# Triazine Resistance in *Phalaris paradoxa:* Physiological and Molecular Analyses

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Triazine resistance in a mutant biotype of *Phalaris paradoxa* is accompanied by changes in the chlorophyll fluorescence induction curve, and by reduced quantum yield for electron transport, indicating altered photosystem II activity. However, light-saturated rates of electron transport in isolated chloroplasts, rates of $CO_2$ uptake in leaves and dry weight production of the triazine resistant biotype, are equal or superior to those of the wild type. A single mutation in the *psbA* gene, leading to a serine to glycine shift at position 264 of the thylakoid membrane 32 kDa $Q_B$-protein, was found in the herbicide resistant mutant. The results indicate that triazine resistance is not necessarily linked to inferior photosynthetic and growth performance.

## Introduction

Triazines and phenyl urea herbicides inhibit photosynthetic electron transport at the level of $Q_B$, the second stable electron acceptor of photosystem II (PS II) [1–3]. The herbicide binding site has been identified as the 32 kDa apoprotein of $Q_B$ (the $Q_B$-protein) [4]. This polypeptide is synthesized in the chloroplast from the chloroplast gene – *psbA* [5, 6]. A point mutation in this gene, leading to a change of a single amino acid in the $Q_B$ protein, confers atrazine resistance in *Amaranthus hybridus* [7] and in *Solanum nigrum* [8, 9]. Similar mutations were also identified in the green alga *Chlamydomonas reinhardii* [10], and in the cyanobacterium *Anacystis nidulans* R2 [11, 12]. The alteration in the $Q_B$-protein was found to be associated with a slower electron transport in PS II from $Q_A$ to $Q_B$ [13], lower quantum yield for $CO_2$ fixation [14] and reduced efficiency of growth [15–18].

Triazine resistant biotypes of several agronomically important grass weeds were recently found in Israel [19, 20]. Similar to previously reported cases, triazine resistance in these weeds is also evident at the level of electron transport in isolated chloroplasts. Further experiments conducted with one of these weeds, *Phalaris paradoxa,* indicated that the triazine resistant (R) biotype may surpass the triazine susceptible (S) biotype in photosynthetic rates and growth [20, 21]. These findings prompted us to further investigate the physiological and molecular basis for triazine-resistance in *P. paradoxa.*

## Materials and Methods

Seeds of R and S biotypes of *P. paradoxa* were collected from roadsides and adjacent fields respectively, and plants were grown and propagated as previously described [21]. Plants for photosynthesis and genetic experiments, were grown in a phytotron (17 °C/12 °C day/night temperatures, and an 8 h photoperiod).

Envelope-free chloroplasts were isolated from R and S leaf blades [19], and photoreactions were assayed as previously described [21]. Electron transport was measured with an oxygen electrode, $CO_2$-uptake with a Licor LI-6000 portable infra-red gas analyzer, and chlorophyll fluorescence with a laboratory-built apparatus.

Chloroplast DNA was isolated from 6-weeks-old plants according to Palmer [22]. Methods for restriction endonuclease digestion and electrophoresis,

*Abbreviations:* $Q_A$, $Q_B$, primary and secondary quinone acceptors of photosystem II, respectively; bp, base pair; Kb, Kilobase pairs; kDa, kilodalton; PS II, photosystem II; Atrazine, 2-chloro-4-ethylamino-6-isopropylamino-s-triazine; e.t., electron transport; R, triazine-resistant; S, triazine-susceptible; PPFD, photosynthetic photon flux density; $F_I$, intermediate chlorophyll-fluorescence level.

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southern hybridization and molecular cloning were as described by Maniatis *et al.* [23]. Nucleotide sequencing was performed according to Maxam and Gilbert [24].

## Results and Discussion

Resistance to atrazine in the R biotype of *Phalaris paradoxa*, is expressed both in whole plants and in isolated chloroplasts. R plants survived pre-emergence application of 10 kg/ha of atrazine (Table I), which was about 40 times the concentration necessary to completely control the S plants [29]. Fifty percent inhibition of electron transport in isolated S chloroplasts was obtained with 1 μM atrazine while even a 100-fold higher concentration was not sufficient for such inhibition in S chloroplasts (Table II).

As previously shown for other weed species [13], triazine resistance in *P. paradoxa* resulted in a typical alteration of the fluorescence induction curve of isolated chloroplasts. The intermediate fluorescence level – $F_I$, which is evident as a shoulder in the fluorescence-rise from its initial level – $F_O$ to its maximum – $F_M$, was significantly higher in R compared to S chloroplasts (Table II). An increase in the $F_I$ level is the result of inhibition of electron transfer from $Q_A$ to $Q_B$ [13]. Measurements of electron transport at different photon flux densities, with methylviologen as electron acceptor, indicated a 30% reduction in quantum yield for R compared to S *P. paradoxa* chloroplasts (Table II).

In spite of the reduction in quantum yield, there was no decline in the maximal, light-saturated, rate of electron transport in R chloroplasts. The maximal rate was actually 20% higher for R compared to S chloroplasts (Table II). Also, light-saturated rates of $CO_2$ uptake by intact leaves were largely similar for R and S plants even though the quantum yield was lower in R plants (Table I). Dry weight production by R plants grown under noncompetitive conditions was higher in R compared to S *P. paradoxa* plants.

Electron flow from $Q_A$ to $Q_B$ is not the rate limiting step in photosynthetic electron transport under light-saturating conditions. Even in R chloroplasts, where the transfer rate from $Q_A$ to $Q_B$ is found to be 10 times slower compared to S chloroplasts [25], it is still much faster than the rate-limiting electron flow out of the plastoquinone pool [26]. This consideration could explain our observation of reduced rates of whole chain electron transport in R chloroplasts at low but not at high PPFD levels [21]. Similar results were obtained by Ort *et al.* [14] and by Jansen *et al.* [27]. Holt *et al.*, however, reported decreased rates of electron transport in *Senecio vulgaris* at all PPFD levels tested [28].

Differences between R and S biotypes in nuclear and chloroplastic genome, other than the triazine resistance trait, are most probably involved in determining the maximal rates of e.t. and of photosynthesis. Such genetic polymorphism is probably responsible for the variable differences in photosynthetic capacity found between R and S biotypes of different higher plants [15–21, 27, 29]. Even where R and S biotypes are nearly nuclear-isogenic (*e.g.* [30]), their chloroplasts which control a large part of

Table I. Comparison of photosynthesis and growth in triazine resistant (R) and susceptible (S) plants of *P. paradoxa*.

| Parameter measured | R | S | R/S |
|---|---|---|---|
| Plant survival of pre-emergence treatment with 10 kg/ha atrazine [%] | 100 | 0 | – |
| $CO_2$ uptake: light-saturated rate[a] | 13.3 | 12.7 | 1.05 |
| $CO_2$ uptake: quantum yield[b] | 0.38 | 0.46 | 0.83 |
| Dry weight/plant (gr)[c] | 4.9 | 4.6 | 1.06 |

[a] The rate (in $\mu mol \times m^{-2} \times s^{-1}$) was obtained from the *y*-axis intercept of a plot of $(CO_2 \text{ uptake})^{-1}$ *vs.* $(PPFD)^{-1}$.
[b] The quantum yield was obtained from the slope of the same plot [1]. Being based on incident rather than absorbed PPFD, it is given in relative units.
[c] Total shoot dry weight was determined 150 days after sowing.

Table II. A comparison of photosynthetic reactions in triazine resistant (R) and susceptible (S) chloroplasts of *P. paradoxa*.

| Parameter measured | R | S | R/S |
|---|---|---|---|
| Inhibition of e.t. by atrazine ($I_{50}$)[a] | $> 10^{-4}$ M | $10^{-6}$ M | > 100 |
| Light-saturated rate of e.t.[b] | 43.1 | 35.7 | 1.21 |
| Quantum yield for e.t.[c] | 0.26 | 0.37 | 0.70 |
| $F_I$-fluorescence ratio[d] | 1.0 | 0.2 | 5.0 |

[a] $I_{50}$-atrazine concentration required for 50% inhibition of electron transport.
[b] The rate (in microequivalents $\times$ mg chl$^{-1} \times$ h$^{-1}$) was obtained from the *y*-axis intercept of a plot of $(e.t.)^{-1}$ *vs.* $(PPFD)^{-1}$.
[c] The quantum yield was obtained from the slope of the same plot.
[d] The $F_I$-fluorescence ratio is defined by $(F_I\text{-}F_O)/F_O$. $F_O$ was similar for R and S chloroplasts.

the photosynthetic characteristics are of different sources and therefore likely to be polymorphic.

The reduction in quantum yield and the increase in $F_I$ in R-type *P. paradoxa* suggest that resistance to atrazine in this weed is due to an alteration in PS II, as reported for other triazine resistant mutants [13]. In order to identify the mutation responsible for this change, we have cloned and sequenced the chloroplast *psbA* gene from *P. paradoxa.*

Chloroplast DNA, isolated from R and S biotypes of *Phalaris paradoxa,* was cleaved with the restriction endonucleases *Hind*III, *Eco*RI and *Bam*HI and was subjected to a southern hybridization analysis using the cloned *psbA* gene of *Amaranthus hybridus* [7] as a probe. The results indicated that the *psbA* gene of *P. paradoxa* is located within a 5 kb *Eco*RI DNA fragment (Fig. 1). This fragment from the S biotype was cloned in the plasmid pBR328 and the recombinant plasmid was designated pPPCS5. An *Eco*RI-*Bam*HI fragment of 2.25 kb from the R biotype which contained the *psbA* gene was also cloned in the plasmid pBR322 and the recombinant plasmid was designated pPPCR225.

The nucleotide sequence of a 1.4 kb *Taq*I-*Eco*RI fragment of plasmids pPPCS5 and pPPCR225 was determined. This fragment contained the whole coding region of the *psbA* gene, plus 130 bp upstream and 110 bp downstream to it. The deduced amino acid sequence of the $Q_B$-polypeptide [31] showed a high degree of homology (ca. 98–99%) to the polypeptides from other higher plants [6, 7]. However, the $Q_B$-protein of *P. paradoxa* differed significantly from all plants and algae examined so far by containing a lysine, rather than arginine, at position 238.

A comparison of the amino acid sequence of the $Q_B$-protein from R and S biotypes, revealed only one difference. Serine in residue 264 in the S type was replaced by glycine in the R biotype (Fig. 2). This is exactly the same change that has been identified in four cases of atrazine resistant mutants of other high-

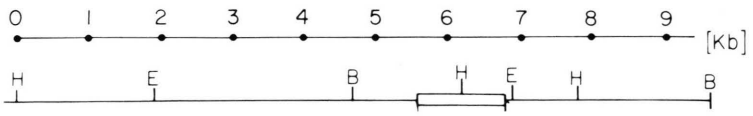


Fig. 1. Location of the *psbA* gene (box) on a partial restriction map of a chloroplast DNA of *P. paradoxa.* H – *Hind*III; E – *Eco*RI; B – *Bam*HI.

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AAT GAG GGT TAC AAA TTT GGT CAA GAG GAA GAG ACT TAT AAT ATT GTG GCT GCT CAT GGT
Asn Glu Gly Tyr Lys Phe Gly Gln Glu Glu Glu Thr Tyr Asn Ile Val Ala Ala His Gly
                240                                                       250

                                                        GGT
                                                        Gly
TAT TTT GGC CGA TTA ATC TTC CAA TAT GCT AGT TTC AAC AAC TCT CGT TCT TTA CAC TTC
Tyr Phe Gly Arg Leu Ile Phe Gln Tyr Ala Ser Phe Asn Asn Ser Arg Ser Leu His Phe
                260                                                       270

TTC TTG GCT GCT TGG CCT GTA GTA GGT ATC TGG TTC ACT GCT TTA GGT ATT AGT ACT ATG
Phe Leu Ala Ala Trp Pro Val Val Gly Ile Trp Phe Thr Ala Leu Gly Ile Ser Thr Met
                280                                                       290
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Fig. 2. Nucleotide sequence and deduced amino acid sequence of a 180 bp region of the *psbA* gene from *P. paradoxa.* Numbers refer to amino acid residues from the initiation Met. The boxed codon indicates the only difference found between *psbA* genes of the R and S biotypes. The dotted box marks the lysine residue at position 238.

er plants [7–9, 32]. The same serine residue is substituted by alanine in diuron-resistant mutants of *Chlamydomonas* [10], and in the cyanobacterium *Anacystis nidulans* R2 [11, 12]. In all these cases, except for cyanobacteria, the mutation also alters the electron flow in PS II. Unlike higher plants and algae the $Q_B$-protein of *Anacystis nidulans* contains lysine at position 238. We have found a lysine residue at the same position also in *Phalaris paradoxa*. Since this region of the polypeptide is assumed to be part of, or close to the herbicide binding site [33], it is possible that the substitution of lysine for arginine at position 238 in the $Q_B$-protein somehow reduces the effect that the mutation at serine 264 has on the $Q_A$ to $Q_B$ electron transport in PS II.

## Conclusions

Triazine resistance caused by a point mutation in the *psbA* gene was previously shown to be accompanied by detrimental effects on photosynthesis and plant growth. It was accordingly assumed that these characteristics are inherent features of the resistance trait. The results presented here indicate that the very same mutation in the *psbA* gene of *P. paradoxa* resulted in triazine resistance but was not accompanied by low photosynthesis rates or reduced growth. It is concluded that this type of triazine resistance is not necessarily linked to lower photosynthetic performance.

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