Atrazine Resistance in Chenopodium album

LOW AND HIGH LEVELS OF RESISTANCE TO THE HERBICIDE ARE RELATED TO THE SAME CHLOROPLAST PSBA GENE MUTATION

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ABSTRACT

In Chenopodium album two different levels of atrazine resistance have been found according to following criteria: lethal dose and leaf fluorescence curve. The intermediate (I) phenotype is represented by a low level of resistance and a typical I fluorescence curve. It arose at high frequency, within one generation, after self-pollination of particular plants displaying a susceptible (S) phenotype. The resistance phenotype (R) has a high level of resistance and presents a typical resistant fluorescence curve. It appeared after self-pollination of chemically treated I plants. The I, R, and also R (resistant plants found in atrazine treated fields) phenotypes contain a serine to glycine mutation at amino acid position 254 in the chloroplast psbA gene product. The steady state level of the psbA gene transcript is not modified between S, I, R, and R phenotypes.

High levels of resistance to atrazine (2-chloro-4-ethylamine-6-[isopropylamino]-s-triazine) have been correlated with a single amino acid substitution (Ser to Gly) in higher plants at position 264 in the M, 32,000 chloroplast thylakoid membrane protein (16, 20, 21). A change of Ser to Ala at the same position in this protein is correlated with resistance to lower levels of atrazine and diuron in an algal mutant (7) and a cyanobacterial transformant (15). Atrazine competes with plastocyanine for binding to a 32 kD thylakoid protein (termed the Qh-protein) where an azido-derivative of atrazine has been shown to bind specifically (27). Thus, atrazine inhibits photosynthetic electron transport in PS II by preventing the formation of Qh. In resistant plants and algae, atrazine does not bind to the Qh-protein (7, 17, 27). Two mutants of Chlamydomonas (10) displaying lower levels of resistance to either atrazine or diuron (DCMU) have been correlated with two different amino acid substitutions in the 32-kD protein (8). Lower levels of atrazine resistance and cross-resistance to different herbicides have also been observed in some higher plants (18). Three levels of resistance to atrazine have previously been found in Chenopodium album: low (S), intermediate (I), and highly resistant (R) (Table 1). Intermediate I plants have originated spontaneously from particular susceptible genotypes and display a 10-fold increase of resistance at the plant level. However, isolated I thylakoids showed a 1000-fold increase of resistance, similar to that of the R plants (14). Moreover, the fluorescence curve of I leaves having absorbed atrazine showed a secondary peak called M, 2 min after onset of illumination (12). These characteristics were maternally inherited but I plants treated with a sublethal dose of atrazine produced 100% true R descendants (12). Such R-induced plants showed more than 400 times higher resistance at the seedling stage and the typical R fluorescence curve without the M peak. Since these plants are isogenic, genealogically related, and differentially resistant to atrazine, they represent an ideal model to study atrazine resistance. In this paper, by using genetic and molecular approaches, we show that the I to R transition most probably involves an extrachloroplastic genetic mechanism.

MATERIALS AND METHODS

Plant Isolation and Culture. A total of 371 plants of Chenopodium album L. were collected at the seedling stage in a garden never treated with atrazine or any herbicide, near Dijon (Burgundy, France), then transferred to a greenhouse. One leaf per plant was cut and left to absorb atrazine in the dark before recording the Chl fluorescence which indicated that all the plants were susceptible (S curve: 6, 13, 14). Another leaf was used for the isozyme analysis as previously reported: a total of 35 phenotypes were found in this population (1). Chromosome counts were carried out on root tips using the technique of Feulgen; all the 371 plants examined were haploid (2n = 54). Thereafter the plants were bagged and left to flower by self-pollination. Part of the progeny of each plant was (a) treated with 1 kg ha−1 atrazine, (b) treated with 0.5 kg·ha−1 atrazine, (c) kept free from herbicide contact and studied by leaf Chl fluorescence. Treatment with 1 kg ha−1 atrazine resulted in the death of all seedlings. In contrast, when progenies were treated with 0.5 kg·ha−1 atrazine, up to 12% of the descendants survived in 25 out of the 371 progenies. Fluorescence studies on individual leaves of non-treated plants showed that within the 25 plant lines which produced I plants with resistance to atrazine several descendants had fluorescence patterns characteristic of the I phenotype. The 25 plants from which the I descendants were obtained (with an average frequency of 4%) displayed the same isoenzymatic pattern (phenotype No. 3) (1) and were called Sp (susceptible precursor). The other susceptible plants of the population were used as the S control. Resistant plants (R) were found in a maize
The different biotypes (S–R), their origins, the concentrations of atrazine that are lethal on soil growing seedlings, their leaf fluorescence curves, the nature of their progenies and the atrazine dose necessary for 50% inhibition (DS50) of thylakoid activity (Hill reaction) are described. S, Sp, I, R, and R biotypes of *C. album* were isolated as previously described (14) and summarized in the “Materials and Methods” section. Fluorescence curves (S, M, R) are labeled according to Ref. 13.

<table>
<thead>
<tr>
<th>Biotype</th>
<th>Origins</th>
<th>Atrazine Lethal Dose kg/ha</th>
<th>Leaf Fluorescence Curve</th>
<th>DS 50% of Thylakoids</th>
<th>Nature of the Progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>S (95% of the wild population)</td>
<td>Garden (never treated with chemicals)</td>
<td>0.15</td>
<td>S</td>
<td>$2.0 \times 10^{-7}$</td>
<td>100% S</td>
</tr>
<tr>
<td>Sp (5% of the wild population)</td>
<td>Same garden</td>
<td>0.15</td>
<td>S</td>
<td>95% Sp</td>
<td>5% I</td>
</tr>
<tr>
<td>I</td>
<td>Self-pollination of Sp</td>
<td>1.00</td>
<td>M</td>
<td>$0.9 \times 10^{-4}$</td>
<td>100% I</td>
</tr>
<tr>
<td>li</td>
<td>I treated with a sublethal concentration of Atrazine</td>
<td>4.00</td>
<td>R</td>
<td>$1.1 \times 10^{-4}$</td>
<td>100% Ri</td>
</tr>
<tr>
<td>Ri</td>
<td>Self-pollination of li (greenhouse)</td>
<td>4.00</td>
<td>R</td>
<td>$1.1 \times 10^{-4}$</td>
<td>100% R</td>
</tr>
</tbody>
</table>

Field treated for 5 years with atrazine. Intermediate plants isolated as in (c) bred true when selfed (their progeny were 100% I) and their atrazine resistance characteristics were maternally inherited in crosses with S plants (14). Seedlings of the I genotype were sprayed at the two-leaf stage with atrazine, monolinuron (3-[pchlorophenyl]-1-methoxy-1-methylurea) and hydroxyatrazine (2-hydroxy-4-ethylamino-6-isopropylamine-s-triazine) at doses shown in Table II. All plants were bagged during flowering to prevent cross-pollination. Progeny of these treated plants was sprayed with 1 kg·ha⁻¹ atrazine to discriminate between I and R phenotypes. The resistant phenotype obtained from a treated I plant (li) was called Ri (induced resistant). All these biotypes (S, Sp, I, li, R, Ri) of *C. album* were cultivated in a greenhouse under controlled temperature and light conditions (16 h light at 23°C, 8 h dark at 18°C).

**Inheritance and Chloroplast Properties.** The Ri progeny of I plants treated at the two-leaf stage with a dose of 0.5 kg·ha⁻¹ atrazine was grown in a greenhouse and self-pollinated. The subsequent progenies were then selfed until the fourth generation (G₄). Fifteen progenies, of 1000 seedlings each, were treated at each generation to test the level of resistance (I or R). Reciprocal crosses between Ri and S plants were performed by open pollination. Because no distinct genetic marker exists between I plants and their direct isogenic Ri descendant, these crosses were performed by using Ri plants previously obtained from a different population (Loire Valley, France) (12) with a distinct isozymic genotype. Hybrids were identified by isoenzyme analyses as shown by the combination of the two parental isozyme patterns (14). The rapid phase of the initial fluorescence rise of dark adapted leaves of the Sp, I, and Ri plants was recorded as previously described (13, 26). Results were expressed as the ratio of the initial fluorescence rise ($F - F_o$) to the original fluorescence ($F_o$).

**Chloroplast DNA Isolation and psbA Gene Cloning.** Chloroplasts were isolated from leaves of *Chenopodium album* as previously described (5). The purified chloroplast pellets, after treatment with DNAse I, were lysed in 50 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1 mg/ml autopurified pronase, 2% n-lauroyl sarcosine, and left to stand overnight at 4°C. Ethidium bromide (300 μg/ml) and solid CsCl (up to 1.5 g/ml) were added to the lysates before centrifugation at 20°C for 48 h at 42,000 rpm (Beckman rotor 50 Ti). The band exhibiting UV fluorescence was recovered and dye removed with CsCl saturated butanol. The DNA was then precipitated with ethanol, centrifuged and vacuum dried, and the pellet was finally redissolved in 50 mM Tris-HCl (pH 8.0), 0.5 mM EDTA. A shotgun cloning in plasmid pBR325 of the Hind III chloroplast DNA fragments from the five different biotypes of *C. album* was performed as described (19). Recombinant clones were identified by their positive hybridization with a spinach psbA probe. This probe is a subclone from a larger *Bam*HI 5 kb fragment from spinach kindly provided by H. Bohnert (Tucson, AZ). Restriction mapping has been performed as previously described (5).

**DNA Sequence Strategy.** Nucleotide sequencing of the two strands was performed by M13 cloning and dyeideoxy sequencing methods as already described (25). The cloned HindIII 900 bpfragments were further restricted with *Hae*III and the 350 bp *Hind*III-*Hae*III and the 65 bp *Hae* III fragments were eluted from acrylamide gel and cloned into the appropriate sites of phage M13mp18 (23). DNA was electrophoresed on 8% polyacrylamide gels (0.4× 550×250 mm) in TBE buffer (89 mM Tris, 89 mM boric acid, 20 mM EDTA) at 18 to 25 mA using an LKB 2010-001 Macrophor Electrophoresis Unit.

**Northern Analysis.** RNA was prepared from young green untreated seedlings of *C. album* Sp, I, Ri biotypes. In the case of the li biotypes, RNA was extracted from leaf chloroplasts 2 weeks after treatment with 0.5 kg·ha⁻¹ atrazine on I plants. Leaves were immersed in a 5% sodium hypochlorite solution, rinsed extensively with distilled water, and dried on 3MM filters. Chloroplasts were purified as previously described except that experiments were performed using sterile glassware and media. The isolated chloroplasts were resuspended in 4 M guanidinium thiocyanate, 0.2 M Tris-HCl (pH 8.0), 2% n-lauroyl sarcosine, and 0.1% mercaptoethanol. RNA was pelleted by centrifugation through a cushion of 5.2 M CsCl, 0.1 M EDTA for 12 h at room temperature in a Ti50 rotor at 40,000 rpm. The RNA pellet was resuspended in 10 mM Tris HCl (pH 7.4), 5 mM EDTA, 1% SDS, and precipitated with 2 volumes of ethanol. RNA was finally resuspended in 10 mM Tris HCl (pH 8.0), 1 mM EDTA. For Northern experiments, 3 μg of RNA for each sample was electrophoresed in denaturing agarose-formaldehyde gels as previously described (29) and transferred onto a Genescreen mem-

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1 Abbreviation: bp, base pair.
brane filter (Genescreen, New England Nuclear). Hybridization with the spinach HindIII 900 bp probe and rinsing of filters were performed as described by the manufacturer. Filters were then exposed to Kodak x-ray films.

**Mae I Restriction Site as a Marker for the Ser264 to Gly264 Mutation.** Chloroplast DNA from individual, well-defined biotypes was isolated as previously described. Chloroplast DNA (3 μg) was digested by MaeI (C TAG) in the presence of 250 mM NaCl, 5 mM spermidine for 4 h at 45°C. Eppendorf tubes were submerged in a water bath to prevent evaporation during digestion. Prior to loading on the 1.5% agarose gel, the samples were diluted 2-fold with distilled water. After electrophoresis the Mae I digested ctDNA was transferred to Genescreen membrane filters, baked at 80°C, and subsequently hybridized to the radio-labeled 900 bp spinach probe.

**RESULTS**

**Genetic Analysis and Chloroplast Properties.** The progeny of I plants treated with different chemicals (Table II) all exhibit the R phenotype. In contrast, neither I nor R plants appeared as progeny of S when treated with sublethal doses of the chemicals. The R phenotype was maintained in Ri progenies at least through four generations of self-pollination in the absence of selective pressure, and no revertants were observed. The Ri characteristics were maternally inherited in crosses with the S genotype, exactly as was found with R plants growing in maize fields. However, a genetic disjunction was found in the F2 generation of the I x Ri crosses (Table III). Maternal inheritance was first observed in the F1 of these crosses, but the F2 segregated I and R phenotypes in such a way that the maternal phenotype had the highest frequency. The S phenotype has never been found in these F2. This inheritance pattern suggests either an incomplete cytoplasmic

**Table II. Progeny Phenotypes Obtained after Treatment of I Plants with Atrazine at the Seedling Stage**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>No. of I Plants Treated</th>
<th>Frequency of I and R Descendants in Progeny</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemical</td>
<td>Dose (kg ha⁻¹)</td>
<td>I</td>
</tr>
<tr>
<td>Atrazine</td>
<td>0.5</td>
<td>22</td>
</tr>
<tr>
<td>Hydroxyatrazine</td>
<td>0.5</td>
<td>12</td>
</tr>
<tr>
<td>Hydroxyatrazine</td>
<td>4.5</td>
<td>12</td>
</tr>
<tr>
<td>Monolinuron</td>
<td>0.5</td>
<td>20</td>
</tr>
</tbody>
</table>

**Table III. Inheritance of Atrazine Resistant Phenotype after Reciprocal Crosses**

Number of plants obtained for each phenotype are indicated. Phenotypes were determined by the fluorescence test for all the F1 and the F1 of the I x Ri crosses, by seedlings treatment with atrazine at 0.5 kg ha⁻¹ for the F2 of the S x R crosses.

**Fig. 1.** Fluorescence records of dark adapted leaves of the Sp (- - -), I (-- -), and Ri (---) genotypes of *C. album*. Arbitrary units for the fluorescence yield; two scale for the time: ms during 500 ms, then s.

**Fig. 2.** Restriction map of the *C. album* chloroplast DNA region containing the psbA gene. The start and termination codons are labeled by NH₂ and COOH, respectively. *Atr* marks the location of the atrazine resistance mutation site affecting amino acid residue 264 in *Amaranthus* (20) and *Solanum* (16, 21). A detailed Mae I and Hae III restriction map of the *Chenopodium* HindIII 900 bp fragment is represented below. The MaeI (AtrI') restriction site is only present in susceptible S and Sp biotypes. In I, R, and Ri biotypes the single bp change of A to G (see Fig. 2) prevents MaeI digestion at this site. Arrows represent the sequencing strategy for 3 clones of each (S, Sp, I, Ri) biotype.
divergence although the proteins encoded are identical (Fig. 3). Sequences from the different Chenopodium biotypes differ only in the first base pair of codon 264 where an adenine (AGT/GGT) in I and Ri plants. This is the fourth demonstration of this Ser264 to Gly264 point mutation in higher plants (16, 20, 21). Both I and Ri plants possess the same Ser264 to Gly264 change, that is in agreement with their chloroplast properties (reduced Qₐ to Qₚ electron flow, thylakoids 1000-fold more resistant and maternal inheritance), but not with their level of resistance to atrazine at the whole plant level. The I or Ri plants maintained by self-pollination still contain the amino acid 264 mutation after four generations in absence of atrazine selective pressure.

Transcript Analysis. The step corresponding to the production of complete herbicide resistant Ri genotypes requires induction with any one of the previously described chemicals (Table II). The amount and size of psbA gene transcripts were analyzed in a Northern experiment using the spinach HindIII 900 bp DNA fragment as a probe. Data (Fig. 4C) show that there is no apparent modification in the amount and size of the psbA gene transcript (1.1 kb) in the different biotypes. This result was expected since it has already been reported that the psbA gene product is post transcriptionally regulated by light (9) and that no apparent modification of this product is detectable between susceptible and resistant plants (22). We therefore suggest that the I to Ri transition is not directly related to the chloroplast psbA gene activity.

Mae I Restriction Analysis. Another way to check for psbA gene codon 264 mutation is to use the restriction enzyme MaeI which recognizes a sequence overlapping the mutation site (C TAG, Fig. 3). As shown in Figure 4A, the results confirm the sequence analysis (Fig. 3) and furthermore provide good evidence for an isogenic chloroplast situation in each phenotype. A 350 bp restriction band and a doublet at 120 bp are visible in S phenotype while only two bands (350 and 240 bp) are present in I and Ri lanes. The 120 bp band is not visible in I and Ri; conversely no 240 bp fragment can be seen in S plants. A reconstitution experiment to assess heteroplasmacy, using 1 μg of HindIII 900 bp psbA gene insert and different dilutions, has estimated the level of heteroplasmacy as lower than 10⁻³ if it exists (not shown).

**DISCUSSION**

Our results provide evidence for the same psbA gene mutation in the two I and Ri phenotypes of Chenopodium album that

**FIG. 3.** Nucleotide sequence of a part (codons 212–280) of the Spinacia oleracea psbA gene is given in line 1. Represented below are the nucleotide differences present in C. album susceptible (line 2 = S, Sp) and resistant biotypes (line 3 = I, Ri, and R). There are no differences at the amino acid level except in the resistant biotypes where ser 264 is changed to glycine (open star). The positions of other known amino acid changes in the psbA gene product of different diuron and atrazine mutants from Chlamydomonas reinhardtii which have an unaltered electron transport are indicated with black stars (8; J Erickson, personal communication).

**FIG. 4.** A and B, investigation of a possible heteroplasmic state in Sp, I, and Ri biotypes. Chloroplast DNA (5 μg) of the Sp, I, and Ri biotypes was restricted by MaeI (C TAG) and the resulting fragments were transferred to Genescreen membrane filters. The autoradiogram (A) represents hybridization of the Southern blot with the psbA gene probe (HindIII 900 bp fragment) from spinach. The map of the resulting fragments is shown on C. B, Autoradiogram of the Northern analysis of the chloroplast psbA gene transcripts in the different biotypes Sp, I, II, and Ri. The probe was the spinach HindIII 900 bp fragment.
possess very different and stable levels of resistance to the atrazine herbicide. The appearance of I plants from the S phenotype is still unclear genetically: within a single field population the I phenotype appears from susceptible plants without apparent atrazine selection (12). We cannot determine if I plants are generated by selection from previously mutated cDNA copies or resulted from a specific mutator system as proposed for Solanum nigrum (2). The mutated I plants lack a high level of resistance at the seedling stage: they are only 10-fold more resistant than the S plants and the fluorescence kinetics of leaves that have absorbed atrazine show a toxic effect of the herbicide (presence of a secondary peak: M). This is probably due to an extrachloroplastic mechanism since R and I plants have an identical pbsA gene sequence, Qα to Qβ electron transfer and thylakoid response to atrazine. Moreover, the erratic segregation of I and R phenotypes in the F₂ of I × R crosses suggests the interaction of a foreign genetic system. Evidence is given here that this genetically encoded difference between I and R plants may be due to the control of environmental factors such as chemical treatments. The progeny of I plants sprayed at the seedling stage with three different chemicals (atrazine, monolinuron, hydroxyatrazine) was phenotypically R, i.e., with a R fluorescence curve and a 40-fold increased resistance at the whole plant level. Thereafter, these new characteristics were stable and maternally inherited, except for the I × Ri crosses. This heritable change may resemble those induced by environmental growth conditions in flax (4). Since hydroxyatrazine is a nonherbicidal metabolite of atrazine (3), the possibility of a selective destruction of susceptible chloroplasts, as would be supposed in the case of a mixed R/S chloroplast population, can be ruled out. Additional mutation due to atrazine and its metabolites could hardly account for 100% mutated progeny since they are known to have little mutagenic potential (28). It could be due to atrazine toxicity at another site different from the chloroplast (e.g. mitochondria), induction of a detoxification system, change in the membrane permeability, or other possibilities. The very rapid (two generations) appearance of the resistant phenotype in the Chenopodium system may be of considerable importance in understanding the wide distribution of atrazine resistant species.

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