

Analysis of genomic DNA methylation patterns in regenerated and control plants of rye (*Secale cereale* L.)

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Abstract Rye (*Secale cereale* L.) is a species that has shown high rates of somaclonal variation when plants obtained by in vitro culture were analysed using different techniques. In this study, using methylation-sensitive amplified polymorphism (MSAP) markers, we analysed the cytosine methylation status at genomic level of regenerated plants of rye that were obtained by somatic embryogenesis. Such plants were originated from three different cell lines and the results were compared with the data obtained from the control plants grown from seeds of the same cultivar and lot. A similar total number of MSAP markers was observed in the regenerated (937) and control plants (1,022), while the mean number detected per plant was significantly higher in regenerated (554.43) than in control plants (356.00). The analysis indicated conservation of the number of partially-methylated CCGG/GGCC sites for all type of plants. However the mean number of non-methylated sites was near twofold in the regenerated plants (442.48) than in control plants (248.19). Methylation changes have been detected in all the regenerated plants when compared within cell lines, with an average frequency of 9.01 % of the detected markers. We also observed that regenerated plants from one or several cell lines shared methylation changes at the same locus pointing to a non-random behaviour of the changes in genomic methylation.

Keywords Somaclonal variation · Epigenetic instability · Methylation · MSAP · Rye

Introduction

The occurrence of somaclonal variation was observed and reported, for the first instance, more than 50 years ago (Braun 1959) and still it remains one of the major problems of many tissue-cultured plants. Somaclonal variation was defined later by Larkin and Scowcroft (1981) as the variation originating in cell and tissue cultures and today it refers to tissue culture-induced stable genetic, epigenetic or phenotypic variation (Neelakandan and Wang 2012). Such variation is reported in varying proportions for many plant species when it is analysed using different detection methods, including molecular markers with high power of resolution (Bairu et al. 2011, and references therein).

Recent studies of somaclonal variation are focused on the analysis of the epigenetic instability that is generated by the in vitro growth of plant cells. One of the epigenetic modifications of the hereditary material is DNA methylation (the addition of a methyl group to a cytosine) that occurs in plants mainly on transposons and other repetitive elements (Zhang et al. 2006). Higher plants DNA contain large quantities of 5-methylcytosine (m^5C) and very often its content is comparable to that of cytosine. The DNA methylation patterns analysed in regenerated plants of several species are variable indicating that such epigenetic mechanism contribute to the process of somaclonal variation. The methylation changes may activate transposable elements and may also be involved in cytogenetic instability through modification of heterochromatin (Kaeppler et al. 2000).

Unlike in mammals, where it occurs almost exclusively in the symmetric CG context, cytosine methylation in plants is located in symmetric CG and CHG context (H = A, T, or C) and the asymmetric CHH context (Henderson and Jacobsen 2007). The use of isoschizomers

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that differ in their sensitivity to methylation of their recognition sequence allowed the development of several methods to detect the methylation polymorphisms, such as restriction landmark genomic scanning (Hatada et al. 1992), methylation-sensitive arbitrary PCR (Gonzalzo et al. 1997) or methylation-sensitive representational difference analysis (Ushijima et al. 1997). But it is the methylation-sensitive amplified polymorphism (MSAP), a modification by Reyna-López et al. (1997) of the amplified length polymorphism (AFLP) technique (Vos et al. 1995), the chosen method for the analysis of epigenetic instability in regenerated plants of different species such as *Musa acuminata* (Peraza-Echeverría et al. 2001; James et al. 2004), *Codonopsis lanceolata* (Guo et al. 2007), *Humulus lupulus* (Peredo et al. 2006, 2009) *Hordeum brevisubulatum* (Li et al. 2007), *Pisum sativum* (Smykal et al. 2007), *Solanum tuberosum* (Sharma et al. 2007), *Vitis vinifera* (Schellenbaum et al. 2008; Baránek et al. 2010), *Doritaenopsis* hort. (Park et al. 2009), *Arachis villosulicarpa* (Pacheco et al. 2008), *Cymbidium hybridum* (Chen et al. 2009), *Freesia hybrida* (Gao et al. 2010), *Ocotea catharinensis* (Hanai et al. 2010), *Clivia miniata* (Wang et al. 2012), *Gardenia jasminoides* (Wu et al. 2012), or *Agave tequilana* (Díaz-Martínez et al. 2012). Those studies have demonstrated that the MSAP technique is highly efficient and reliable for large-scale detection of cytosine methylation changes at unbiased loci in plant genomes.

Rye (*Secale cereale* L.) is an excellent species for studies on somaclonal variation as it has shown high mutation rates when plants obtained by in vitro culture procedures were analysed, using different techniques, for the detection of somaclonal mutants. Chromosomal variants have been described for rye calluses (Asami et al. 1976) and other cytogenetic and biochemical mutants were also detected in regenerated plants (Bebeli et al. 1990). Amplification of repeats sequences from telomeric heterochromatin has been observed in regenerated plants of rye using in situ hybridization (Karp et al. 1992). Cytogenetic variants were also detected among regenerated plants and in the progeny of normal diploid regenerated plants (Linacero and Vázquez 1992). Linacero and Vázquez (1993) reported a high (50.75 %) mutant frequency in plants regenerated from immature embryo cultures and other characteristics typical of somaclonal variation were observed in that study in rye such as the obtaining of dominant mutations, the presence of more than one mutation per plant, the obtaining of homozygous mutants and the detection of high mutation rate in particular loci. Hot spots for genetic instability have been reported for rye genome in regenerated plants using RAPD, AFLP, and ISSR markers (Linacero et al. 2000; de la Puente et al. 2008; Linacero et al. 2011), and the transposition of the mobile element *RYS1* have been related to the generation

of somaclonal variants using RAPD markers (Alves et al. 2005). High mutant rates, mutational hot spots, and the relation between telomeric repeat regions, mobile elements, and somaclonal variation were also observed in regenerated plants using AFLP markers (de la Puente et al. 2008). The correlation between genetic and epigenetic changes within somatic embryo-derived plants has been studied previously in rye using ISSR fingerprinting (Linacero et al. 2011).

In this study we evaluated, for the first time using the MSAP technique, the cytosine methylation status at genomic level of regenerated plants of rye that were obtained by somatic embryogenesis from three different cell lines and we compared the results with those in control plants from the same cultivar of origin. The aim was to analyse in a large number of loci if methylation variation shows the same non-random distribution that has already been detected for nucleotide variation in regenerated plants of rye, pointing to the existence of common mechanisms connecting both types of variation.

Materials and methods

Plant material and DNA extraction

Seeds of rye (*Secale cereale* L. cv. “Ailés”) were used to obtain donor plants of immature embryos. Somatic embryogenesis was induced in 3–4-month-old immature embryo-derived calli obtained according to Linacero and Vázquez (1993) procedures. Emerging regenerated plantlets were potted into soil when they reached 13–15 cm in height. Then the development of new leaves was controlled. A 7-day-old leaf fully developed in ex-vitro conditions, was collected for DNA extraction. Cell lines were established for all embryogenic calli derived from the same immature embryo and were named L6, L7, and L8, which included six (L6.1–L6.6), seven (L7.1–L7.7), and eight (L8.1–L8.8) regenerated plants each, respectively. Twenty-one plants, grown in soil and obtained from the same seed lot of Ailés cultivar, were used as control plants and were named P01–P21. When the control plants had reached 13–15 cm in height, the development of new leaves was controlled. Then a new leaf that had grown for 7 days was collected from each control plant for DNA extraction.

MSAP protocol

The original MSAP protocol that involved the use of the methylation-sensitive isoschizomers *HpaII* and *MspI* as ‘frequent-cutter’ enzymes (Reyna-López et al. 1997) was used with modifications. Both endonucleases recognize and cut at the same tetranucleotide 5'-CCGG-3' sequence but

they are blocked if the two cytosines are methylated at both DNA strands. *HpaII* and *MspI* have differential sensitivity to other methylation status of the cytosine residues (Butkus et al. 1987; Ben-Hattar and Jiricny 1988; McClelland et al. 1994; Hou et al. 2004). The adapter and pre-selective primer (*E+A*) for the ‘rare-cutter’ *EcoRI* endonuclease were the same as that used in standard AFLP analysis (Vos et al. 1995), while the *HpaII/MspI* adapter and the basic primer sequence (*HM+0*) were designed according to Xiong et al. (1999).

To detect MSAP markers, two restriction-ligation reactions were set up in parallel. In the first reaction, 100 ng of DNA was digested with 25 U of *HpaII* plus 25 U of *EcoRI* (NEB, New England Biolabs) for 2 h at 37 °C in a final volume of 11 µl containing 1 U of T4 DNA ligase (NEB), 0.5 µM *EcoRI* adapter, 0.5 µM *HpaII/MspI* adapter, 1× T4 DNA ligase buffer (NEB), 50 mM NaCl, and 100 ng/µl BSA. The second reaction was carried out in exactly the same way, except that *MspI* was used in place of *HpaII*. Reactions were stopped by incubating at 65 °C for 8 min and diluted in Milli-Q water to 200 µl for PCR amplification.

Pre-selective amplifications were conducted independently by using 4 µl of each of the above diluted restriction-ligation reactions with *E+A/HM+C* primers in a final volume of 20 µl containing 15 µl AFLP Core Mix (Applied Biosystems, Foster City, CA) and 0.5 µl, 10 µM solution of each primer. PCR reactions were performed with the following profile: 72 °C for 2 min, 20 cycles of 20 s denaturing at 94 °C, 20 s annealing at 56 °C and 2 min extension at 72 °C, ending with 30 min at 60 °C. After checking for the presence of a smear of fragments by agarose electrophoresis using half of the reaction volume, the remaining amplification products were diluted 20 times in Milli-Q water.

Selective amplifications were performed in a final volume of 20 µl by using 3 µl of the diluted pre-amplification products with 15 µl AFLP Core Mix (Applied Biosystems, Foster City, CA), 1 µl of 5 µM *EcoRI* primer with three selective bases (*E+AGG* or *E+AGC*), and 1 µl of 5 µM *HpaII/MspI* primer with three selective bases (*HM+CCG*, *HM+CCC*, or *HM+CTG*). *EcoRI* selective primers were 5′-labelled with JOE (*E+AGG*) or NED (*E+AGC*) fluorescent dyes. PCR parameters included an initial hold at 94 °C for 2 min, 10 touchdown cycles (94 °C for 20 s, 66 °C for 20 s, and 72 °C for 2 min) during which the annealing temperature was decremented 1 °C each cycle, followed by 20 cycles of 94 °C for 20 s, 56 °C for 20 s, and 72 °C for 2 min, a final hold at 60 °C for 30 min.

The denatured products of selective amplifications were separated by capillary electrophoresis on an ABI Prism 310 Genetic Analyzer running Data Collection v.3.1 software (Applied Biosystems, Foster City, CA). Injection samples

were prepared by adding 2 µl of JOE-labelled selective PCR reaction, 4 µl of NED-labelled selective PCR reaction, and 1 µl of GeneScan 500 ROX size standard (Applied Biosystems, Foster City, CA) to 18 µl of deionised formamide. The samples were denatured for 5 min at 95 °C and then snap cooled on ice before injection at 15 kV for 12 s. Each MSAP procedure was reproduced at least twice for each DNA sample.

Data analysis

The electropherograms were analysed using GeneMapper 4.0 software (Applied Biosystems, Foster City, CA), which automatically quantified and sized the amplified fragments for each DNA sample, digestion reaction, and selective primer combination. Six matrices were created, one for each selective primer pair combination, where all the amplified fragments between 50 and 500 bp were compared across all the samples and scored as either present (1) or absent (0) for each DNA sample and digestion reaction. All the polymorphic fragments annotated at the GeneMapper-generated matrices were validated or corrected by visual inspection of the corresponding electropherograms.

MSAP markers were classified, for each DNA sample, as type 11, type 10, type 01, or type 00 accordingly to the presence (1) or absence (0) of the corresponding amplified fragment in the digestion reactions with *HpaII* and *MspI*, respectively. The six MSAP-marker matrices were grouped in a single matrix to allow the sample comparison analysis. A methylation identity rate for MSAP markers (IRM) was calculated for all pairs of DNA samples based on Jaccard’s coefficient of similarity comparing the presence codes 11, 10, 01, and 00 for each marker, but excluding code 00 as shared marker. The unweighted pair group method using arithmetic averages (UPGMA) procedure was used for cluster analysis in order to show the phenetic relationships between genotypes.

Results

MSAP markers detection in control and regenerated plants

The analysis of 42 rye plants (21 plants regenerated via somatic embryogenesis and 21 control plants) revealed a total number of 1,226 different MSAP markers that were obtained from the comparison of 504 electropherograms, twelve for each analyzed plant as six selective-primer combinations and two independent double-digestion reactions were used. Each amplified fragment obtained from at least one plant DNA and one double-digestion reaction (*HpaII+EcoRI* or *MspI+EcoRI*) was considered as a

MSAP marker that reveals the presence of a CCGG/GGCC site. The number of different markers detected in the control population was 1,022 while cell lines L6, L7, and L8 showed 515, 585 and 590 markers, respectively, and 937 markers when all the 21 regenerated plants were considered as a single group. However, the number of markers detected per plant was higher in regenerated (from 507 in plants L6.3, L6.4, and L6.5–583 in plant L8.5) than in control plants (from 291 in plant P06–429 in plant P10) with significant different mean values of 554.43 and 356.00 markers, respectively (Student's *t* test, $p < 0.0001$).

MSAP markers classified as type 11 (fragment detected in both restriction reactions) reveal non-methylated CCGG/GGCC sites and they were the more frequent for all the analyzed plants, whereas that several kinds of partially-methylated CCGG/GGCC sites are detected by markers classified as type 10 or 01. The total number of non-methylated (type 11) and partially-methylated (type 10 plus type 01) sites for each analysed plant is shown in Fig. 1. All the regenerated plants showed a higher number of non-methylated sites than control plants with significant different mean values when comparing both groups of plants (Student's *t* test, $p < 0.0001$); the mean number of non-methylated sites was near twofold in regenerated plants (442.48) than in control plants (248.19). Non-significant differences were observed for the mean number of partially-methylated sites observed in regenerated (111.95) and control plants (107.81) which correspond to 20.19 and 30.28 % of the mean number of detected markers in each group of plants, respectively.

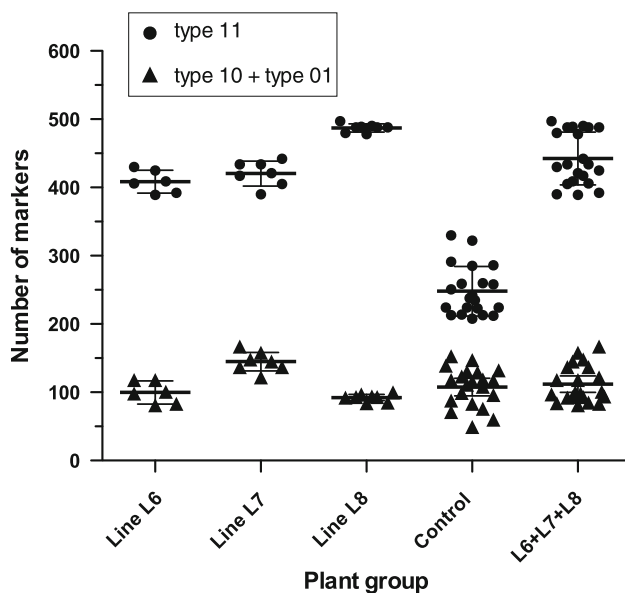


Fig. 1 Grouped column scatter graph for the number of different types of markers detected in each analyzed plant. *Thick horizontal lines* represent mean values and *fine horizontal lines* represent standard deviations

The markers type 10 were more frequent than type 01 in most of the analyzed plants (14 out of 21 control plants and 19 out of 21 regenerated plants) but non-significant differences were observed for mean values in the plants of the control population (58.19 and 50.57, respectively) while the type 10 (hemi-methylation of the external cytosine) was significantly more frequent than type 01 (full-methylation or hemi-methylation of the internal cytosine) in each of the three cell lines and also when all the regenerated plants were grouped (67.48 and 45.48 mean number for types 10 and 01, respectively; Student's *t* test, $p < 0.0001$). However, non-significant differences were observed when comparing the number of type 10 and type 01 markers between control and regenerated plants.

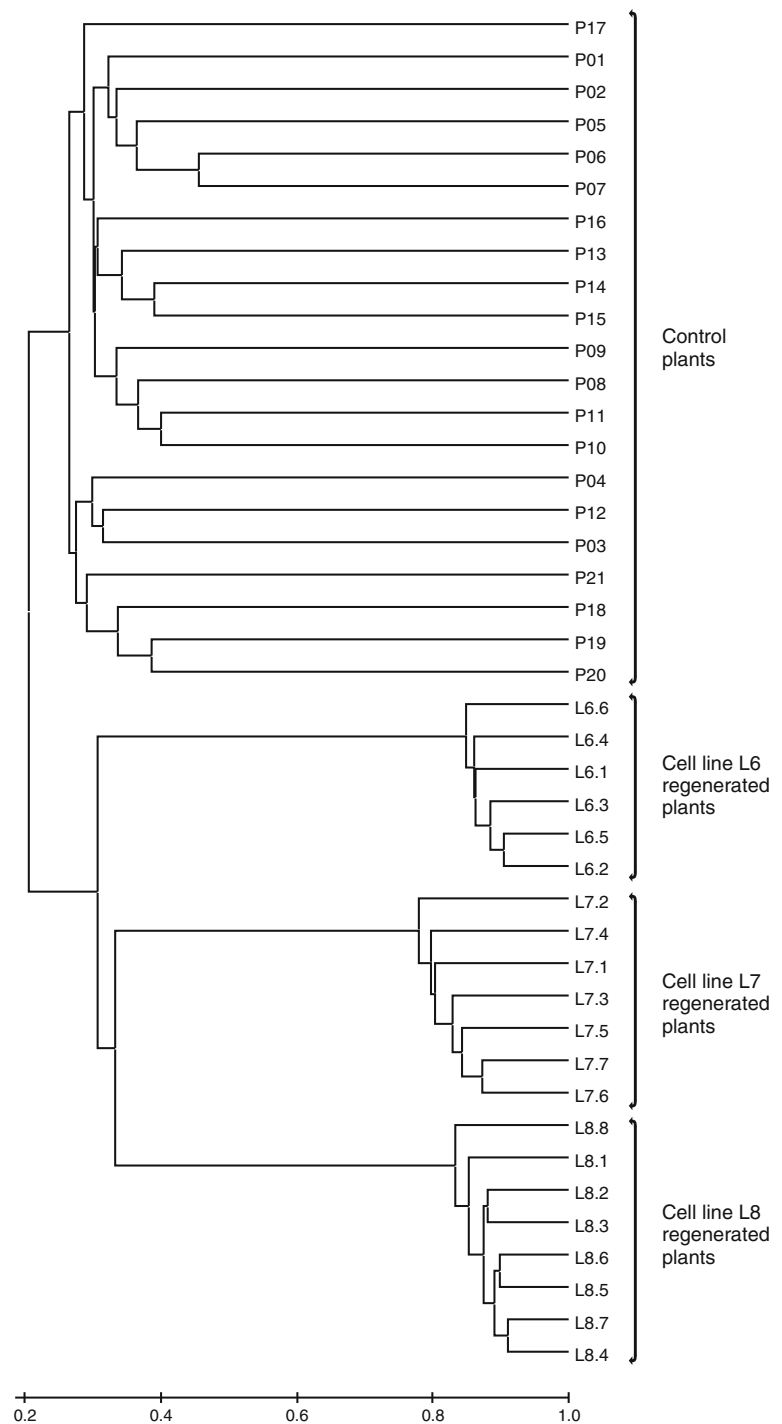
The identity rate for MSAP markers (IRM) was obtained for all pairs of plants and ranged from 0.138 (plants P12 and L6.4) to 0.910 (plants L8.4 and L8.7), with mean values of 0.861, 0.783, and 0.834 between regenerated plants from cell lines L6, L7, and L8, respectively, 0.318 between regenerated plants from different cell lines, and 0.288 between plants from the control population. The relationships among the 42 plants are shown graphically by the dendrogram of Fig. 2.

Methylation polymorphism

In order to assess the variability of each of the MSAP markers (Table 1), we compared the corresponding presence codes across all the plants from the same cell line or group of plants (control or regenerated plants). If they showed the same code we classified the marker as monomorphic in such line or group. Otherwise the marker was annotated as polymorphic. Two kinds of polymorphic markers were considered, those that were always detected in at least one digestion reaction (types 11, 10, or 01) for all the comparing plants and those that were not detected at all (type 00) for at least one plant. The absence of a fragment in both digestion reactions may be also due to a different reason (polymorphism in the nucleotide sequence) than changes in the methylation pattern, while the first kind of polymorphic markers are only due to methylation status modification (epigenetic polymorphism). Most of the polymorphic markers detected into the three cell lines can be assigned to epigenetic changes (92.86 % in L6; 89.43 % in L7; 94.59 % in L8). This is not the case when comparing the group of control plants (68.99 %) or the whole group of regenerated plants (54.92 %), two groups of plants where original variation (genetic plus epigenetic) is expected to be present.

The analysis of the number of epigenetic polymorphic markers detected in the regenerated plants of each cell line (Table 2) revealed a variation ranging from 3.72 to

Fig. 2 Phenogram obtained using the identity rate for MSAP markers and UPGMA cluster analysis for the 42 analysed plants



13.64 % of the detected markers in each plant with a mean value of 9.01 %. All possible types of variations were detected, however, the status of partial methylation of a site (types 01 and 10) for the corresponding plant while most or all of the plants from the same cell line showed non-methylation in the same site (type 11) was the more frequent variation. Singletons, variations detected in a single plant from the corresponding cell line, ranged from 1.85 to

44.19 % of the polymorphic markers detected in each plant with a mean value of 21.13 %. Therefore two or more regenerated plants from each cell line shared most of the observed variations. We observed epigenetic polymorphisms that were shared between regenerated plants obtained from different cell lines in 102 markers, 10.9 % of the total number of detected markers in this group of plants.

Table 1 Total number and percentages (in parenthesis) of detected markers in each cell line or group of plants

Cell line or plant group	Total markers	Monomorphic markers			Polymorphic markers	
		Type 11	Type 10	Type 01	Total	Epigenetic
Line L6	515	327 (63.5)	25 (4.9)	9 (1.7)	154 (29.9)	143 (27.8)
Line L7	585	295 (50.4)	31 (5.3)	13 (2.2)	246 (42.1)	220 (37.6)
Line L8	590	375 (63.6)	19 (3.2)	11 (1.9)	185 (31.3)	175 (27.8)
L6 + L7 + L8	937	92 (9.8)	2 (0.2)	0 (0.0)	843 (90.0)	463 (49.4)
Control	1,022	48 (4.7)	0 (0.0)	0 (0.0)	974 (95.3)	672 (65.8)

Table 2 Number and types of epigenetic polymorphic markers in the regenerated plants of each cell line

Cell line	Plant	Detected markers	Type of variation ^a						Total (%)	Singleton ^b
			01 (11)	10 (11)	11 (01)	11 (10)	10 (01)	01 (10)		
L6	L6.1	510	15	19	0	3	0	0	37 (7.25)	15 (40.54)
	L6.2	511	2	5	8	4	0	0	19 (3.72)	7 (36.84)
	L6.3	507	15	11	6	7	0	1	40 (7.89)	8 (20.00)
	L6.4	507	17	26	6	4	1	0	54 (10.65)	13 (24.07)
	L6.5	507	6	18	1	6	1	0	32 (6.31)	9 (28.12)
	L6.6	508	11	9	1	22	0	0	43 (8.46)	19 (44.19)
L7	L7.1	571	12	19	12	19	0	1	63 (11.03)	16 (25.40)
	L7.2	557	42	17	5	11	1	0	76 (13.64)	25 (32.89)
	L7.3	562	24	13	10	9	10	0	57 (10.14)	9 (15.79)
	L7.4	563	36	13	10	8	1	0	68 (12.08)	16 (23.52)
	L7.5	571	16	17	13	11	2	1	60 (10.51)	9 (15.00)
	L7.6	569	26	9	9	6	2	2	54 (9.49)	1 (1.85)
	L7.7	564	13	7	19	9	0	1	49 (8.69)	7 (14.29)
L8	L8.1	580	16	25	10	16	1	2	70 (12.07)	14 (20.00)
	L8.2	577	19	13	8	7	2	0	49 (8.49)	9 (18.37)
	L8.3	574	12	12	5	10	1	0	40 (6.97)	7 (17.50)
	L8.4	581	16	15	5	8	0	0	44 (7.57)	4 (9.09)
	L8.5	583	9	19	6	6	1	0	41 (7.03)	4 (9.76)
	L8.6	582	17	16	4	12	1	0	50 (8.59)	4 (8.00)
	L8.7	581	5	16	7	7	2	0	37 (6.37)	1 (2.70)
	L8.8	578	31	16	8	12	1	2	70 (12.11)	25 (35.71)

^a Presence code for the corresponding plant followed by the more frequent presence code (in parenthesis) in the cell line plants

^b Variations only observed in the corresponding single plant; the percentages of polymorphic singletons appear in parenthesis

Discussion

Methylation detection using *HpaII* and *MspI* isoschizomeres

MSAP markers are highly reproducible and have been frequently used to identify DNA methylation alterations among regenerated plants. Therefore the number of species analysed with this methodology is increasing every year, as previously stated. In the majority of such studies, MSAP markers are detected using gel electrophoresis. In contrast, we used a capillary electrophoresis system that enabled a

precise estimation of fragment mobility (1 bp resolution) and allowed the reduction of homoplasmy (co-migration of non-homologous fragments), which is the major issue in the analysis and interpretation for AFLP derived data (Meudt and Clarke 2007). We also manually checked the electropherogram comparisons for all the polymorphic markers in order to reduce the scoring errors. The maximum size of 500 bp for the analyzed fragments reduced the detection of false large type 10 and 01 markers, that can be originated by the presence of internal CCGG/GGCC sites (Xu et al. 2000, 2004; Li et al. 2002). We have not found significant differences in mean fragment size for type 10

and 01 markers and either in the frequency of type 10 and 01 markers in the samples if only fragments smaller than 250 bp are used (data not shown).

The original MSAP technique involves the use of *HpaII* and *MspI* endonucleases that recognize the restriction site CCGG/GGCC. Both isoschizomers are blocked by full methylation of both cytosines at both DNA strands so they do not allow a precise estimation of genomic methylation levels. Both *HpaII* and *MspI* cleave when there is no methylation on either cytosine, *HpaII* (but not *MspI*) also cuts if only the external cytosine is hemimethylated, and *MspI* (but not *HpaII*) also cleaves if only the internal cytosine is fully or hemimethylated (Butkus et al. 1987; Ben-Hattar and Jiricny 1988; McClelland et al. 1994; Hou et al. 2004). Although the above published studies are conclusive, several authors consider that *MspI* does not cleave when the internal cytosine is hemimethylated (Baurens et al. 2003; Guo et al. 2007; Li et al. 2007; Salmon et al. 2008) or that *HpaII* also cleaves when the internal cytosine is hemimethylated (Li et al. 2002; Bardini et al. 2003; Chakrabarty et al. 2003; Portis et al. 2004; Hao et al. 2004; Hanai et al. 2010). Therefore, the total number of detected MSAP markers using *HpaII* and *MspI* for a single plant is proportionally related to the presence of partially-methylated (presence of methyl-cytosine in a single position; types 10 and 01) plus non-methylated CCGG/GGCC sites (type 11).

When a MSAP marker is not detected for one sample, using *HpaII* and *MspI* endonucleases, it is not possible to discern whether it is due to a nucleotide sequence change (genetic polymorphism) or to the complete methylation of the target (epigenetic polymorphism). In this work we have considered only as polymorphic epigenetic markers those that can be detected in all samples by at least one of the two endonucleases, although this implies an underestimation of epigenetic variation. Most (89.43–94.59 %) of the total variations detected in the regenerated plants analysed in this work can be attributed undoubtedly to methylation changes, pointing to a higher level of epigenetic deregulation in the in vitro environment (Neelakandan and Wang 2012).

Methylation polymorphism in regenerated plants of rye

We detected a significant higher number of non-methylated plus partially-methylated sites in the genome of the regenerated plants than in the control plants, due to the presence of near twofold non-methylated sites in the regenerated plants. The control population showed a wider variation in the number of such sites (from 291 to 429 detected markers per plant) than regenerated cell lines (from 507 to 583 detected markers per plant). Control and regenerated plants showed similar numbers of partially-

methylated sites as has also been reported for donor and regenerated plants of *C. lanceolata* (Guo et al. 2007). This homogeneity for partially-methylated sites across populations and lines has also been described in rice (Takata et al. 2005), wheat (Zhang et al. 2008), and *Brassica oleracea* (Salmon et al. 2008). The average percentage of partially methylated sites detected in the control population of Ailés cultivar (30.28 %) was similar to the value (32.25 %) reported for the rye inbred line L155 (Zhang et al. 2008), slightly higher than the observed value in rice (25 %; Takata et al. 2005), and lower than the value obtained in wheat (41.81 %; Zhang et al. 2008).

Several studies using different plant regeneration systems, including somatic embryogenesis and axillary bud proliferation, have also reported hypomethylation in tissue culture regenerants of different species as detected either by AFLP-based methods or HPLC (Renau-Morata et al. 2005; Peredo et al. 2006; Li et al. 2007; Schellenbaum et al. 2008). The comparison of controls and regenerated hop somaclones using MSAP markers revealed that 83 % of the changes observed in the polymorphic loci were demethylation of the recognition sites (Peredo et al. 2006). In tobacco, Koukalova et al. (2005) reported that hypomethylation of particular rDNA gene families was initiated as early as 2 weeks after callus induction and that remethylation took place upon plant regeneration. However, no obvious difference in methylation levels has been observed in *C. lanceolata* (Guo et al. 2007), pea (Smykal et al. 2007) or *G. jasminoides* (Wu et al. 2012).

Rye is an allogamous species and every embryo used as a culture explant was a genetically unique result of a cross. Regenerated plants were obtained by somatic embryogenesis from 1 to 2 mm long immature embryos that were too small to remove a sample. Therefore polymorphic MSAP marker comparisons were only made within a cell line and it was not possible to establish which methylation status was the initial for each locus. Methylation changes have been detected in all the twenty-one regenerated plants analysed in this work while it was reported for only 50 % of the twenty rye (cv. Ailés) regenerated plants analysed using ISSR fingerprinting on *HpaII/MspI*-digested DNA (Linacero et al. 2011), although the number (176) of markers analyzed in the ISSR study was notably lower. The frequency of modified MSAP markers in the cell lines, an average of 9.01 %, was higher than the 2.90 % of ISSR markers reported in rye by Linacero et al. (2011) but close to 10.5 % reported for a similar number of MSAP markers in *C. lanceolata* among regenerated plants (Guo et al. 2007).

The low mean number (21.13 %) of variations observed for a single plant (singletons) reveals the high frequency of shared polymorphic loci between regenerated plants from the same cell line. We also observed in 102 markers that

plants obtained from different cell lines shared methylation changes at the same locus. This non-random behaviour of the changes in genomic methylation as response to tissue culture has also been reported in rye previously (Linacero et al. 2011) and also in barley (Bednarek et al. 2007), hop (Peredo et al. 2006), sorghum (Zhang et al. 2009), *Gentiana* (Fiuk et al. 2010), and rice (Shan et al. 2012).

One possible reason for the non-random distribution of methylation changes is the presence of a large amount of repetitive DNA sequences in the genome of rye, including transposons and tandem repeats (Flavell 1986) that are common in methylated regions of the eukaryote genomes. Tandem repeats in retrotransposons are essential for their mobility and are frequently transcriptionally silenced by DNA methylation (Lippman et al. 2004). But tandem repeats are also necessary and sufficient for de novo methylation in a two-step recruitment of RNA-directed DNA methylation machinery (Chan et al. 2006) and therefore could be hot spots for methylation changes. On the other hand, promoter methylation of specific single-copy genes and consequent gene repression has been reported to contribute to the regulation of the undifferentiated state in *Arabidopsis* callus and suspension cultures (Berdasco et al. 2008). Other example is the promoter of *LEC1* gene that undergoes hypomethylation prior to somatic embryo formation, whereas these methylation levels increase during subsequent developmental events leading to vegetative growth (Shibukawa et al. 2009). Auxin signaling components, which are crucial for de novo adventitious shoot initiation and regeneration, are regulated by DNA methylation and histone modification of regulatory sequences (Li et al. 2011). This preferential methylation changes at both repetitive sequences and specific genes could explain the non-random distribution of methylation changes observed in regenerated plants of rye and other species.

Our study provides evidence that methylation changes occur at a sufficiently high frequency to be an important source of tissue culture-induced variation in rye. It is possible that decreases in methylation could affect chromatin structure (Tanurdzic et al. 2008). This could lead to changes in gene expression due to position effects, changes in recombination rates, and changes in the timing of DNA replication, perhaps leading to chromosome breakage (Phillips et al. 1994). Our results show that certain loci show more variation than others, suggesting that the variation may not be random. A more extensive study, using a different pair of methylation sensitive endonucleases that allow the simultaneous detection of genetic and epigenetic modifications at the same loci, is now underway and could provide us with valuable data about the correlation between epigenetic and genetic instability induced by tissue culture in rye and the genomic regions involved in such variation.

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