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An in vitro molecular study of the *Nicotiana tabacum* L. genome in the presence or absence of the herbicide atrazine

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Abstract *Nicotiana tabacum* L. somaclones both selected and not selected for tolerance to the triazine herbicide atrazine were used to compare tissue culture-induced variability in the presence or absence of stress. Two types of repeated sequences (rDNA and a randomly cloned, anonymous sequence) were analysed both qualitatively and quantitatively, and overall genome variation was assessed by RAPDs. Multiplicity differences were found for the two sequences both between the tolerant and susceptible group and within each group with respect to leaf DNA, but no qualitative differences were detected with either RFLPs or RAPDs. Moreover, we investigated whether stress-induced variation in the atrazine target gene, the chloroplast *psbA* gene, was responsible for herbicide tolerance by analysing two possible resistance mechanisms: the presence of a specific point mutation in the gene and its amplification and/or increased expression. Some somaclones were shown to be a mosaic for *psbA* gene mutation, but the number of cells or plastid genomes involved seemed too low to account for tolerance in the whole tissue. Atrazine tolerance could then be due to an increase in the number of plastids/plastid genomes or/and to a permanent response to respiration inhibition whose basis is, up to now, unknown.

Key words *Nicotiana tabacum* L. · Atrazine · Somaclonal variation · Stress

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Introduction

Somaclonal variation is the term attributed by Larkin and Scowcroft (1981) to genetic variability released during in vitro culture of plant tissues and regenerated plants. The frequency of somatic genetic changes in vitro is variable, depending on several factors like the culture conditions, the duration of the dedifferentiated state, the source of the initial explant and the genotype of the donor plant. This last factor, although not sufficiently investigated, seems to play a key role as mutation frequencies are very low in some species like barley (Shimron-Abarbanell and Breiman 1991) and very high in others like maize and rice (Brown et al. 1991; Muller et al. 1990). At the molecular level, somaclonal mutations have been attributed to several classes of genome modifications such as point mutations (Dennis et al. 1987), variation in copy number (Grisvard et al. 1990), methylation or demethylation of DNA sequences (Kaeppler and Phillips 1993), activation of transposable elements (Peschke et al. 1987) and retrotransposons (Hirochika 1993) whose relative frequencies differ from those observed in vivo, and whose underlying mechanisms remain mostly to be elucidated. Once the possibility is ruled out that somaclonal variation reflects only pre-existing variation in the original explant (Brown et al. 1991; de Paepe et al. 1982), several hypotheses can be proposed to explain the occurrence of this phenomenon. Firstly, the fact must be taken into account that, at variance with what occurs in animals (Walbot and Cullis 1985), plants are endowed also in vivo with a very high spontaneous somatic variability, only part of which is allowed to pass through the meiotic filter (D'Amato and Hoffmann-Ostenhof 1956). The conditions of in vitro culture may thus release the ontogenetic constraints acting at the whole plant level on cells, which are generally derived from differentiated tissues, resulting in a relaxed selection pressure on mutations. Secondly, the possibility of a real increase in

mutation rates due to the presence of stress conditions has to be considered. According to McClintock (1984), the genome can respond to a situation of "shock" through the activation of programmed cellular responses, but in some cases this may lead to genome instability, resulting in reorganization of the genome itself to overcome a threat to its survival. Within this framework, Lebel et al. (1993) have demonstrated that stress conditions can increase the frequency of intrachromosomal recombination in *Nicotiana tabacum* somatic cells from two- to nine-fold, depending on the stress source.

The purpose of the investigation presented here was twofold. In the first place, the culture stability of a species, *Nicotiana tabacum*, which does not seem to be very variable (Dulieu 1986), was tested with molecular tools in tissue cultures challenged or unchallenged with an additional stress agent (the triazine herbicide atrazine) in addition to the in vitro culture itself. Tobacco somaclones selected for tolerance to the herbicide under non-photoautotrophic conditions were scored for variation at the level of polymorphism by means of restriction fragment length polymorphisms (RFLPs) and randomly amplified polymorphic DNAs (RAPDs) (Williams et al. 1990), multiplicity of repeated sequences (rDNA and one randomly cloned, anonymous repeated sequence) and methylation. Secondly, we investigated whether stress-induced variation in the target gene could eventually be responsible for herbicide tolerance by checking two possible resistance mechanisms. Specific atrazine resistance in most higher plants, grown both in vivo and in vitro, and algae is due to a single point mutation in the chloroplast *psbA* gene, which codes for the herbicide target protein, P32, the second stable electron acceptor of photosystem II (Hirschberg and MacIntosh 1983; Erickson et al. 1984; Sato et al. 1989). The resulting mutated protein has a lower affinity for the herbicide than the wild type. On the other hand, resistance to other herbicides, such as glyphosate (Hollander-Czytko et al. 1988) and L-phosphinothricine (Donn et al. 1984), is achieved through the amplification of the gene coding for the target enzyme. The *psbA* gene was therefore analysed in somaclones either selected or not selected for herbicide tolerance both for the presence of the gene mutation and for the occurrence of amplification and/or increased expression.

Materials and methods

Plant material and culture conditions

Nicotiana tabacum L. cv 'White Burley' callus formation and maintenance have been described in a previous paper (Bogani et al. 1985).

Selection of atrazine-tolerant clones

The selection of atrazine-tolerant clones was carried out by agar plating, as previously described (Bettini et al. 1992), at a concentration of 1 μ*M*, which gave a plating efficiency of 2% under our conditions. Plating efficiency for the control without atrazine was 10%. Atrazine-tolerant clones were recovered after 1 month and routinely transferred on atrazine-supplemented medium. For the isolation of susceptible clones, single cells and clumps were plated on medium without atrazine.

DNA probes

The DNA probes used in this study were: pAT250, corresponding to the entire rDNA unit of wheat (8 kb; R. Flavell, Plant Breeding Institute, Norwich, UK); pNtrH70, an anonymous tobacco repeated sequence (0.6 kb; this paper); H900, a fragment of the *Chenopodium album* chloroplast *psbA* gene (0.9 kb; Bettini et al. 1987); pS6, a SalI fragment of wheat chloroplast DNA (1.4 kb; kindly provided by Dr. C. Hartmann, Institut de Biotechnologie des Plantes, Orsay, France). The insert fragments were isolated by elution from agarose gels (Geneclean kit, BIO 101, La Jolla, Calif.) and labelled by primer extension with random hexanucleotides (Feinberg and Vogelstein 1983) using either α-[^{32}P]dCTP or DIG-dUTP as described.

DNA extraction

Nuclear DNA extraction was carried out as described in Bettini et al. (1988) from the leaves of axenically grown tobacco plants. Total DNA extraction from callus (at least four different callus pieces for each somaclone) and leaf tissue (several leaves from different axenically grown plants) was performed according to Bogani et al. (1995). Plasmid DNA was isolated by a standard miniprep procedure (Sambrook et al. 1989).

Cloning of a *Nicotiana tabacum*-repeated sequence

*Hin*dIII-digested nuclear DNA was shotgun-cloned in the corresponding site of the plasmid pUC19. A total of 157 clones were analysed for the presence of repeated sequences by dot blot hybridization using digoxigenin-labelled total leaf DNA as a probe. One of the clones giving stronger signals was arbitrarily chosen and denoted pNtrH70; it contained an insert of 600 bp which did not hybridize with the rDNA probe.

DNA dot blot hybridization

For dot blot analysis, total DNA at a concentration of 100 ng/μl in sterile distilled water was denatured at 100°C for 5 min and then immediately transferred onto ice. After adding 20 × SSC (Sambrook et al. 1989) to a final concentration of 2×, we spotted aliquots of 200 and 400 ng in a final volume of 4 μl on Hybond N$^+$ membranes (Amersham). Dots were in replicas. DNA was fixed to the membrane by UV cross-linking (Spectrolinker XL-1000, Spectronics Corp, USA). For probe preparation, inserts were labelled with α-[^{32}P]dCTP (Amersham). Membranes were prehybridized in 2 × SSPE (Sambrook et al. 1989), 1% SDS, at 46°C for at least 6 h, and hybridizations were carried out at 42°C for 12–18 h in 5 × SSPE, 5 × Denhardt, 1% SDS, 50% formamide and 100 μg/ml salmon sperm DNA. Filters were then washed 2 × 20 min in 2 × SSC, 0.5% SDS at room temperature and 2 × 20 min in 0.1 × SSC, 0.1% SDS at 42°C. After the autoradiography (Agfa Curix), dot blots were quantified by cutting each dot and counting radioactivity in a Betamatic scintillator (Kontron Scientific Instruments) with the addition of 1 ml per sample of Opti-Fluor (Hewlett Packard). Each experiment was independently repeated at least twice. Moreover, to assess the reliability of the method, we carried out a series of preliminary

experiments with serial dilutions of different preparations of leaf DNA; no significant differences between repetitions were found. ANOVA was used to compare the results of dot blot hybridizations.

Southern hybridization

For Southern analysis, 5 μg total DNA was digested overnight with 30 units of restriction endonuclease (Boehringer Mannheim) in the presence of spermidine, separated on agarose gels (Sambrook et al. 1989), alkaline-blotted onto Hybond N^+ membranes and fixed to the membrane by UV cross-linking. The rDNA, pNtrH70 and H900 inserts were digoxigenin-labelled, and hybridization was carried out using the "Nonradioactive DNA labelling and detection kit" (Boehringer Mannheim) with CSPD® as the chemioluminescent substrate, following the manufacturer's instructions. Autoradiography was carried out using X OMAT AR Kodak film (Eastman Kodak, Rochester, N.Y.). Each experiment was repeated twice.

RAPD analysis

RAPD analysis was performed using eight primers of different lengths; i.e. one 17 mer (pUC/M13 reverse sequencing primer, Promega), one 16 mer ($GATA_4$) and six 10 mers, both alone and in combination. The sequences of the 10-mer primers was: 5′ATCGCACACT3′ (AI2); 5′TGGTCACTGT3′ (AH30); 5′TGGTGACTGA3′ (AH29); 5′AGGTCACTGA3′ (AG1); 5′TGGCCAGTGA3′ (CD11); 5′TCCGAGTCTG3′ (CD12). Oligonucleotides were custom-synthesized by Genenco (Florence, Italy). The reaction conditions were those described by Williams et al. (1990), with 50 ng template DNA. Amplification was carried out in a Perkin Elmer Cetus GeneAmp 9600 for 50 cycles of 1 min at 92°C, 2 min at 35°C and 2 min at 72°C with an applied temperature ramp of 1°C/3 s for the 35°–72°C transition.

Mutation analysis of the *psbA* gene

A 945-bp fragment corresponding to the *psbA* gene (based on Sugita and Sugiura 1984) was amplified by polymerase chain reaction (PCR) using as primers two 21-mers with the following sequence: 5′GAAAACCGTCTTTACATTGGA3′ and 5′AGTTGTGAGCATTACGTTCAT3′. The PCR reaction mixture contained 1 μg template DNA, 100 μ*M* dNTPs, 0.1 μ*M* primers, 2.5 units *Taq* polymerase and 1 × polymerase buffer supplied by the manufacturer (Boehringer Mannheim) in a final volume of 50 μl. Amplification was carried out for 30 cycles of 1 min at 94°C, 2 min at 40°C, 2 min at 72°C in a Perkin Elmer Cetus GeneAmp 9600. The identity of the PCR products was confirmed by hybridization with the digoxigenin-labelled H900 insert. Amplified *N. tabacum psbA* gene fragments (20 μl) were digested overnight with the restriction endonuclease *Mae*I (Boehringer Mannheim), which enables the sequence of the wild type and those of the atrazine-resistant biotypes to be distinguished (MacNally et al. 1987). One of the target sequences for *Mae*I (C/TAG) in the *psbA* gene is changed to CTGG by the mutation conferring resistance to the herbicide, thus leading to the loss of a restriction site (Fig. 3A). Digestion products were finally electrophoresed on a 8% acrylamide – TBE gel (Sambrook et al. 1989) for 14 h at 9 mA and visualized by ethidium bromide staining.

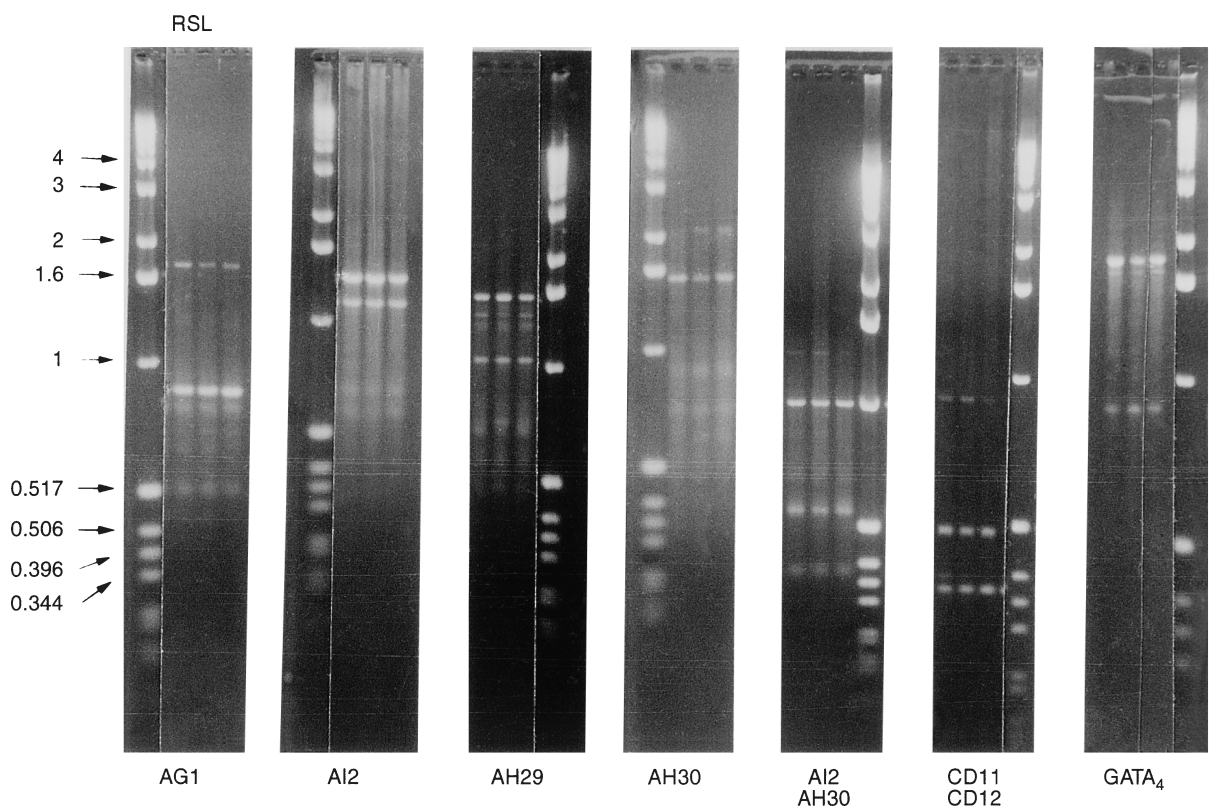

Fig. 1 RAPD patterns obtained with the primers AG1, AI2, AH29, AH30, AI2 + AH30, CD11 + CD12, and $GATA_4$ on total DNA from the *Nicotiana tabacum* somaclones adapted (*R*) or not (*S*) to the herbicide atrazine, and on leaf DNA (*L*). The sequences of the primers are reported in the Materials and methods. Molecular weights (1-kb DNA ladder, Gibco BRL) are expressed in kilobasepairs (kbp)

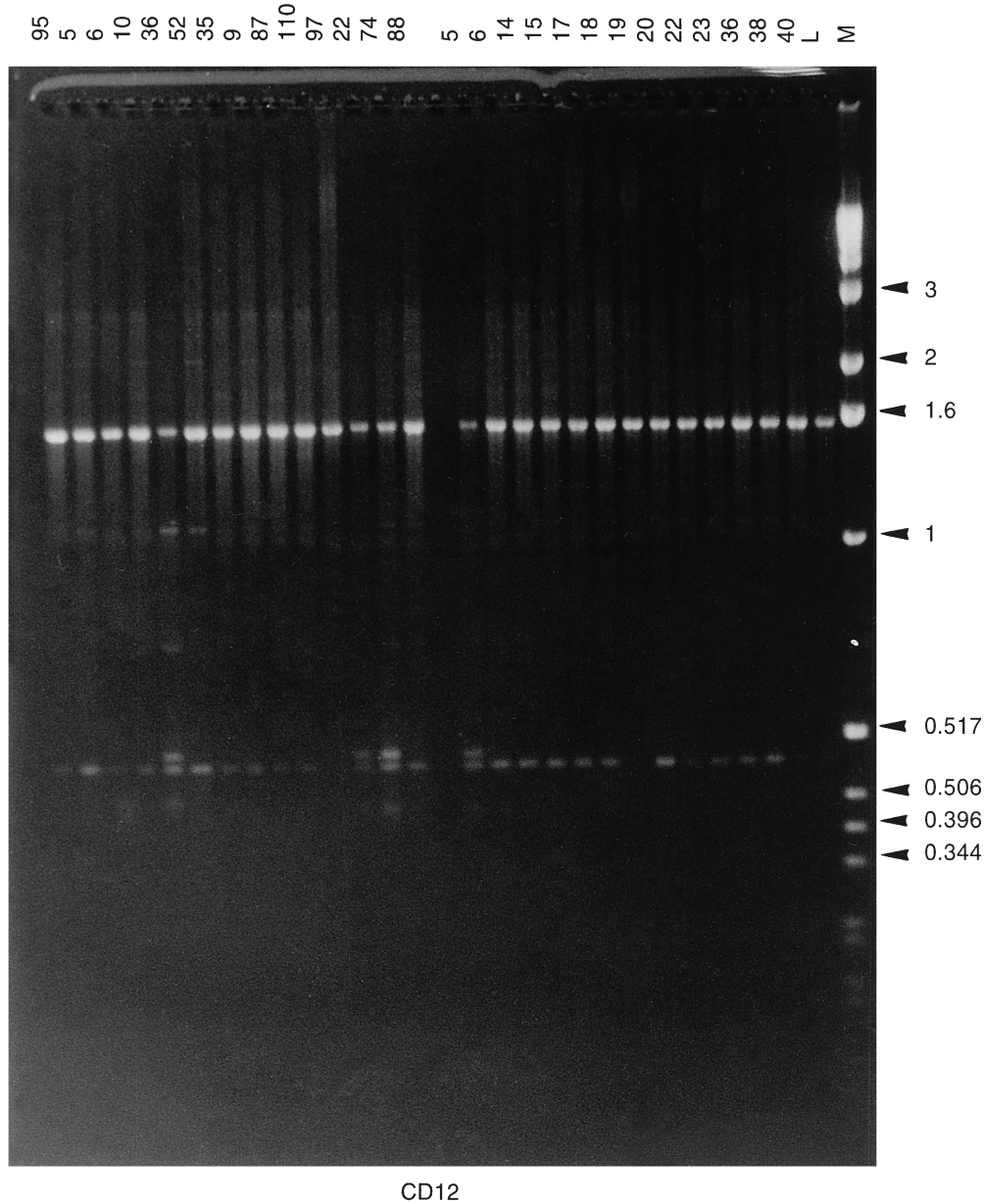

Fig. 2 Amplified DNA polymorphism for the 10-mer primer CD12 on DNA from the atrazine-tolerant (*from 95 to 88*, on the *left*) and susceptible (from *5 to 40*, on the *right*) tobacco somaclones, and leaf DNA (*L*). Molecular weights (*M* = 1-kb DNA ladder, Gibco BRL) are expressed in kilobasepairs

RNA extraction and Northern hybridization

RNA extraction was performed according to Mahé et al. (1992). A 20-μg aliquot of RNA per sample was fractionated on denaturing formaldehyde-agarose gels (Sambrook et al. 1989), blotted onto nylon membranes (Boehringer Mannheim) and hybridized to the digoxigenin-labelled H900 probe, according to the manufacturer's instructions. The filters were exposed to X OMAT AR Kodak film.

Results

Genetic and epigenetic variability in control and atrazine-stressed cultures: polymorphism analysis, multiplicity and methylation

Qualitative variations were scored as RFLPs, for rDNA and the anonymous repeated sequence pNtrH70, and by RAPDs. For RFLP analysis, total DNA (5 μg) from 12 tolerant and 12 sensitive clones was digested with the restriction endonucleases *Hin*dIII, *Bam*HI and *Eco*RI and hybridized with the digoxigenin-labelled rDNA or pNtrH70 insert. No polymorphism was detected in either simple or double (*Eco*RI + *Bam*HI; *Eco*RI + *Hin*dIII; *Bam*HI + *Hin*dIII) digestions, both in the atrazine-adapted and in the susceptible clones (data not shown).

RAPD analysis was performed on DNA from 14 tolerant and 14 susceptible somaclones. Eight primers, both alone and in combination, were used to obtain 12 different amplification profiles, some of which are shown in Fig. 1. Only primer CD12 was found to give a reproducible polymorphism (Fig. 2), no correlation being observed between the RAPD pattern and atrazine tolerance.

Table 1 Multiplicity of the different probes in the atrazine-tolerant tobacco somaclones with respect to leaf (*n.d.* not determined)

Probe Clone no.	rDNA x/leaf	rDNA cpm	pNtrH70 x/leaf	pNtrH70 cpm	H900 x/leaf	H900 cpm	pS6 x/leaf	pS6 cpm
Leaf	1	226.3	1	201.3	1	124.3	1	43
1.36	1	231.4	2.4	488.7*	3.2	395.3**	2.4	103.9**
1.52	1.1	244.75	2.9	584.6**	3.6	453**	3.7	158.6**
1.35	1.2	269.75*	3.3	658.5**	1.5	192.2	0.9	39.2
1.9	0.4	99.4**	1.2	237.8	1.1	141	1.4	61.6**
1.110	–	n.d.	3.3	657.3**	13.5	1676**	8.2	354.2**
1.22	0.8	183.1	1.9	394.1*	2.2	268.2**	1.6	70.3**
1.97	1.1	244.5	2.8	558.3*	3.3	407.2**	2.6	111.7**
1.74	1.1	246.1	3.1	633**	3.5	433.1**	2.3	100.8**
1.88	1.7	383.2**	4.1	831.4**	1.4	177.7	1.6	67.1*
1.6	0.3	78.7**	1.7	354	1.9	235.5**	1.65	70.7**
1.95	0.6	133*	2	403.1*	1.9	231.9*	1.9	82.9**
1.10	0.8	183.75	2.2	434.9*	2	248.3**	1.9	82.5**
1.87	1.03	234	1.3	255.6	0.8	95.7	1	44.9
1.5	1.05	238.9	2.2	435.9*	1.3	160.5	1.5	63.3**

* Significant at $P \leqslant 0.05$; ** $P \leqslant 0.01$

Table 2 Multiplicity of the different probes in the atrazine-susceptible tobacco somaclones with respect to leaf (*n.d.* not determined)

Probe Clone no.	rDNA x/leaf	rDNA cpm	pNtrH70 x/leaf	pNtrH70 cpm	H900 x/leaf	H900 cpm	pS6 x/leaf	pS6 cpm
Leaf	1	189.9	1	227.5	1	168.5	1	56.7
wt 2	1.7	315.2*	2.4	559.3**	1.1	185.3	1.2	69.9
wt 5	0.4	84.2**	0.5	115.85*	0.6	96.4*	0.75	42.7
wt 10	1	183.9	1.5	334.3	–	n.d.	0.8	47.1
wt 14	2.5	470**	3.3	741.9**	4.3	723.8**	3.8	218.6**
wt 15	2	376*	1.5	329.8	1.7	280	2.2	126*
wt 18	–	n.d.	1.9	426.6*	1.8	299.5*	2.1	119.2*
wt 19	2.3	443.6**	2.2	499.8**	1.75	294.8*	1.6	92.8*
wt 20	1.5	286.65**	2.3	514.2**	1.03	173.4	1.1	63.9
wt 22	0.7	133.2*	0.7	168.1	0.4	73*	0.6	32.7
wt 23	1.6	299.5**	1.7	392.8*	1	167.4	1.1	64.2
wt 17	1.6	312.3**	2.2	505*	2.2	367.7**	2.2	125**
wt 36	1.9	368.95**	2.2	491.4*	0.85	144	1.9	109.2*
wt 38	1.7	317.6**	1.9	441*	1	169	1.25	70.8
wt 40	1.3	248.5	1.8	417.5*	0.8	127.4	1	59.7

* Significant at $P \leqslant 0.05$; **$P \leqslant 0.01$

To detect copy-number variation, we prepared series of dot blots with 200 and 400 ng total DNA from 14 atrazine-tolerant and 14 atrazine-sensitive clones and hybridized with the radioactively labelled rDNA or pNtrH70 inserts. For rDNA, a significant variation in copy number was shown to occur in 5 out of the 14 tolerant clones (Table 1) when compared to the leaf DNA, while 11 out of the 14 sensitive clones showed a difference in rDNA multiplicity with respect to the leaf (Table 2). The *F*-statistic test (Snedecor 1956) was used to evaluate the differences between the two groups of clones. On the whole, the atrazine-tolerant clones showed a significant ($P = 0.01$) decrease in copy number for rDNA with respect to the susceptible ones (Table 3). The anonymous repeated sequence pNtrH70 showed a significant increase in copy number both in the tolerant (11 clones out of 14) (Table 1) and in the sensitive (10 clones out of 14) somaclones (Table 2) with respect to leaf DNA. When the average values for the two groups of clones were compared, the herbicide-tolerant clones showed a higher copy number than the susceptible ones ($P = 0.01$, Table 3).

Previous work (Brown 1989) had shown that differences in methylation patterns for repeated sequences can occur between plant tissues and in vitro cultures; it therefore seemed interesting to analyse rDNA methylation in our system. The two isoschizomeric restriction endonucleases *Hpa*II and *Msp*I were used: both enzymes cleave the target 5′CC/GG 3′ sequence, but only *Msp*I can cut DNA when the internal cytosine is methylated. Five micrograms of total DNA from 12 tolerant and 12 susceptible clones were digested with

Table 3 Effect of the presence of atrazine stress on the multiplicity of the probes used in this study. Values are the average of at least two independent experiments for each probe (*atr-T* atrazine-tolerant · *atr-S* = atrazine-susceptible)

Probe	Atrazine-tolerant	Atrazine-susceptible	*F*-value	Variance
rDNA	213	295.4	378**	atr-T = 22801.2 atr-S = 18760.3 $F = 1.21^{n.s.}$
pNtrH70	495	424	305.8**	atr-T = 204023.2 atr-S = 98695.3 $F = 2.06$**
H900	365.4	238.6	98.7**	atr-T = 256201.5 atr-S = 55454 $F = 4.62$**
pS6	100.8	88.7	127.7**	atr-T = 8890.95 atr-S = 4375.2 $F = 2.03$**

* Significant at $P \leqslant 0.05$; ** $P \leqslant 0.01$; n.s., not significant

*Hpa*II or *Msp*I and hybridized with the digoxigenin-labelled rDNA probe. The results showed that there were no differences in rDNA methylation pattern between the tolerant and susceptible clones examined (data not shown). Moreover, total DNA from all the clones was also digested almost to completion by *Msp*I but not by *Hpa*II (data not shown), suggesting a high methylation level; but again no differences were found between the two groups of clones.

Analysis of atrazine resistance: *psbA* gene mutation, copy number and expression

As mentioned in the Introduction, a point mutation in the chloroplast *psbA* gene, leading to a single amino acid substitution at position 264 in the mature protein, has been shown to be responsible for atrazine resistance in vivo in higher plants. We therefore analysed our somaclones in order to assess if the tolerance to the herbicide could be ascribed to this phenomenon. A 945-bp region of the *psbA* gene spanning the mutation site was thus amplified by PCR and the products digested with the restriction endonuclease *Mae*I. Upon digestion of the amplified *psbA* fragment, a unique 162-bp fragment was detected in the presence of the mutation (Fig. 3A). The results showed that a major 127-bp band, characteristic of the non-mutated form of the gene, was present in all clones, and a very faint band at 162 bp was apparent in some lines (Fig. 3B), thus suggesting that some somaclones may be a mosaic for the mutation. The digestion pattern obtained, however, was not in complete agreement with the map reported in Fig. 3A. As the published sequence (Sugita and Sugiura 1984) is obtained from a tobacco cultivar ('Bright Yellow 4') different from the one used in this study ('White Burley'), it is possible that the first recognition site for *Mae*I is missing in the latter. This *Mae*I site is also absent in another *Nicotiana* species, *N. debneyi* (Zurawski et al. 1982). The sequencing of the *psbA* gene of *N. tabacum* var 'White Burley' could solve this problem, but it does not seem to be necessary in our case as there is no ambiguity regarding the presence of the bands that allow the presence or absence of the mutation to be determined.

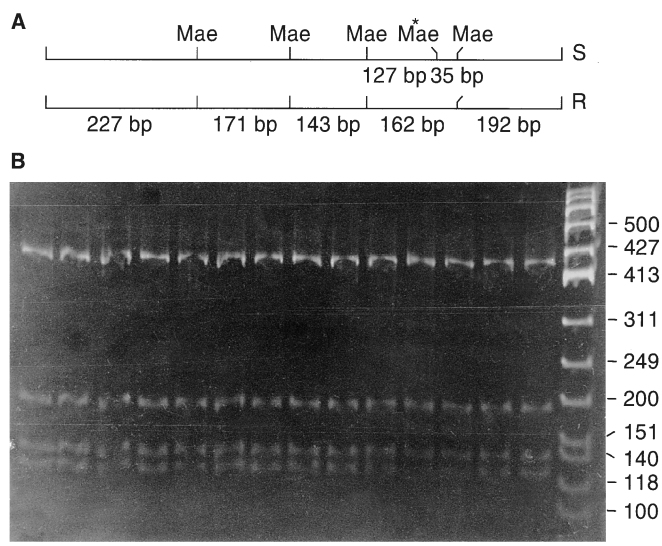

Fig. 3 **A** *Mae*I restriction map of the *Nicotiana tabacum psbA* gene (cv 'Bright Yellow 4'; Sugita and Sugiura 1984). The point mutation conferring atrazine resistance leads to the loss of the restriction site marked by the *asterisk*. **B** *Mae*I restriction pattern for the PCR-amplified *psbA* gene fragment in the tolerant tobacco somaclones. Sample order (*left to right*) is: 1.87, 1.35, 1.9, 1.95, 1.10, 1.52, 1.36, 1.22, 1.88, 1.5, 1.74, 1.6, 1.97, leaf, MW marker ($\Phi \times 174$ *Hin*fI, Promega). Fragments were separated on a 8% acrylamide-TBE gel. Molecular weights are expressed in basepairs

psbA gene amplification was analysed by dot blot hybridization using as a probe the radioactively labelled insert from the H900 clone. The results showed that 9 out of 14 atrazine-adapted clones had a significantly higher copy number for H900 than control leaf DNA (Table 1), while an increase in multiplicity for this sequence was present only in 4 out of 14 sensitive clones (Table 2). A highly significant increase ($P = 0.01$) in H900 copy number in the tolerant group with respect

to the susceptible one was shown by ANOVA (Table 3). To assess if this increase in multiplicity was due to the true amplification of the target sequence or to an increased number of chloroplasts/chloroplast genomes in the tolerant clones, we carried out the same experiment using as a probe the 1400-bp insert from the pS6 clone, which comprises a region of the wheat chloroplast genome not containing the *psbA* gene. The results (Table 1 and 2) showed that the multiplicity for pS6 was the same as for H900, except for the susceptible clone wt36 and the tolerant 1.35 and 1.22 clones, thus demonstrating that the differences in copy number were probably due to an increase in the number of chloroplasts and/or chloroplast genomes rather than to the specific amplification of the target gene. These data were confirmed by regression analysis that showed a very high correlation coefficient between the two probes: $r = 0.98$ ($P = 0.01$) for the atrazine-adapted clones and $r = 0.95$ ($P = 0.01$) for the atrazine-susceptible ones.

Finally, the possibility of an increase in *psbA* gene expression was checked by Northern hybridization on 9 tolerant and 11 susceptible clones with the digoxigenin-labelled H900 insert. No significant differences in *psbA* gene expression were shown between the two groups of clones (data not shown).

Discussion

The aim of the present work was to analyse the effect of applying an additional stress, besides tissue culture itself, on somaclonal variation in *Nicotiana tabacum* by comparing cell clones selected or not selected for tolerance to the herbicide atrazine and to test for the presence of molecular evidence of a mechanism that could be responsible for herbicide tolerance.

The results obtained showed a surprisingly high stability of the *Nicotiana* genotype at the molecular level. RFLP analysis carried out on a generally variable sequence (the entire rDNA unit) and on an anonymous repeated sequence with three restriction enzymes and their combinations did not show any variability. A similar result was obtained for rDNA through the use of the isoschizomers *Msp*I and *Hpa*II, thus showing in this case the absence of a variation in methylation patterns, variations being a rather frequently found feature in plant tissue cultures. Moreover, only in 1 RAPD profile out of 12 was a single, reproducible polymorphism observed (Fig. 2), which is at variance with results obtained with other plant species (Brown et al. 1993). It should be stressed that such a stability could not be impaired by the presence of atrazine, a powerful toxic agent in non-photoautotrophic cultures as well, where it induces a strong anaerobiosis, as previously shown by our laboratory (Bettini et al. 1992).

Molecular analysis of the atrazine-target gene revealed the existance of mosaic somaclones in which a minority of cells, or plastid genomes, seemed to have a mutated *psbA* gene (Fig. 3B). The stoichiometric amount of the 162-bp band being very low, it seems nevertheless difficult to account for the herbicide tolerance in the relevant somaclones on the basis of the reduced affinity between atrazine and the target protein resulting from the mutation of the *psbA* gene. No differences between the two groups of clones were detected for both *psbA* copy number (Table 1 and 2) and expression. Another possible mechanism for atrazine resistance, i.e. herbicide detoxification by conjugation with glutathione through the activity of the glutathione *S*-transferase enzyme family (GST, EC 2.5.1.18) (Shimabukuro et al. 1971), has been tested in our system (Bettini et al. 1992), but no increase in constitutive or induced GST activity was detected in the tolerant clones with respect to the control. Taken together, these data suggest that cell tolerance may be due, in our case, to an increase in plastids/plastid genomes, as demonstrated by the hybridization with the H900 and pS6 probes (Table 1 and 2), or/and to a permanent response to respiration inhibition whose basis is, up to now, unknown.

In partial contrast with their apparent qualitative stability, both of the repeated sequences analysed seemed to respond to in vitro culture conditions and to stress by showing an increase or a decrease in the number of copies in the presence of the herbicide (Tables 1–3). Moreover, atrazine-adapted and susceptible clones both showed a high multiplicity variation with respect to leaf, thus confirming that quantitative variation for repeated sequences is a common characteristic of somaclonal variants (Flavell 1989; Bennett and Smith 1991; Cavallini et al. 1996). Multiplicity changes at the level of repeated sequences are recognized as having a great adaptive and evolutionary significance (Flavell 1985), contributing to the adaptation of plants to stress and disequilibrium conditions. In flax, for example (Cullis 1986), the amplification of repeated sequences induced by unfavourable environmental conditions is transmitted to the progeny and may contribute to the evolutionary change through an increase in DNA complexity and its effect on developmental modulation, thus leading to phenotypic variation with a minimal frequency of lethal events.

The comparison of variance data (Table 3), also showed a higher variability in the atrazine-selected somaclones than in the susceptible ones for all the probes used in this study except rDNA.

In conclusion, our results, besides suggesting a surprising stability of the *N. tabacum* genome in vitro, a situation which resembles closely that found in *Hordeum* (Shimron-Abarbanell and Breiman 1991), and its maintenance in the presence of an additional stress agent, do confirm the response of plant cells in vitro to changes in physiological conditions through modifications

in the copy number of repeated sequences. Such a feature, frequently found in somatic cells in vivo to be correlated with differentiation and dedifferentiation processes (for extensive reviews on the subject see Buiatti 1977; Bassi 1990; Nagl 1990), seems to suggest a regulatory role of multiplicity variation in plant development.

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