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# UNIPARENTAL INHERITANCE OF A CHLOROPLAST PHOTOSYSTEM II POLYPEPTIDE CONTROLLING HERBICIDE BINDING

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## Summary

The ability of atrazine to inhibit Photosystem II electron transport and the rate of electron transfer from the primary to the secondary quinone electron acceptors in the Photosystem II complex were examined in triazine-resistant and -susceptible parental biotypes of *Brassica campestris* L. and their $F_1$ progeny derived from reciprocal crosses. The lack of herbicide inhibitory activity and the presence of functional properties which decreased the $Q^-$ to B electron transport rate constant were inherited in parallel through the maternal parent. We conclude that the herbicide receptor protein is uniparentally inherited through the female parent. These data are discussed in relation to other studies which indicate that the binding site is a 32 000-dalton polypeptide which determines the functional properties of B (the secondary Photosystem II electron acceptor).

## Introduction

The chloroplast is a semi-autonomous organelle; its genome codes for ribosomal components, messenger and transfer RNA, as well as several polypeptides

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Abbreviations: Atrazine, 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine; B, secondary electron acceptor of Photosystem II (bound quinone), a plastoquinone-protein complex; C, 'Candle', commercial rapeseed cultivar of *Brassica campestris* L.; DCIP, dichlorophenolindophenol; Q, primary electron acceptor of Photosystem II (quencher); R, triazine-resistant biotype of *B. campestris*; S, triazine-susceptible biotype of *B. campestris*; Chl, chlorophyll.

which play functional roles in the photosynthetic process [1,2]. Several research laboratories are currently attempting the identification of specific chloroplast gene products, as well as investigating the regulation of their synthesis and the control of their integration into functional photosynthetic components. To date, however, the only polypeptide for which a specific function in the photosynthetic process is known and which has been mapped to the chloroplast genome, is the large subunit of ribulose-1,5-bisphosphate carboxylase [3].

It has been suggested from mutational studies and/or protein synthesis analysis in the presence of selective inhibitors, that some polypeptides comprising the Photosystem II complex are products of chloroplast DNA [4—8]; however, the specific function of the individual proteins has not been elucidated. An approach to identifying at least one of these polypeptides has recently become available through the appearance of plant species which are insensitive to triazine herbicides.

Atrazine, the most carefully characterized chemical in the triazine herbicide family (with respect to mode of biochemical action) has been shown to specifically inhibit electron transport at the level of a bound plastoquinone molecule. which serves as the cofactor of the second electron carrier (B) on the reducing side of Photosystem II [9—11]. Onset of inhibition of electron transport requires the non-covalent binding of the atrazine molecule to a polypeptide thought to be the apoprotein of B [11]. Analysis of binding indicated that only one inhibitor molecule occupies each binding site [12]. Mild trypsin treatment of either intact chloroplast membranes or isolated Photosystem II particles results in the loss of the herbicide-binding site, thus indicating the involvement of a protein receptor [13—16].

Recent studies have demonstrated that chloroplast membranes in triazine-resistant weeds have been modified such that the triazine binding site is selectively lost [11,12]. Chloroplasts containing this genetic alteration continue to function photosynthetically, but a subtle change in their electron transport properties appears; the rate of electron transfer in Photosystem II from the primary electron acceptor (Q) to the secondary electron acceptor (B) in the resistant chloroplasts is reduced by more than a factor of 10 [10,11].

Since the alteration in the Photosystem II complex of chloroplast membranes from triazine-resistant weed biotypes can be characterized by (1) atrazine inhibition of Photosystem II partial reactions [11,17], and (2) measurement of $Q^-$ to B electron transport via chlorophyll fluorescence detection during either induction transients or after flash illumination [10,11,17], we have used these parameters as biochemical markers to characterize the inheritance of the modified atrazine receptor (protein binding site) in chloroplast membranes. In previous studies, 'whole plant' atrazine resistance in progeny from reciprocal crosses between susceptible and resistant *Brassica campestris* L. has shown uniparental inheritance through the female parent [18]. Our goal in the current study was to determine whether atrazine resistance by intact seedlings correlates with inherited resistance to triazines at the level of chloroplast membrane components.

## Materials and Methods

Three biotypes of *Brassica campestris* and $F_1$ progeny from six reciprocal crosses were examined for herbicide sensitivity. The susceptible and resistant weed biotypes were originally collected from fields in Quebec. 'Candle' is an atrazine-susceptible Polish rapeseed cultivar of *B. campestris*. The reciprocal crosses between resistant and susceptible individuals were performed as described earlier [18]. The identification system utilized herein refers to individual plants within each biotype by number.

Seedlings were grown in a soil mixture in a growth chamber (24°C, 16 h photoperiod) for 3—4 weeks. Chloroplasts were isolated by grinding 3—4 fully mature leaves in a Waring blender in 30 ml of isolation solution (100 mM Tricine-NaOH, pH 7.8, 400 mM sorbitol, 10 mM NaCl, 2 mM sodium ascorbate, 3 mM $MgCl_2$). This mixture was filtered through four layers of cheesecloth and centrifuged at 1500 × *g* for 5 min. The pellet was resuspended in a wash solution (10 mM Tricine-NaOH, pH 7.8, 10 mM NaCl, 5 mM $MgCl_2$) to ensure complete lysis of the chloroplast envelope and centrifuged again. The final stroma-free thylakoids were resuspended (10 mM Tricine-NaOH, pH 7.8, 100 mM sorbitol, 10 mM NaCl, 5 mM $MgCl_2$) and the chlorophyll concentration was determined by the method of Arnon [19].

*Inhibition of electron transport*

Photosynthetic electron transport rates from water to DCIP were measured in the presence of various concentrations of atrazine. Twenty μg of chlorophyll was added to a 2-ml volume of reaction solution (50 mM sodium phosphate, pH 6.8, 10 mM NaCl, 5 mM $MgCl_2$, 100 mM sorbitol, 1 mM $NH_4Cl$, $10^{-7}$ M gramicidin D, 0.03 mM DCIP). DCIP reduction was monitored in an Hitachi Model 100-60 spectrophotometer at 580 nm. The suspension was cross-illuminated with actinic light filtered through a red Corning 2-58 filter. The phototube was shielded from scattered actinic light by a blue Corning 4-96 filter. Aliquots of standard atrazine solutions ($10^{-6}$ to $10^{-2}$ M in ethanol) were added to each chloroplast suspension immediately prior to illumination; maximum ethanol concentration in the reaction solution was 0.5%. An extinction coefficient of 18 cm/mM was used to calculate the rates of DCIP reduction.

*Measurement of chlorophyll fluorescence*

Two ml of reaction mix (5 μg/ml Chl, 10 mM Tricine-NaOH, pH 7.8, 100 mM sorbitol, 10 mM NaCl, 5 mM $MgCl_2$) were illuminated with blue actinic light (Corning 4-96 filter). Chlorophyll fluorescence induction transients were monitored at a right angle to the actinic source through a red Corning 2-64 filter. The fluorescence was detected by a photodiode assembly previously described [20]. The changing voltage signal from the diode was recorded using a Nicollet Explorer III digital oscilloscope. Traces were either photographed from the screen or were recorded on a *x-y* plotter.

Measurements of changes in Chl fluorescence yield after flash illumination (30 ns laser pulse) were carried out as previously described [10]. The sample was flashed 4- to 6-times before measurements began to establish a randomized

reduction state of the secondary Photosystem II electron acceptor. Dark intervals between the actinic flash and the weak measuring flash ranged from 50 μs to 10 ms (as indicated in text or figures). The reaction mixture was identical to that used for the measurement of fluorescence induction.

## Results

### *Inhibition of electron transport*

Chloroplasts isolated from atrazine-susceptible but not atrazine-resistant weed biotypes have been shown to exhibit atrazine-sensitive Photosystem II electron transport [11,12,17]. The three parent biotypes of *B. campestris* used in these studies were consistent with this observation (Fig. 1). Both Candle and the susceptible biotype showed 50% inhibition at atrazine concentrations of 0.1 to 0.3 μM. Chloroplasts isolated from the resistant biotype, in contrast, were only approx. 40% inhibited at 100 μM atrazine (the maximum concentration tested due to water solubility of the inhibitor).

The effects of atrazine on Photosystem II mediated electron transport in chloroplasts from $F_1$ progeny derived from one reciprocal cross are shown in Fig. 2. A summary of the $I_{50}$ values (the atrazine concentration giving 50% inhibition of the stated reaction) from other crosses are summarized in Table I. Chloroplasts from the progeny with susceptible maternal parents all showed a low $I_{50}$ value, while those from plants with resistant maternal parents exhibited only limited inhibition and high $I_{50}$ values. Variation existed in the $I_{50}$ values of susceptible $F_1$ progeny, particularly with Candle. This may be due to slight non-uniformity in the genetic background of the parental pools. To overcome problems with self incompatibility, the cultivar Candle comprises a mixture of genetic lines. However, the overall large differences in $I_{50}$ values between resistant and susceptible plants are clearly evident.

The middle curve of Fig. 2 demonstrates the biphasic pattern of inhibition produced by a range of concentrations of atrazine added to a reaction mixture

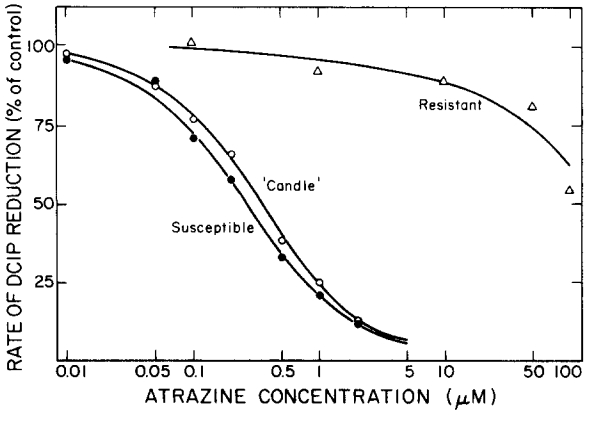


**Fig. 1. Atrazine inhibition of Photosystem II-dependent electron transport ($H_2O \rightarrow$ DCIP) in resistant and susceptible biotypes and the cultivar 'Candle'. The rate of DCIP reduction was expressed as the percentage of the rate in an herbicide-free control. Control rates of DCIP reduction (μmol · $mg^{-1}$ Chl · $h^{-1}$) were: S, 389; C, 432; R, 397.**

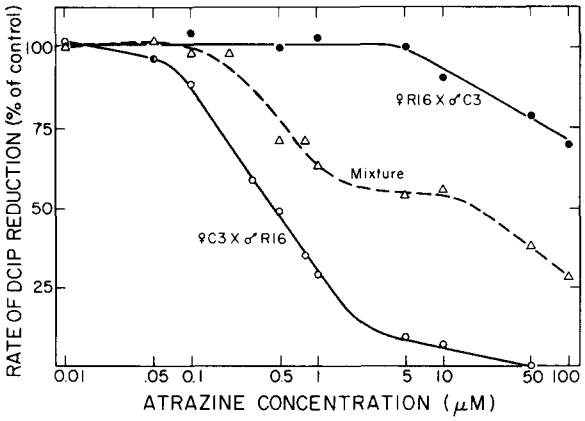


**Fig. 2. Atrazine inhibition of Photosystem II-dependent electron transport ($H_2O \rightarrow$ DCIP) in a reciprocal cross. The rate of DCIP reduction was calculated as in Fig. 1. R, resistant; C, Candle. The sample labeled 'mixture' consisted of 50% susceptible and 50% resistant chloroplasts, on a chlorophyll basis. Control rates of DCIP reduction ($\mu$mol · mg$^{-1}$ Chl · h$^{-1}$) were C3 X R16, 396; R16 X C3, 280; mix, 343.**

containing equal amounts of resistant and susceptible chloroplasts. This implies that a heterogenous population of chloroplasts does not produce a simple monophasic inhibition curve. It therefore appears that the $F_1$ plants contain a homogeneous population of chloroplasts all with sensitivity toward atrazine similar to that of the female parent (Fig. 1).

*Chlorophyll fluorescence induction measurements*

Measurements of room temperature chlorophyll fluorescence inductions in

TABLE I

ANALYSIS OF PHOTOSYSTEM II PROPERTIES IN ISOLATED CHLOROPLASTS

$I_{50}$ values were determined from herbicide dosage assays as shown in Fig. 1. $\Delta F_I/\Delta F_M$ values were determined from records of induction transients. Genetic nomenclature: R16 = individual 16 from the resistant biotype. $I_{50}$, the atrazine concentration giving 50% inhibition of the stated reaction.

| Sample | $I_{50}$ (M) | $\Delta F_I/\Delta F_M$ |
|---|---|---|
| A. Parential plants | | |
| Resistant (R) | $>1 \cdot 10^{-4}$ | 0.44 |
| Susceptible (S) | $1.8 \cdot 10^{-7}$ | 0.18 |
| Candle (C) | $3.0 \cdot 10^{-7}$ | 0.20 |
| B. Reciprocal crosses | | |
| R16 X C3 | $>1 \cdot 10^{-4}$ | 0.38 |
| C3 X R16 | $4.5 \cdot 10^{-7}$ | 0.22 |
| R17 X C4 | $>1 \cdot 10^{-4}$ | 0.41 |
| C4 X R17 | $7.5 \cdot 10^{-7}$ | 0.20 |
| R19 X C6 | $>1 \cdot 10^{-4}$ | 0.43 |
| C6 X R19 | $2.0 \cdot 10^{-7}$ | 0.17 |
| R24 X S1 | $>1 \cdot 10^{-4}$ | 0.49 |
| S1 X R24 | $3.5 \cdot 10^{-7}$ | 0.20 |
| R25 X S2 | $>1 \cdot 10^{-4}$ | 0.44 |
| S2 X R25 | $2.5 \cdot 10^{-7}$ | 0.18 |
| R26 X S3 | $>1 \cdot 10^{-4}$ | 0.44 |
| S3 X R26 | $4 \cdot 10^{-7}$ | 0.15 |

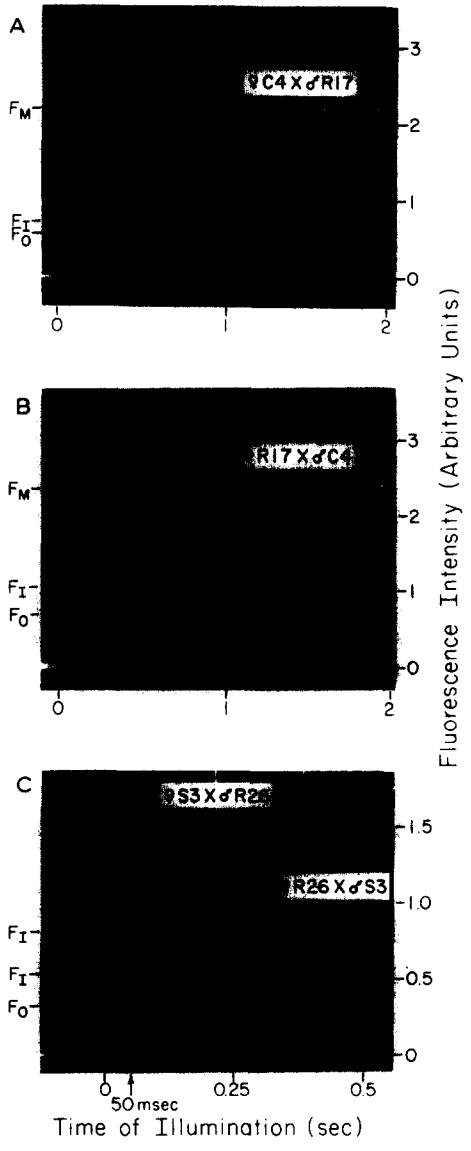


**Fig. 3. Fluorescence transients in resistant and susceptible $F_1$ progeny. R, resistant; S, susceptible; C, Candle. (A and B) Complete transient records of progeny of a reciprocal cross. These transients were recorded on a Nicollet Explorer III oscilloscope and photographed. $F_O$, $F_I$ and $F_M$ levels are noted. (C) Superimposed and expanded transients from progeny of another reciprocal cross. The arrow on the X axis indicates the point at which $F_I$ was measured.**

chloroplasts isolated from the susceptible and resistant plants yield strikingly different transients [11,17]. The resistant plants show a significantly higher intermediate level of fluorescence ($F_I$) than do the susceptible plants. A comparison of the fluorescence rise in chloroplasts isolated from the $F_1$ progeny of reciprocal crosses between resistant and susceptible biotypes is shown in Fig. 3. The data shown are photographs of the transient signals recorded on an oscilloscope screen. In Fig. 3A, the maternal parent was triazine-susceptible; in Fig.

3B the maternal parent was resistant. Both transients show nearly equivalent $F_O$ (original) and $F_M$ (maximal) fluorescence as well as a similar area above the rise curves. These characteristics indicate a high degree of similarity in the light-harvesting pigment bed and electron acceptor pools in the two samples (for discussions relating to interpretation of fluorescence transients, see Refs. 17, 21 and 22). The only obvious difference in the two transients was in the intensity of fluorescence at the $F_I$ (intermediate) level; this is shown in the expanded transients of Fig. 3C. It should be noted that while a higher $F_I$ level is attained in the progeny from a resistant maternal parent, there is a slower rise in the final phase of the transient to a $F_M$ level. This results in a cross-over of the rise-curve at a point indicated in Fig. 3C by the intersection of the vertical and horizontal cursors.

A quantitative evaluation of fluorescence rise curves is possible by calculating the ratio of intensity of fluorescence at $F_I$ to that at $F_M$. The value generated by the expression:

$$\Delta F_I/\Delta F_M = (F_I - F_O)/(F_M - F_O)$$

represents the proportion of the total variable fluorescence yield which occurs during the rapid (intermediate) phase of the fluorescence rise. $F_I$ in this equation is taken as the fluorescence intensity at the beginning of the linear intermediate portion of the transient. The $\Delta F_I/\Delta F_M$ values for chloroplasts from all plants examined in this study are presented in Table I. In all cases where the samples showed resistance to atrazine in electron transport assays, the $\Delta F_I/\Delta F_M$ value was greater than 0.4. In contrast, the triazine-susceptible samples gave values near 0.20.

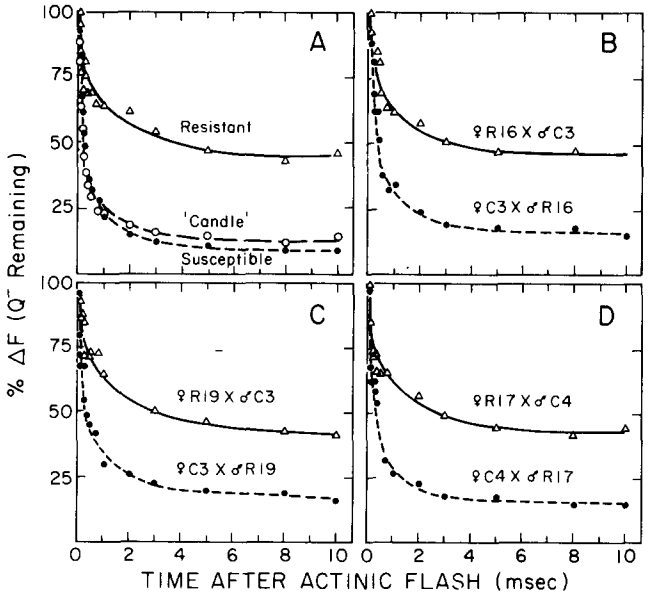


**Fig. 4. Fluorescence intensity at intervals after short flash excitation. Data points shown are variable fluorescence expressed as percentage of fluorescence at the earliest time point (0.05 ms). (A) The decay of fluorescence in resistant and susceptible biotypes and the cultivar Candle. (B—D) similar fluorescence decay measurements in three resistant (R) X Candle (C) reciprocal crosses.**

*Analysis of chlorophyll fluorescence after short flash excitation*

The level of chlorophyll fluorescence detected at various time intervals (50 μs—10 ms) after an intense, saturating flash has previously been shown to be a direct measure of the rate of reoxidation of the primary Photosystem II electron acceptor [23—26]. We have reported that the rate of Q to B electron transport assayed by this technique in chloroplasts isolated from atrazine-resistant biotypes of *Amaranthus retroflexus* L. [10], *Ambrosia artemisiifloria* L. and *Chenopodium album* [11] is slower than the rate of Q to B electron transport in chloroplasts isolated from susceptible biotypes of the same species. A similar trend was observed with the three parental types of *Brassica campestris* (Fig. 4A).

Analysis of Q to B electron transfer in chloroplasts from progeny of reciprocal crosses between triazine-resistant and susceptible biotypes (Fig. 4 B—D) showed similar kinetic characteristics. For any given sample, the kinetics of electron transfer paralleled that of the maternal parent.

## Discussion

The appearance of triazine resistance in numerous weed biotypes, including *Brassica campestris*, has been shown to be the result of a dramatic decrease in herbicide affinity at the triazine-binding site in the Photosystem II complex [11,12]. This alteration results in insensitivity of Photosystem-II mediated photochemistry to triazines in isolated chloroplast thylakoid preparations from the resistant weeds (Fig. 1 and Refs. 11, 12, 17). In addition, the thylakoid alteration responsible for the decreased herbicide binding has been correlated with a subtle change in the functioning of electron carriers on the reducing side of Photosystem II; this can be detected via chlorophyll fluorescence analysis. A decrease in the rate of $Q^-$ to B electron transfer is indicated by a high intermediate ($F_I$) level during chlorophyll fluorescence induction transients (Fig. 3B; Refs. 11, 17). The $Q^-$ oxidation rate was quantitatively shown to be more than 10-fold slower in resistant chloroplasts via measurement of onset of chlorophyll fluorescence quenching after flash illumination (Fig. 4A and Refs. 10, 11). We have previously concluded that this correlation between loss of herbicide-binding site and altered electron transport kinetics of B indicates that the herbicide-binding site is contained on the apoprotein of B [11]. An alteration in this polypeptide could simultaneously change the triazine-binding site as well as modify the microenvironment of the bound plastoquinone cofactor of B, thereby altering its redox properties (see further discussion in Ref. 10).

In reciprocal crosses, examined herein, we have monitored atrazine inhibition of electron transport as a measure of herbicide binding. In all cases, the $F_1$ progeny displayed herbicide sensitivity nearly identical to that of the maternal parent (Fig. 2, Table I). It should be noted that all chloroplasts from a given plant displayed a uniform phenotype with respect to triazine sensitivity; heterogeneous populations of plastids in the assays would have shown biphasic or multiphase patterns in herbicide dosage response assays. This was observed in mixed preparations of isolated chloroplasts (Fig. 2).

We have used the two methods for analysis of chlorophyll fluorescence described above to characterize Q to B electron transfer in the chloroplasts

from $F_1$ progeny of reciprocal crosses. Direct measurement of $Q^-$ oxidation was used in analysis of Candle (C) × resistant (R) biotype crosses; in all cases the chloroplast samples which displayed atrazine resistance in electron transport assays (Table I) showed a much slower reoxidation rate of $Q^-$ (i.e., 50% reappearance of the fluorescence quencher, Q at times $\geqslant 3$ ms in resistant chloroplasts vs. $\leqslant 0.5$ ms in the susceptible chloroplasts) (Fig. 4). This phenomenon was more easily analyzed by monitoring the $\Delta F_I/\Delta F_M$ value of the fluorescence induction transients in all $F_1$ progeny. Again, without exception, the slow reoxidation of $Q^-$, which results in an elevated $F_I$ fluorescence level, was detected in all chloroplasts from plants derived from a resistant maternal parent (Table I).

On the basis of these studies, inheritance of atrazine resistance at the chloroplast membrane appears to be uniparental through the female parent. This agrees with the studies of inheritance of 'whole plant' atrazine resistance [18, 27] where no segregation was observed in the $F_2$ and backcross progeny of these crosses. We conclude that inheritance of this trait both in whole plants and at the level of the chloroplast membrane, is strictly maternal.

In recent studies, we have utilized a radioactive, photoaffinity-labeled atrazine-analog to covalently tag a polypeptide which creates the triazine-binding sites [28]. This polypeptide has an apparent molecular weight of 32 000. It is present in both susceptible and resistant biotypes of chloroplasts but binds the analog only in susceptible membranes [28]. Based upon protease sensitivity, the polypeptide is surface-exposed in intact chloroplast membranes; trypsin cleavage of a 32-kdalton polypeptide is accompanied by onset of an irreversible block in electron transport at the level of B and concomitant loss of atrazine binding sites [13]. This evidence leads to the conclusion that the 32-kdalton polypeptide, which contains the triazine binding site, is a functional component of the Photosystem II complex. This protein may be either the apoprotein of B or a closely associated polypeptide constituent of the Photosystem II complex that directly influences the functional properties of B.

Our genetic analyses indicate that atrazine resistance is inherited uniparentally through the female parent. This indicates that the structural gene encoding the 32-kdalton protein, identified as the triazine receptor, is maternally inherited. Although on the basis of these data the effect of maternal nuclear DNA cannot be conclusively eliminated, these observations suggest that this 32-kdalton protein is coded for by chloroplast DNA.

It will be of interest to determine if there are similarities between the atrazine-binding protein and the previously described chloroplast synthesized 'peak D' polypeptide, the 'photogene-32' product, or the 'rapidly turned-over chloroplast thylakoid protein' which have previously been reported to be of approx. 34—32 kdaltons in chloroplast-directed protein synthesis studies [30—34]. In the case of maize, the structural gene for a 34-kdalton polypeptide has been localized to chloroplast DNA [34].

It has not escaped our attention that the identification of a specific chloroplast gene-product coding for herbicide resistance has both agronomic and basic research implications. The triazine-resistance trait should prove to be a useful positive-selection tool in future genetic modification experiments using cell cultures. In addition, ongoing studies in which the resistance trait has been trans-

ferred to commercial *Brassica* species (cross-fertilized with the resistant biotypes) have shown persistence of the trait over seven generations [35]. The success of gene transfer via traditional techniques demonstrates that the trait has sufficient stability to warrent attempts at novel gene transfer to other species.

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