting segment would be aligned in the thylakoid as shown in Fig. 5 to make a pocket into which the quinone fits. This model is based primarily on an alignment of D1 with the L protein of the photocentre of the purple bacterium *Rhodopseudomonas viridis* for which the complete 3D structure and that of its associated components is known (Deisenhofer et al. 1985). Although the similarity between D1 and L is substantial (Fig. 6), there are some obvious differences. For example, in the alignment presented here, the putative D-E connection in D1 is 42 amino acids but it is only 25 in L, indicating that the similarity between the two proteins should not be over emphasized. In contrast, there is an extraordinarily high level of sequence conservation in D1 in organisms as different as *Poa, Euglena, Chlamydomonas* and the cyanobacteria (as shown in Fig. 6) especially in the region of the D and E helices.

Despite having some reservations about the precise configuration of the Q_B pocket in D1, one can be quite confident about its existence. The pocket is at a hydrophobic/hydrophilic interface so that electrons can travel rapidly from the hydrophobic interior of the

HELIX D

```
                 Chl                                   Fe
         170      ↓                                     ↓           199
     L   N P G H M S S V S F L F V N A M A L G L H G G L I L S V A N
Lumen                                                                  Stroma
    D1   H P F H M L G V A G V F G G S L F S A M H G S L V T S S L I
         195                                    ↑       ↑           224
                                             Azido     Dr2
                                                       219
```

D-E CONNECTION

```
   200                                                                     224
L  P G - - - - - - - - - D G D K V K T A E H E - - N - - Q - - - Y F - R D V V G Y - S I -
D1 R E T T E N E S A N E G Y K F G Q - E E E T Y N I V A A H G Y F G R L I F Q Y A S F N
   225                                                    ↑       ↑                ↑   266
                                                         MZ2    Ar207           DCMU4
                                                         251     255              264
```

HELIX E

```
                       Fe
           225          ↓                                      251
       L   G A L S I H R - L G L F L A S N I F L T G A F G T I A S
Stroma                                                               Lumen
      D1   N S R S L H F F L A A W P V V G I W F T - A L G - I S T
           267              ↑                                  292
                           275
```

Fig. 6. Alignment of amino acid sequences of the L subunit of *Rps. viridis* and that of the D1 protein from *P. annua* in the region of the D1 and E transmembrane helices and the D-E connection. *Vertical lines* between the aligned sequences indicate where there are identical residues, and dots the positions of amino acids with similar properties but which are not identical. The single letter amino acid code is given in Fig. 4. The alignment within the helices is based on one of those proposed by Michel et al. (1986a) and, as such, differs slightly from that of Trebst (1986) and Sayre et al. (1986). The residues in the D-E connection have been positioned to maximise correspondence of similar residues and this alignment emphasizes the dissimilarities between L and D1 in this region. To show which are the most highly conserved regions in L and D1 respectively, the horizontal lines above the alignments shows residues conserved in the purple bacteria *Rps. viridis, Rb. sphaeroides* and *Rb. capsulatus* (see Michel et al. 1986) and below, the conserved residues in all known D1 sequences including those of *P. annua, C. reinhardii, Euglena gracilis* and the cyanobacteria. The histidine residue which is a ligand for the Mg^{2+} of one of the special pair of chlorophylls is indicated (Chl) as are other histidine residues (Fe) which are possible ligands of an iron atom. The position of the methionine residue which reacts with azido-atrazine (Wolber et al. 1986) is also shown (Azido). Altered amino acids in *C. reinhardii* mutants resistant to herbicides which block PSII electron transport (e.g. atrazine, DCMU) are also shown by arrows. Dr2 is a valine to isoleucine change, Ar207 a phenylalanine to tyrosine change, DCMU4 a serine to alanine change (Erickson et al. 1985) and the change at position 275 (Erickson, personal communication) is from a leucine to phenylalanine residue. In MZ2 there is a change from an alanine to valine residue (Johanningmeier et al. 1987)

membrane for reduction of the quinone yet the quinone still has access to the stroma from which it extracts protons once it is reduced. The quinone is loosely held in this pocket and freely exchanges with a pool of plastoquinone in the adjacent thylakoid matrix. It is thus easily displaced by herbicides which take its place in the pocket and bind relatively tightly thus disrupting electron flow. The methionine at position 214 and somewhat buried in the membrane reacts with the azido group on azidoatrazine (Wolber et al. 1986) and all known mutants of D1 that are resistant to herbicides which block electron tansport in photosystem II occur in this pocket, either in the D-E connection or towards the stromal surface of the D and E helices (Fig. 6).

Of particular interest is the serine residue at position 264 in D1 aligned here (Fig. 6) to correspond in position to a similar residue in the bacterial L protein. Crystallographic studies have shown (Michel et al. 1986b) that a hydrogen bond is possible between the side chain oxygen of serine of this protein and the ethylamino nitrogen of the atrazine analogue terbutryn. Mutation of this serine to alanine abolishes this hydrogen bond and thereby decreases binding of the herbicide. If these serines of L and D1 are indeed homologous then there is the clear implication that it is the abolition of a hydrogen bond which decreases atrazine sensitivity in plants as well. Mild trypsin digestion of the thylakoids abolishes the sensitivity to some PSII-inhibiting herbicides (Mattoo et al. 1981), presumably by disrupting the D-E connection which forms the pocket thus destroying the herbicide binding site.

Several herbicide resistant mutants of D1 other than those changed at position 264 have been identified in *Chlamydomonas* (Erickson et al. 1985; Johanningmeier et al. 1987; Fig. 6). These result in a reduced sensitivity to atrazine and/or to diuron. It is somewhat puzzling therefore that the only alterations to D1 which have been observed in higher plants are at position 264. Perhaps other changes do also occur but have not been selected for so far by prevailing herbicide treatments.

From a practical point of view, the fact that the mutation which results in atrazine resistance in higher plants is at the heart of PSII, where both the primary excitation of electrons occurs and quinone is reduced by the excited electrons, helps explain why electron transport and overall plant performance is affected by this change (Radosevich and Holt 1982). However, the other changes found in *C. reinhardii* which also confer resistance, albeit to a lesser extent, do not appear to have the same adverse effect on electron transport (Erickson et al. 1985), possibly because their effect on the quinone pocket are less pronounced. Equivalent changes, if introduced into the genes of crop plants, might confer sufficient herbicide resistance for them to be of use while not having a negative effect on plant performance.

Despite this problem, naturally occurring atrazine resistance has been introduced into a Canadian variety of oilseed rape (canola) which is in commercial use (Beversdorf and Hume 1984). In this particular case any yield loss is compensated for by being able to spray the crop with atrazine to kill off the wild mustard with which the rape would otherwise outcross with a resulting increase to an unacceptable level of erucic acid in its oil. Because even this type of atrazine resistance is of practical use in particular instances, efforts to induce it by mutagenesis (Cseplo et al. 1985) or introduce it into new varieties by transformation or cell fusion seem worthwhile.

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