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Isolation and molecular characterization of atrazine tolerant barley mutants

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Abstract Two atrazine-tolerant barley mutants were isolated from atrazine-selection experiments performed on barley chloroplast-mutator plants. Genetic analysis demonstrated that atrazine tolerance was maternally inherited. Molecular characterization of the mutants was performed by PCR amplification of an internal fragment of the chloroplast gene *psb*A. The *Bst*XI restriction patterns of the amplified fragments showed two bands in both tolerant barley mutants and only one in the atrazine-sensitive control. The 277-bp amplified fragments from the parental line and both atrazine-tolerant mutants were cloned and sequenced. Sequence analysis showed a single nucleotide substitution in both barley atrazine-tolerant mutants, i.e. A to G at the +790 position of the *psb*A gene-coding sequence. This point mutation corresponds to an amino-acid change of serine- to -glycine and creates a *Bst*XI restriction site. Our results confirmed the conservative variability involved in atrazine tolerance which was previously reported for several other species. To our knowledge this is the first report on the obtention of atrazine-tolerant barley. This finding provides support to the hypothesis that, in addition to a wide variety of chlorophyll deficiencies, the barley chloroplast mutator genotype induces variability in other traits, which could include agronomically valuable mutants.

Keywords Barley mutants · Atrazine tolerance · Chloroplast mutator

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Introduction

A wide diversity of chlorophyll deficient (CD) types was observed, after four generations of self-pollination, in one barley grain mutant selected from a mutagenic treatment (Prina 1992). From that material, CD seedlings were observed in most of the plant progenies and the majority of them showed cell-lineage variegation. Genetic analysis indicated that chlorophyll deficiencies were maternally inherited, while it was concluded that the instability originated in a nuclear mutator genotype, which would produce an outburst of variability localized on the otherwise highly conservative chloroplast genome (Prina 1992). The selection and genetic characterization of families carrying chlorophyll deficiencies were easily performed in genotypes carrying the mutator gene (Prina 1996). In contrast, only a few families with morphological abnormalities or sterility were obtained (unpublished data). In an effort to isolate barley stocks carrying changes in other traits, we focused on the potential application of the mutator gene to gain tolerance to herbicides. Atrazine was chosen as a model herbicide for selection experiments because of the well-understood genetic basis of the tolerance to triazines.

Triazine herbicides inhibit photosynthesis by binding to D1, a thylakoid membrane protein of photosystem II (PSII) (Mazur and Falco 1989). This 32-kDa protein, also known as Q_B, binds to a plastoquinone and passes electrons to the free plastoquinone pool (van Rensen 1982). PSII herbicides bind to D1 protein and displace the plastoquinone; therefore they block photosynthetic electron transport to PSII (Vermaas et al. 1984). The D1

protein is encoded by the *psb*A gene in the chloroplast genome (Steinback et al. 1981). The *psb*A gene was first sequenced in *Spinacea oleracea* and *Nicotiana debneyi* by Zurawski et al. (1982), and since then it has been cloned and sequenced in several photosynthetic organisms revealing that it is highly conserved (Holt et al. 1993). In several plant species, tolerance to triazines is due to a point mutation in the *psb*A gene at codon 264 (Botterman and Leemans 1988).

In this paper, we present a novel strategy to isolate atrazine-tolerant barley mutants that involves exposure of barley chloroplast-mutator plants to atrazine selection. In addition, we report the genetic and molecular characterization of two atrazine-tolerant mutants.

Materials and methods

Mutant isolation

Two-rowed barley (*Hordeum vulgare* L.) plants homozygous for the recessive allele of the chloroplast-mutator gene, previously obtained on a *MC182* (INTA Castelar accession number) background (Prina 1992, 1996), were used as starting material to select atrazine-tolerant mutants. Two different selection experiments were performed (Fig. 1). At the onset of each experiment, approximately 6,000 plants were watered once with 250 ppm of atrazine aqueous solution (2.5 l/m^2) at tillering stage in the field nursery. Experiment I consisted of two generations of field selection, each one followed by a generation of seed multiplication without selection. Experiment II consisted of an initial field selection, followed by a second generation of selection performed on seedlings, at the second-leaf stage, that were grown in pots in the greenhouse and watered once with 25 ppm of atrazine aqueous solution (2.5 l/m^2). In both experiments the hydroponic growing-rack method (Myhill and Konzak 1967) was used as the final test to assay atrazine tolerance in a 4.5 ppm atrazine aqueous solution. All selection doses were chosen after dosimetry experiments with normal non-mutator plants of *MC182*, the parental line.

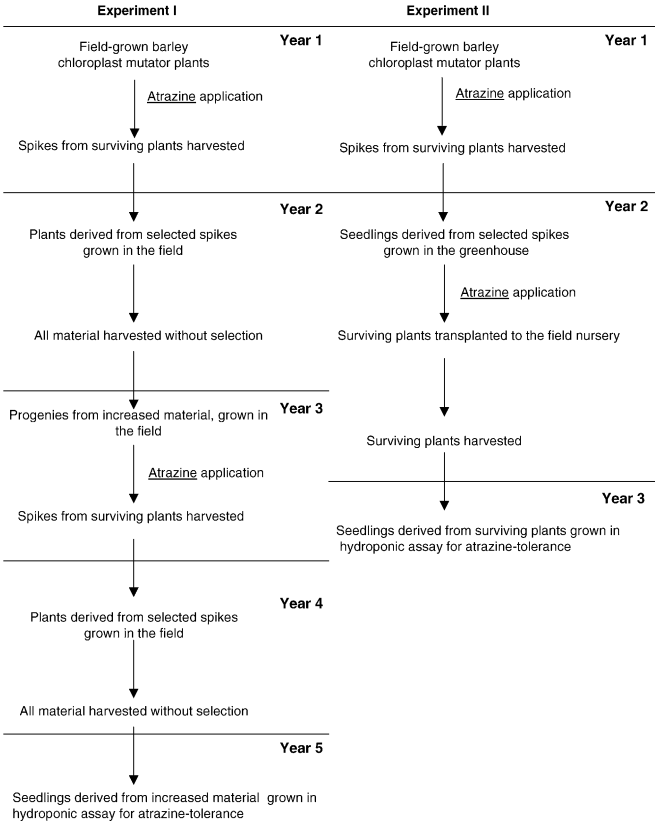

Fig. 1 Flow chart showing the steps followed for the isolation of atrazine-tolerant mutants in barley

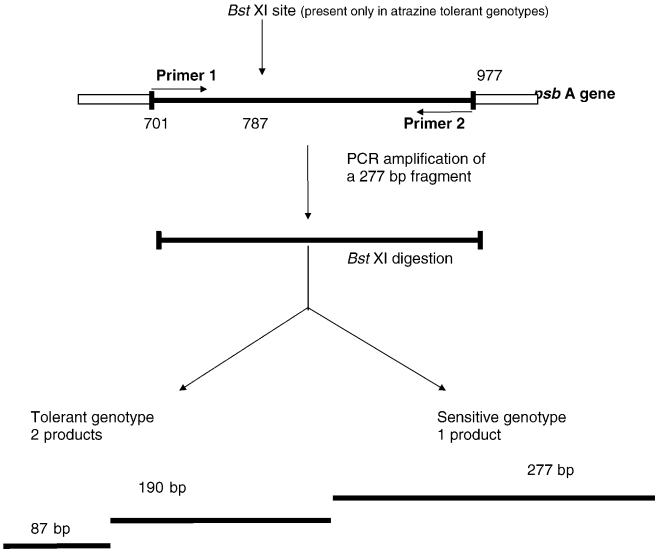

Fig. 2 Experimental procedure used to identify atrazine-tolerant/sensitive genotypes

Molecular characterization

Molecular analyses were carried out on two atrazine-tolerant barley mutants, isolated as indicated above, and on the parental line. Rapeseed (*Brassica napus* L.) cultivars *OAC Triton* (triazine tolerant, Beversdorf and Hume 1984) and *Topas* (triazine sensitive) were used to set up a protocol for the detection of a point mutation which confers tolerance to triazines. Several chlorophyll-deficient chloroplast-mutator derived lines (Prina 1996), an unrelated normal barley line *MC20* (INTA Castelar accession number), *Leones INTA* wheat (*Triticum aestivum* L.) cultivar, a maize (*Zea mays* L.) line and two alfalfa (*Medicago sativa* L.) clones, were also analyzed using the method described here.

Analysis of an internal *psb*A gene fragment was performed by a PCR-RFLP protocol (Fig. 2) that was based on the method described by Cheung et al. (1993). For this purpose, total genomic DNA was obtained from leaves as indicated by Saghai-Maroof et al. (1984) with minor modifications. The quantity and quality of DNA were determined by ethidium bromide fluorescence by comparing against lambda DNA digested with *Hind*III, after electrophoresis in a 0.8% w/v agarose gel in TPE 1× buffer (0.09 M Tris-phosphate pH 8.0 and 0.002 M EDTA).

The PCR amplification reaction was performed in a final volume of 25 µl. The reaction components were as follows: 25 ng of genomic DNA, 1× Gibco BRL REact 2 Buffer (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl), 125 µM each of dATP, dCTP, dGTP and dTTP (Pharmacia LKB), 0.5 µM each 21-bp oligomer primers (forward primer 5′ATGAGGGTTACAGATTTGGTC 3′, reverse primer 5′AGATTAGCACGGTTGATGATA 3′; Cheung et al. 1993) and 1 U of *Taq* DNA polymerase (Gibco BRL). Amplifications were carried out in a Techne Progene or in Biometra Uno-Thermoblock heated-lid thermal cyclers, using the following temperature profile: 94 °C for 2 min; 30 cycles of 94 °C

Table 1 Results obtained at different steps of the selection experiments. S: atrazine selection. M: seed multiplication

Experiment I

Year[a]	Greenhouse	Field	Harvested material[b]
1	–	Approximately 6,000 plants (S)	91 spikes
2	–	91 Spike progenies (380 plants) (M)	380 individual plants
3	–	380 plant progenies (approximately 7,600 plants) (S)	135 individual plants
4	–	135 plant progenies (approximately 1,350 plants) (M)	Individual plants corresponding to 127 progenies[c]

Experiment II

Year[a]	Greenhouse	Field	Harvested material[b]
1	–	Approximately 6,000 plants (S)	95 spikes
2	95 spike progenies (S)	six surviving progenies	Individual plants corresponding to 6 progenies[d]

[a] Each year is equivalent to a generation
[b] Harvested material consisted in seeds grouped per spike or per individual plant basis
[c] No evidences of tolerance were found among the 127 progenies when tested in hydroponics
[d] Two of the six progenies tested in hydroponics showed atrazine tolerance

for 5 s, 50 °C for 10 s, 72 °C for 30 s (autoextension 2 s/cycle) and a final extension at 72 °C for 5 min. The samples were stored at –20 °C prior to analysis. The amplified samples were digested by the addition of 8 units of *Bst*XI restriction enzyme (Promega) and 4-h 50 °C incubation. The PCR amplified and digested products were visualized with ethidium bromide after constant voltage electrophoresis (4 V/cm) in a 2% w/v agarose gel in 1× TPE buffer. The gel documentation was performed with a UVP GDS 5000/GDS 7500. Sizes of bands were estimated by comparing with a 1-kb ladder (Gibco BRL).

Further characterization of the two atrazine-tolerant barley mutants and the parental line was performed analyzing a 1.8-kb fragment encompassing the entire *psb*A gene. For this purpose, PCR amplification was done using *trn*H (5′ACGGGAATTGAACCCGCGCA3′) and *trn*K (5′CCGACTAGTTCCGGGTTCGA3′) primers (Demesure et al. 1995). The PCR amplification reaction was performed in a final volume of 25 µl. The reaction components were as follows: 50 ng of genomic DNA, 1× Gibco BRL PCR Buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 2 mM of $MgCl_2$, 125 µM each of dATP, dCTP, dGTP and dTTP (Promega), 0.5 µM of each primer and 1 U of *Taq* DNA polymerase (Gibco BRL). Amplifications were carried out in a Techne Cyclogene with a heated lid, using the following temperature profile: 94 °C for 3 min; 30 cycles of 92 °C for 45 s, 55 °C for 1 min, 72 °C for 2 min and a final extension at 72 °C for 10 min. The samples were stored at –20 °C prior to analysis. Amplification products were run on 8% acrylamide gels, using 1× TBE buffer (0.089 M Tris-borate pH 8.0 and 0.002 M EDTA) and constant voltage (100 V). Gels were stained during 20 min by immersion in 10 mg/ml of ethidium bromide in 1× TBE buffer. The amplified samples were digested by the addition of 10 units of each of the restriction enzymes *Hae*III and *Eco*RV (Gibco BRL) and overnight incubation at 37 °C. Digestion products were analyzed by electrophoresis in a 2.5% (w/v) agarose gel in 1× TAE buffer (0.04 M Tris-acetate pH 8.0 and 0.001 M EDTA). Sizes of bands were estimated by comparing as described above.

For molecular cloning, PCR amplifications of the internal fragment of the *psb*A gene were performed as described previously with the following reaction buffer: 10 mM of Tris-HCl (pH 9), 50 mM of KCl, 0,1% Triton X-100, 4 mM of $MgCl_2$. Promega *Taq* DNA polymerase was used (1 U). The amplified products from the two atrazine-tolerant barley mutants, the parental line and the rapeseed cultivars *OAC Triton* and *Topas* were purified using centri-spin 40 minicolumns (Princeton separations) and cloned using pGem-T vector following the manufacturer's instructions (Promega). The ligation product was electroporated into electrocompetent *Escherichia coli* strain JM109 cells. Recombinant white colonies were selected in Ampicilin 100 Xgal/IPTG LB plates and PCR screened using M13 universal primers (Hoisington et al. 1994). Plasmid DNA was isolated from positive clones by the alkaline-lysis method and purified using Wizard minicolumns (Promega). Sequencing was performed by PCR using the Sanger method with fluorescent labeling. The sequencing reaction products were run and read in an automatic sequence equipment (Applied Biosystems ABI 363). Both strands of the cloned amplified fragments were completely sequenced and the sequences were analyzed using the Sequence Editor program.

Results

Isolation and genetic characterization of two atrazine-tolerant barley mutants

Results obtained from two types of selection experiments are shown in Table 1. In Experiment I, 127 individual-plant progenies were obtained after four generations (two for atrazine selection and two for seed multiplication). When tested in hydroponics for atrazine tolerance, these progenies, showed no noticeable differences as compared with the control. In Experiment II, only six plants survived after a single generation of field atrazine selection followed directly by a generation of atrazine selection at the greenhouse. Progenies of these six plants were hydroponically tested with atrazine solution and two of these plant progenies were still green and healthy by day 20, while seedlings of the control turned yellow and died. A view of an hydroponic assay by day 17 is shown in Fig. 3.

Genetic characterization of the two atrazine-tolerant mutants was done analyzing the hydroponic atrazine tolerance of progenies obtained by self-pollination and reciprocal crosses (Table 2). The statistical analysis of the results from reciprocal crosses showed a clear dependence on the female parent of the cross ($\chi^2_{1\ df} = 36{,}98$ ***)

Molecular characterization

For the molecular characterization of the atrazine-tolerant barley mutants, we used previously reported primers which amplify an internal fragment of the chloroplast

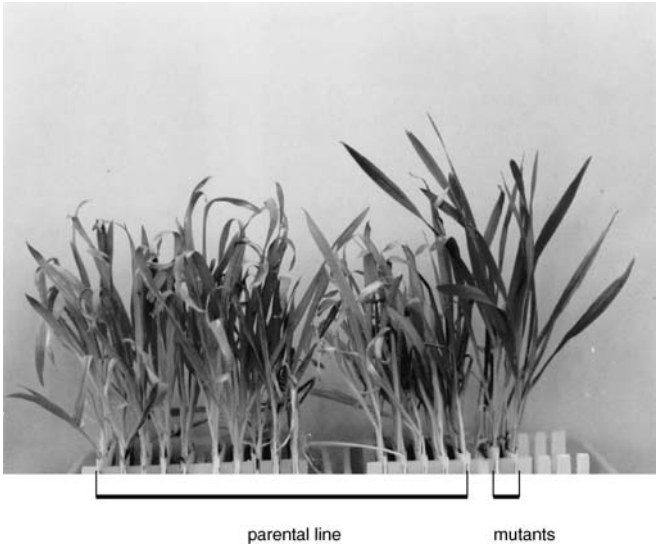

Fig. 3 Hydroponic assay, in a 4.5 ppm atrazine aqueous solution, used to detect atrazine tolerance in barley. By day 17, in comparison with mutant seedlings, those of the parental line showed reduced growth and a lighter color

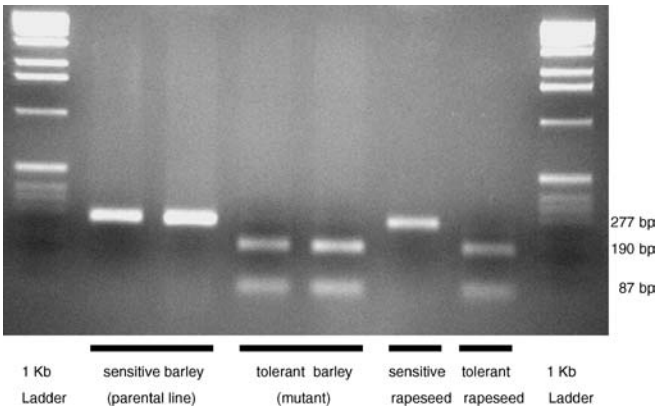

Fig. 4 PCR-RFLP detection of a *psb*A gene mutation conferring tolerance to atrazine. Internal 277-bp PCR amplification products were digested with *Bst*XI and run on 2% agarose gels. Atrazine-tolerant genotypes from barley and rapeseed showed a two-band pattern whereas atrazine-sensitive genotypes from barely and rapeseed showed a single-band pattern. Atrazine-tolerant and -sensitive rapeseed genotypes were used as controls

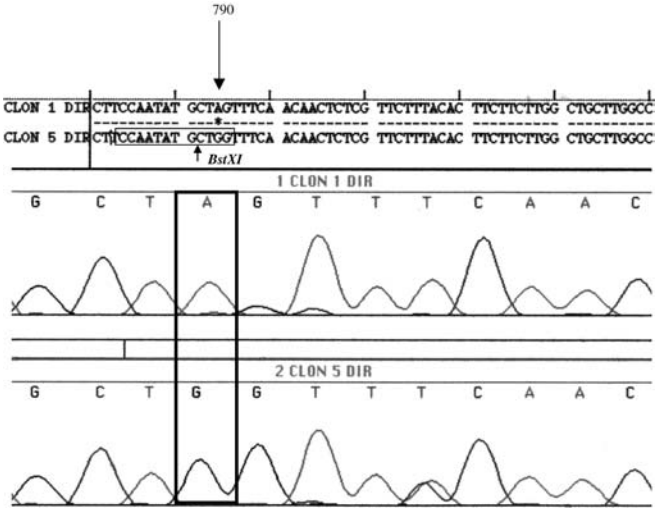

Fig. 5 Partial sequence comparison of a *psb*A gene fragment between the atrazine-sensitive parental line (clone 1) and an atrazine-tolerant barley mutant (clone 5). A single nucleotide substitution (*boxed*), A by G, created a new *Bst*XI restriction site (*boxed*) in the nucleotide sequence of the barley mutant

gene *psb*A in rapeseed, *Chenopodium* spp. and *Amaranthus* spp. (Cheung et al. 1993). In order to develop a simple method to identify atrazine-tolerant mutants we replaced the conventional PCR *Taq* buffer with a restriction enzyme buffer (Gibco BRL REact 2 Buffer), amplified a 277-bp fragment in barley, wheat, corn, alfalfa and rapeseed (data not shown), and subsequently digested the PCR products with *Bst*XI to positively detect a point mutation that confers atrazine tolerance. *Bst*XI restriction patterns of the amplified fragments showed two bands (190 and 87 bp) in both tolerant barley mutants and only one (277 bp) in the control (Fig. 4). Similarly, tolerant and sensitive rapeseed genotypes were also differentiated on the basis of the aforementioned patterns (Fig. 4). Other non-atrazine selected chloroplast-mutator derived lines were examined using this methodology and atrazine-tolerant genotypes were not detected. From other controls, a 277-bp band was obtained after *Bst*XI digestion of the PCR product. Those controls consisted in an unrelated normal barley line, a *Leones INTA* wheat cultivar, a maize line and two alfalfa clones (data not shown).

The PCR amplification of the genomic region encompassing the *psb*A gene showed a 1.8-kb DNA fragment in both barley mutants and the parental line. Furthermore, *Hae*III and *Eco*RV restriction patterns of the amplified product showed four bands (i.e. 970, 380, 320 and 140 bp) in all samples (data not shown), suggesting that no major rearrangements occurred in this region.

Table 2 Genetic analysis of atrazine tolerance in barley

Progeny origin[a]	Number of seedlings	Number of surviving seedlings after 20 days of atrazine hydroponic selection
Normal[a]	52	0
Tolerant mutant 1[b]	8	8
Tolerant mutant 2[b]	8	8
Tolerant mutant 1 (♀) × Normal (♂)[c]	8	8
Tolerant mutant 2 (♀) × Normal (♂)[c]	8	8
Normal (♀) × Tolerant mutant 1 (♂)[c]	9	0
Normal (♀) × Tolerant mutant 2 (♂)[c]	12	0

[a] Seeds were obtained after self-pollination or cross. Seedlings derived from those seeds were assayed for atrazine tolerance
[b] self-pollination progenies
[c] F1 progenies originated in hybridizations between the indicated genotypes

Further molecular characterization of the barley mutants was performed by cloning and sequencing the 277-bp amplified products of the atrazine-tolerant mutants and the parental line. Amplified 277-bp products from rapeseed cultivars were also cloned and sequenced as controls. The sequence of the internal *psb*A gene fragment of the barley parental line fully agreed with previously reported sequence data (Boyer and Mullet 1988; Efimov et al. 1988). A single nucleotide substitution, A to G, at the +790 position of the *psb*A gene coding sequence was observed in both barley atrazine-tolerant mutants (Fig. 5). This point mutation created a *Bst*XI restriction site (Fig. 5).

Discussion

Plastome mutants in higher plants were widely reviewed by Börner and Sears (1986), who pointed out the potential of this variability for physiological, molecular, genetic and biochemical studies of the chloroplast and, also, for plant breeding. The usually highly conservative genetic information encoded in the chloroplast genome (Sears 1983; Melzer and Kleinhofs 1987) can be changed due to the action of plastome mutator genes which dramatically increase plastome mutation rates (Börner and Sears 1983; Prina 1992). Mutator genes were classified by Kirk and Tilney-Bassett (1978) into two groups according to the width of the spectrum of the mutant phenotypes they induce, i.e. narrow-spectrum and wide-spectrum. The barley chloroplast-mutator used in the present investigation belongs to the second group, being the only case described in monocots (Prina 1992). Other cases described as corresponding to this group were reported in *Arabidopsis*, *Epilobium*, *Oenothera*, *Nepeta*, *Petunia* and *Solanum* (see Kirk and Tilney-Bassett 1978; Börner and Sears 1983; Prina 1992). *Oenothera* plastome mutator is, to our knowledge, the best characterized at the molecular level, causing template slippage, deletions and duplications at target sites defined by direct repeats in the plastid genome (Stoike and Sears 1998).

Arntzen and Duesing (1983) proposed the utilization of chloroplast mutator genes for the obtention of atrazine-tolerant mutants. Based on an estimation of a very low probability level of obtaining a gamete-transmissible triazine resistance spontaneously produced, i.e. 10^{-9}–10^{-10}, they theorized that a plastome mutator may have been involved in the appearance of triazine resistance in *Solanum nigrum*. They also proposed the search of plastome mutators in crop species in order to increase the probability of recovering an atrazine-resistant mutant to an acceptable level, and to make this a viable experimental approach. In accordance with that proposal, we used a chloroplast mutator genotype in barley (Prina 1992, 1996), and after atrazine selection we isolated two atrazine-tolerant mutants and accomplished their genetic and molecular characterization. Two different strategies were pursued for isolation of the mutants: in Experiment I the generations of seed multiplication without selection were based on the idea that the weakness of the progenies of atrazine-treated plants could affect their survival when submitted to new atrazine treatments, even though they could carry small tolerant sectors. Experiment II was constructed to impose continuous selection pressure, based on the idea that *psb*A-mediated tolerance could be detrimental in the absence of atrazine as has been reported in other species (Holt et al. 1993). Thus, in rapeseed the comparison between triazine-tolerant and triazine-sensitive genotypes, revealed that the tolerant genotypes exhibited al least 20% lower productivity than the sensitive ones (Gressel 2000). This "fitness penalty" is probably due to the reduced PSII electron transfer efficiency caused by the *psb*A mutant allele (Holt et al. 1993). By this reasoning, in the absence of atrazine, atrazine-tolerant mutant individuals could be displaced by normal ones by plant to plant competition, and atrazine-tolerant mutant sectors could be lost due to negative diplontic selection. Indeed, our atrazine-tolerant barley mutants showed retarded initial growth and reduction in vigour, as compared to the parental line. It is worth mentioning that the aforementioned "fitness penalty" does not mean that such *psb*A-mediated triazine-tolerant crops lack agronomic value. Thus, triazine-tolerant rapeseed is widely grown in Australia because it allows an inexpensive control of important weeds (Gressel 2000).

The approach of Experiment II was successful for the isolation of atrazine-tolerant mutants while none were obtained in Experiment I. Although we believe that this indicates that the strategy upon which Experiment II was based was sound, only two mutants were isolated, and hence the numbers are not sufficient for a definitive judgement about the value of the two selection procedures.

Reciprocal crosses between the tolerant mutants and the normal genotypes were performed to determine the inheritance of atrazine tolerance. The atrazine-tolerance in hydroponic tests clearly showed maternal inheritance as expected for a trait controlled by a plastome gene in barley.

In order to detect the mutation at the molecular level, we modified the method proposed by Cheung et al. (1993) which was based on the PCR amplification of an internal fragment of the *psb*A gene followed by restriction enzyme digestion. In their experiments, PCR amplification products digested with *Mae*I showed a single 277-bp band for atrazine-tolerant genotypes and a two-band pattern (88 and 189 bp) for atrazine-sensitive genotypes. We replaced this restriction enzyme by *Bst*XI, which was successfully used by Thomzik and Hain (1988) for RFLP analysis of the *psb*A gene. The use of *Bst*XI allowed a positive detection of the mutation, i.e. tolerant genotypes would have two bands (87 and 190 bp) whereas sensitive ones would produce a single band (277 bp). This means that the tolerant pattern could not be caused by experimental artifacts, e.g. incomplete digestion or non-restrictable samples. Besides, the replacement of the conventional PCR *Taq* buffer by the *Bst*XI compatible restriction enzyme buffer resulted in a single tube-test procedure particularly suitable for

screening large populations. In addition to the previously mentioned advantages, *Bst*XI is available from several manufacturers which is not the case for *Mae*I.

The molecular analysis affirmed this approach: a new *Bst*XI restriction site was found in the 277-bp internal fragment of the *psb*A gene of the barley mutants (Fig. 4). Subsequently, cloning and sequencing of the aforementioned fragment confirmed a single nucleotide substitution A to G at the +790 position of the *psb*A gene-coding sequence (Fig. 5), that corresponds to an amino-acid change of serine to glycine. This result is in accordance with the molecular basis described for other atrazine-tolerant plant mutants, such as rapeseed (Reith and Straus 1987) and weeds such as *Amaranthus* (Hirschberg and McIntosh 1983), *Solanum nigrum* (Goloubinoff and Edelman 1984) and *Poa annua* (Barros and Dyer 1988), and confirms the conservative variability previously pointed out for this trait (Botterman and Leemans 1988; Holt et al. 1993). Those atrazine-tolerant weeds were observed in areas where atrazine had been extensively used (Botterman and Leemans 1988). Besides, the atrazine tolerance present in some rapeseed cultivars (e.g. *OAC Triton*, Beversdorf and Hume 1984) was introduced from a wild relative (Beversdorf et al. 1980). On the other hand, atrazine-tolerant mutants were isolated from *Nicotiana plumbaginifolia* by treating photoautotrophic protoplast-derived cultures with a mutagen (N-ethyl-N-nitrosourea) and using atrazine as a selective agent (Rey et al. 1990). The frequency of calli tolerant to atrazine was 1.6×10^{-5}. At this point, it is worth making it clear that it is difficult to estimate the number of targets which were imposed to selection when we treated the 6,000 tillering plants at the beginning of each one of the two experiments. This experimental material has been exposed for several generations to the mutator gene activity and consequently it is supposed that the plants carried mutated sectors of very different sizes, i.e. ranging from almost one entire plant to a mutational change in a single molecule of the chloroplast polyploid genome. Moreover, it is not known which is the minimum size of a tolerant sector for being favored by atrazine treatments, either by conferring selective advantage to the carrier plant or, also, by diplontic selection.

The high activity of the barley mutator genotype here used was previously made clear by studying the frequency of chlorophyll deficiencies in crosses using the mutator genotype as male parent (Prina 1992). In this way we started with a normal plastome exposed to the mutator activity in a quarter of the F_2 zygotes. In that experiment the mutator effects were only manifested by three clonal variegated F_2 seedlings out of 1,005 analyzed at the second leaf stage, but interestingly when F_2 plants were analyzed until heading, the number of plants having at least a small chlorophyll-deficient sector approximated to a 1:3 proportion in relation to plants of a wild phenotype. This means that although streaks of small size were observed, somatic mutations are detected in almost all the F_2 plants with a *cpm/cpm* genotype. In the following generations the size of chlorophyll-deficient streaks increased in progenies originated in homozygous F_2 mutator plants, leading to the observation of solid mutant seedlings in the F_4 generation. Those chlorophyll mutants showed a wide spectrum of types, most of them (87.3%) being clonally variegated. Another experiment of hybridization with the mutator genotype was made using Cytoplasmic Line 4 as female (see Prina 1996 for details). That line, was observed to magnify the mutator gene detection in the second leaf of F_2 seedlings for more that 60 times, suggesting the activity of the mutator on cp DNA would be much higher than that finally expressed through the sorting out of chlorophyll-deficient sectors on a normal plastome background. (Prina 1996). Our present results support the hypothesis that, in addition to a wide variety of chlorophyll deficiencies maternally inherited, the barley chloroplast mutator genotype (Prina 1992) induces plastome mutations affecting other traits. In order to test the potential of this mutator genotype in inducing a wide range of variability in the chloroplast genome which could include agronomic valuable mutants, selection for tolerance to different herbicides, antibiotics and other abiotic stresses is in progress.

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