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Original article

Somaclonal variation at the nucleotide sequence level in rice (*Oryza sativa* L.) as revealed by RAPD and ISSR markers, and by pairwise sequence analysis

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Abstract. The nature of somaclonal variation at the nucleotide sequence level was studied in rice cv. Nipponbare. First, we investigated genomic variations by using 2 molecular marker systems: RAPD (random amplified polymorphic DNA) and ISSR (inter-simple sequence repeat). This was followed by sequencing of selected bands that represented genomic variations, and pairwise sequence analysis taking advantage of the whole genome sequence of rice. In addition, transpositional activity of the active MITE, *mPing*, was analysed by locus-specific PCR amplifications. The 2-year-old calli and their regenerated plants, analysed with 24 RAPD and 20 ISSR primers, showed moderate levels of genomic variation (20.83% and 17.04%, respectively). To test whether DNA methylation plays a role in somaclonal variation, the calli were treated with 5-azacytidine, a chemical agent that reduces cytosine methylation by blocking the activity of DNA methyltransferase. Though dwarfism occurred in regenerants from treated calli (a hallmark of the drug treatment), there was only a slight increase in the frequency of somaclonal variation detected in the treated calli and their regenerated plants relative to untreated controls. The transposon *mPing* also remained immobile in both treated and untreated calli. Nevertheless, dendrograms constructed according to the Jaccard coefficient calculated by UPGMA of the ISSR bands revealed that the 5-azacytidine-treated and untreated somaclones were grouped into 2 distinct clusters, suggesting a possible indirect effect of the treatment on the genomic changes, depending on the marker used. Sequence analysis indicated a low level of variation (0.31%), with single-base-pair substitutions predominating.

Keywords: ISSR markers, *mPing* activity, RAPD and ISSR markers, rice, sequence analysis, somaclonal variation.

Introduction

Tissue-culture-induced phenotypic and genotypic variations are collectively termed 'somaclonal variation' (Larkin and Scowcroft 1981). It has relevance in the clonal propagation of valuable or endangered plant germplasm, and in the production of transgenic plants. It may also be an effective means of generating useful mutants. Because of these reasons, somaclonal variation has been intensively studied by using various molecular markers in several plant species, including *Arabidopsis* (Polanco and Ruiz 2002) and rice (Kim et al. 2003). Nonetheless, few studies have addressed the molecular basis or nature of somaclonal variation (Al-Zahim et al. 1999; Yang et al. 1999), though it was found more than a decade ago that alteration in DNA methylation probably plays a role (Muller et al. 1990).

With the near completion of the whole-genome sequencing project on 2 model plants, *Arabidopsis* and rice, it is now feasible to study the nature of somaclonal variation at the nucleotide sequence level. Rice is particularly interesting for this purpose, because apart from being a model plant for the grass family (Poaceae) that includes all cereal crops, it is also a major crop that provides food for more than half of the world's population. Several

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studies of somaclonal variation have been carried out in rice by using cultivars of both subspecies: *indica* and *japonica* (Yang et al. 1999; Kim et al. 2003; Roy and Mandal 2005). The cultivar Nipponbare is a standard genotype for *japonica* rice, whose genome sequence is also the most complete (Sasaki and Burr 2000; Feng et al. 2002). Several earlier studies on somaclonal variation were conducted with the use of this cultivar. By using scutella as a source of calli and random amplified polymorphic DNA (RAPD) as a marker system, it was shown that somaclonal variation increased with culture age (Yang et al. 1999). More recently, a potentially active MITE (miniature inverted transposable element) in rice, called *mPing*, was activated under anther culture conditions in this cultivar, possibly contributing to somaclonal variation (Kikuchi et al. 2003).

The present work was aimed to provide some insight into the nature of somaclonal variation at the nucleotide sequence level in rice. For this purpose, we first analysed the occurrence of genomic variation in calli and regenerated plants by 2 molecular marker systems: RAPD and ISSR (inter-simple sequence repeat). We then isolated and sequenced representative variable bands, and performed a pairwise sequence comparison by taking advantage of the available sequence information for Nipponbare. To test if there is a link between DNA methylation state and the extent or kinds of genomic changes, we also analysed calli treated with 5-azacytidine and their regenerated plants. Finally, we analysed transpositional activity of *mPing* in both treated and untreated calli.

Materials and methods

Plant tissue culture, DNA extraction, RAPD andISSR analysis

Sterilized mature seeds of rice cv. Nippponbare were used in this study. Callus induction, maintenance and plant regeneration were performed according to methods reported earlier (Liu et al. 2004). After 24 months of subculture, portions of the calli were used for regeneration, while other portions of calli were transferred to a medium containing 50 μmol L^{-1} of filter-sterilized 5-azacytidine, a demethylation agent. Two weeks later, portions of the treated calli were subcultured on a maintenance medium, while the other portions were used for regeneration. In both cases, rooted shoots were grown in a greenhouse before DNA extraction and further analysis.

A modified CTAB protocol (Kidwell and Osborn 1992) was used to extract genomic DNA from expanded young leaves of regenerated plants and from 5-azacytidine-treated and untreated calli, and 2 independent samples of the seedlings. ISSR and RAPD amplifications were performed as reported previously (Guo et al. 2006). RAPD and ISSR amplification products were resolved on 1.5% agarose gels stained with ethidium bromide. The GeneRuler^{TM} DNA Ladder Mix (Fermentas Inc, Hanover, MD) was used as a molecular size standard. Gels were visualized and photographed under a UV transilluminator, bands were scored as either present (1) or absent (0), and genetic similarity was estimated on the basis of Jaccard coefficient (Jaccard 1908). Data from the similarity matrix were used for cluster analysis by the unweighted pair-group method with arithmetic averages (UPGMA) and the resulting cluster was represented as a dendrogram (Rohlf 1993). All the calculations were performed by using the NTSYS-pc version 2.02 software package (Numerical Taxonomy System, Exeter Software) (Rohlf 1997). Representative RAPD and ISSR polymorphic bands were isolated and cloned for sequencing. To test whether the observed variations were due to amplification and/or cloning procedures, 10 randomly chosen invariable bands from the control (5 from RAPD and 5 from ISSR) were also isolated, cloned and subjected to sequencing. After removing the RAPD or ISSR primers, the sequences were queried by BlastX at the NCBI website for possible homologies, and by BlastN at the Rice Genome Project (RGP) website to determine changes at the nucleotide sequence level.

Locus-specific PCR assay for *mPing* activity

To detect the possible transpositional activity of *mPing* in the treated and untreated calli, we performed PCR amplification by using 44 pairs of locus-specific primers (Shan et al. 2005) that were designed basing on the whole genome sequence of Nipponbare. The amplification conditions were as reported previously (Shan et al. 2005). PCR products were resolved by electrophoresis in 1.5% agarose gels stained with ethidium bromide, and visualized under a UV transilluminator.

Results

Although there was no discernible difference in appearance between 5-azacytidine-treated and untreated calli, plants regenerated from them showed dramatic morphological differences. The most

striking change in regenerated plants from the treated calli was dwarfism (Figure 1), as was shown earlier by Sano et al. (1990) to be a hallmark of 5-azacytidine-induced demethylation. This demonstrates that our 5-azacytidine treatment was effective, as was confirmed by restriction digestion by *Hpa*II and *Msp*I, a pair of methylation-sensitive isoschizomers that showed much more extensive digestion in the treated calli and plants than in those of the controls (data not shown).

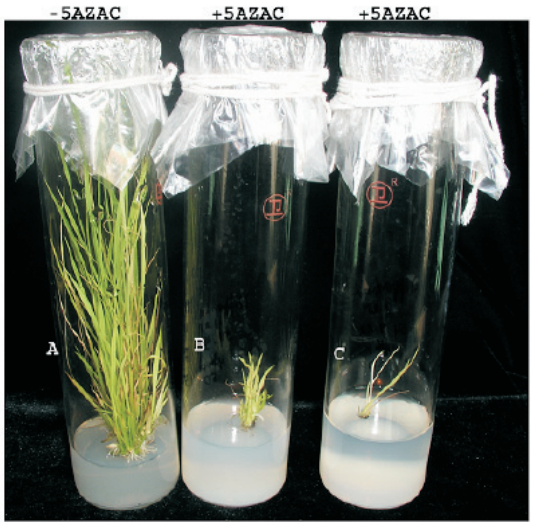

Figure 1. Morphology of regenerated plantlets from untreated callus (A) and from 5-azacytidine-treated callus (B and C) of rice cv. Nipponbare. Dwarfism in B and C is apparent.

To study the effect of tissue culture on genetic variability, DNA from 7 pieces of 2-year-old calli induced from seeds of cv. Nipponbare, 6 regenerated plants and 2 seedlings were analysed by 24 RAPD and 20 ISSR primers (Table 1). Of a total of 168 and 176 bands for RAPD and ISSR respectively, 22 and 16 were polymorphic. That is, 13.09 and 9.09% of genetic variation were detected by RAPD and ISSR markers in these calli and the regenerants. The changed patterns observed with both marker systems included both the loss of original bands and appearance of novel bands (e.g. Figure 2).

To investigate whether demethylation may affect the extent or type of somaclonal variation at the nucleotide sequence level, DNA from 15 pieces of 5-azacytidine-treated calli together with 4 regenerated plants were analysed with the same RAPD and ISSR primers. We found that 35 and 30 bands were polymorphic out of a total of 168 and 176 scored bands in RAPD and ISSR markers, respectively. This indicates a slight increase in the frequency of genetic changes, particularly in ISSR markers, after the 5-azacytidine treatment.

Dendrograms were constructed on the basis of RAPD and ISSR markers. As regards RAPD markers, all somaclones, calli and regenerated plants tended to be grouped into a single cluster and the coefficients of similarity ranged between 0.92 and 1.00 (Figure 3). On the basis of ISSR markers, the clones were grouped into 2 clusters

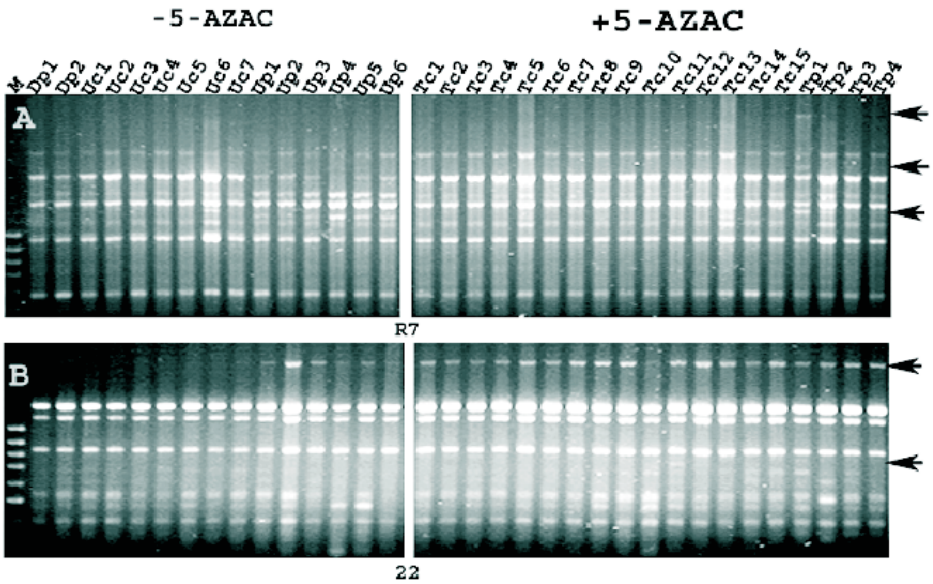

Figure 2. RAPD (A; primer R7) and ISSR (B; primer 22) profiles displaying genomic variations in somaclones of rice cv. Nipponbare. M = DNA ladder; Dp1 and Dp2 = independent samples from 2 seedlings of cv. Nipponbare; Uc1-7 = samples of untreated calli; Up1-6 = regenerated plants from untreated calli; Tc1-15 = samples of 5-azacytidine-treated calli; Tp1-4 = regenerated plants from 5-azacytidine treated calli. Fragments showing somaclonal variation are marked by arrowheads.

Table 1. RAPD and ISSR primers and their sequences used in this study

RAPD primer	Sequence	ISSR primer	Sequence (5`-3`)
R3	5'-GGGCGCCTAG-3'	W1	(AGA)3(GAG)2GG
R6	5'-GGCTGCGGTA-3'	W7	GGG (TGGGG)2TG
R7	5'-GGTCAGGGCT-3'	W8	(CT)8G
R9	5'-CCGAATCACT-3'	W11	(TC)8A
R10	5'-CCCGCAGAGT-3'	W13	(TCT)3(CTC)2CG
R13	5'-CCAGATGCAC -3'	W16	(GAG)3(AGA)2AYT
R17	5'-GTCGTTCCTG-3'	W30	(GGAGA)3
R18	5'-GGGCCACTCA-3'	4	BDB(CA)6
R20	5'-GGTGAACGCT-3'	5	VHV(GT)7
R21	5'-GATGCCAGAC-3'	7	(CT)8RG
R22	5'-TTAGCGCCCC-3'	15	CCC(GT)6
R25	5'-GTGAGGCGTC-3'	16	GSG(GT)6
R26	5'-CACTGGCCCA-3'	17	GSG(GT)6
R32	5'-AAGGCACGAG-3'	18	GCW(GA)6G
R33	5'-TCCGATGCTG-3'	21	GCGA(CA)6
R39	5'-ACCTTTGCGG-3'	22	SSWN(GACA)3
R45	5'-GGCTGGTTCC-3'	32	(AG)8C
R46	5'-TGGGTCCCTC-3'	34	(GA)8C
R49	5'-GAGGTCCACA-3'	36	(AG)8YT
R51	5'-ACAGCCCCCA-3'	37	(AG)8YC
A11	5'-CAATCGCCGT-3'		
RA62	5'-GAAGGCCGTG-3'		
RA71	5´-GAACCACCCC-3´		
D10	5'-AGCCGGCCTT-3'		

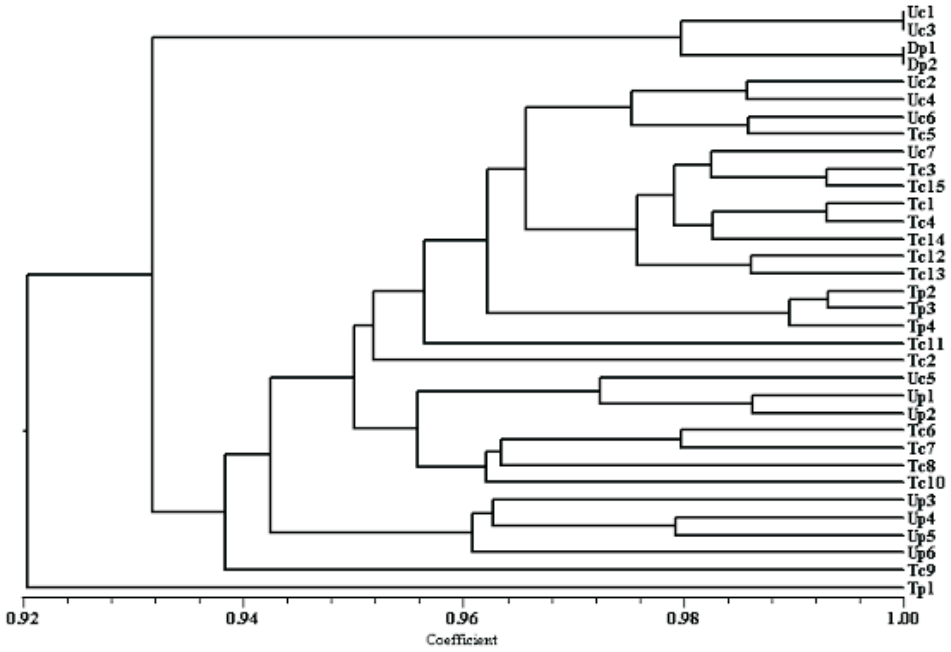

Figure 3. Clusters of rice cv. Nipponbare somaclones generated by the UPGMA method, based on RAPD markers. Dp1 and Dp2 = independent samples from 2 seedlings of cv. Nipponbare; Uc1-7 = samples of untreated calli; Up1-6 = regenerated plants from untreated calli; Tc1-15 = samples of 5-azacytidine-treated calli; Tp1-4 = regenerated plants from 5-azacytidine-treated calli.

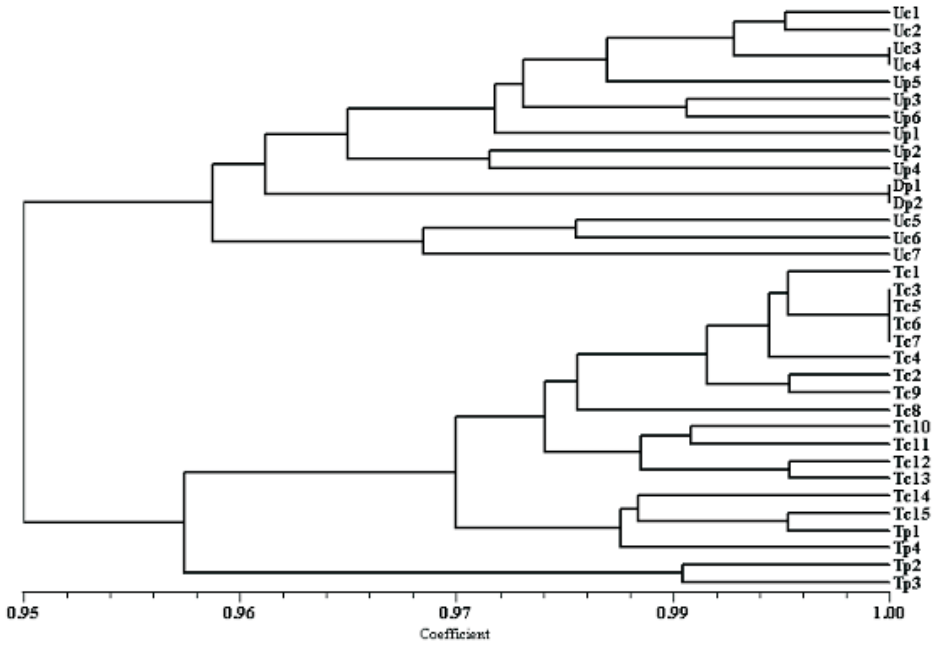

Figure 4. Clusters of rice cv. Nipponbare somaclones generated by the UPGMA method, based on ISSR markers. Dp1 and Dp2 = independent samples from 2 seedlings of cv. Nipponbare; Uc1-7 = samples of untreated calli; Up1-6 = regenerated plants from untreated calli; Tc1-15 = samples of 5-azacytidine-treated calli; Tp1-4 = regenerated plants from 5-azacytidine-treated calli.

Table 2. Pairwise sequence comparison at the primary sequence level and predicted homology of the 19 isolated clones ([a] RAPD fragments; [b]ISSR fragments)

Clone	Changes in nucleotide sequence	Predicted homology
[a]NR3-1	...….G…….(Nipponbare genome = N) ...….A…….(clone sequence = c)	putative leishmanolysin-like protein of *Oryza sativ*a ssp. *japonica*
[a]NR3-2	.....T……C……...(N) .....C……A……...(c)	no similarity
[a]NR3-3	no change	–
[a]NR3-4	no change	–
[a]NR6-2	no change	–
[a]NR7-2	..G..C..T..C..A..G..A..A..T..T..C..A..C..A..C...(N) ..C..T..C..T..T...A..G..T..C..C..T..C..G..G...-…(c)	OSJNBa0009P12.4 of *Oryza sativa* ssp. *Japonica*
[a]NR25-2	.....G……-……...(N) .....A……G…….(c)	no similarity
[a]NA11-2	no change	–
[a]NRA62-1	…...-……(N) .....G…….....(c)	hypothetical protein of *Oryza sativa* ssp. *Japonica*
[a]NRA71-1	…....-……(N) .....A…….....(c)	unknown protein of *Oryza sativa* ssp. *Japonica*
[a]NRA71-4	no change	–
[b]NW8-1	……T..…….....(N) ……C..…….....(c)	no similarity
[b]N15-3	.....A……(N) .....G……(c)	catalase (EC 1.11.1.6) CAT-2 of *Zea mays*, fragment
[b]N16-2	no change	–
[b]N18-2	.....T……C……(N) .....A……G……(c)	hypothetical protein of *Oryza sativa* ssp. *Japonica*
[b]N18-5	no change	–
[b]N32-1	…..T…..……(N) .....-…..……...(c)	no similarity
[b]N32-3	no change	–
[b]N37-2	no change	–

Table 3. Kinds and proportions of genetic variations at the primary nucleotide sequence level of the 19 isolated clones ([a] RAPD fragments; [b] ISSR fragments) and position of corresponding loci

Clone name	Clone size (bp)	Locus position	Transversions		Transitions		Insertions		Deletions	
			No.	bp	No.	bp	No.	bp	No.	bp
[a]NR3-1	442	Chr. 3 (AC103550)	0	0	1	1	0	0	0	0
[a]NR3-2	637	Chr. 3 (AC144426)	1	1	1	1	0	0	0	0
aNR3-3	639	Chr. 6 (AP005466)	0	0	0	0	0	0	0	0
[a]NR3-4	223	Chr. 3 (AC135598)	0	0	0	0	0	0	0	0
[a]NR6-2	641	Chr. 1 (AP003727)	0	0	0	0	0	0	0	0
[a]NR7-2	565	Chr. 2 (AP005408)	5	5	9	9	0	0	1	1
[a]NR25-2	637	Chr. 4 (OSJN00072)	0	0	1	1	1	1	0	0
[a]NA11-2	650	Chr. 6 (AB026295)	0	0	1	1	0	0	0	0
[a]NRA62-1	653	Chr. 7 (AP004264)	0	0	0	0	1	1	0	0
[a]NRA71-1	653	Chr. 1 (AP003727)	0	0	0	0	1	1	0	0
[a]NRA71-4	653	Chr. 5 (AC152969)	0	0	0	0	0	0	0	0
[b]NW8-1	559	Chr. 1 (AP002540)	0	0	1	1	0	0	0	0
[b]N15-3	294	Chr. 8 (AP005620)	0	0	1	1	0	0	0	0
[b]N16-2	525	Chr. 1 (AP002860)	0	0	0	0	0	0	0	0
[b]N18-2	568	Chr. 5 (AC078977)	2	2	0	0	0	0	0	0
[b]N18-5	482	Chr. 2 (AP005285)	0	0	0	0	0	0	0	0
[b]N32-1	583	Chr. 6 (AP003487)	0	0	0	0	0	0	1	1
[b]N32-3	366	Chr. 9 (AP005636)	0	0	0	0	0	0	0	0
[b]N37-2	470	Chr. 4 (AL606652)	0	0	0	0	0	0	0	0
Total	9650		8	8	15	15	3	3	2	2
	Genetic variation (%) = 0.31					28 base pairs				

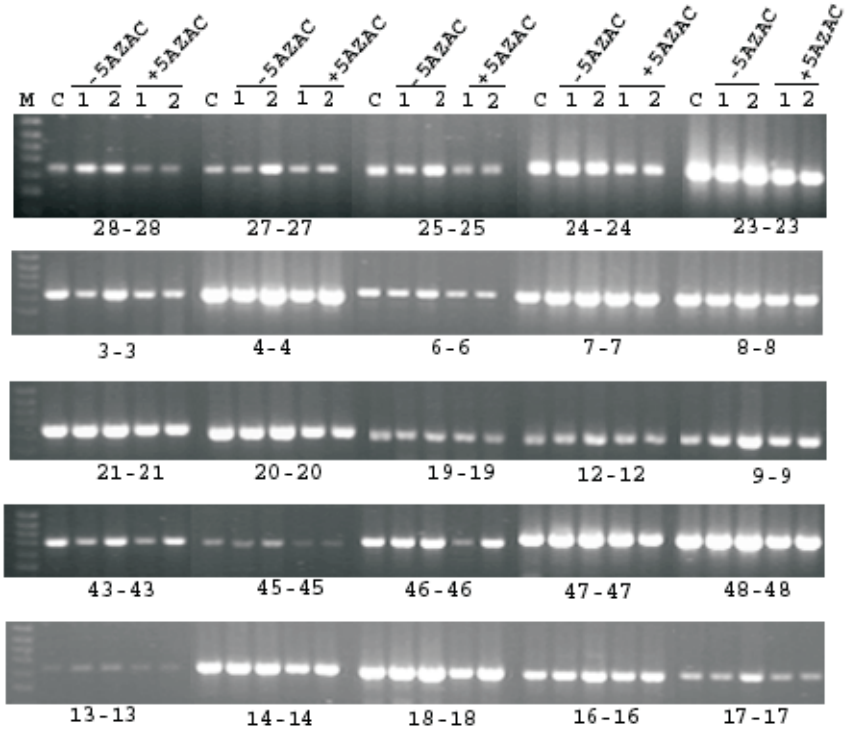

Figure 5. Examples of *mPing* stability in untreated calli and 5-azacytidine-treated calli of rice cv. Nipponbare. M = DNA ladder; C = DNA of a seedling (used as control). The *mPing*-containing, locus-specific primer pairs are 28–28, 27–27, 25–25, 24–24, 23–23, 3–3, 4–4, 6–6, 7–7, 8–8, 21–21, 20–20, 19–19, 12–12, 9–9, 43–43, 45–45, 46–46, 47–47, 48–48, 13–13, 14–14, 18–18, 16–16, and 17–17.

with high similarity (coefficients of similarity ranging from 0.95 to 1.00) (Figure 4). Of a total of 19 cloned sequences generated by RAPD and ISSR markers, 9 did not differ from the Nipponbare genome sequence in the GenBank, indicating that no changes occurred at the primer regions, while the remaining 10 revealed single-base-pair substitutions and some insertions or deletions. Clone [a]NR7-2 showed many single-base-pair substitutions and a single-base-pair deletion (Table 2). Overall, the RAPD markers have revealed more genetic variation than the ISSR markers. Predicted homology showed that 2 altered sequences bore significant homology to known-function genes, 2 to hypothetical proteins, and 1 to an unknown protein, while the rest showed no similarity (Table 2). At the primary nucleotide sequence level, the cloned sequences queried to the GenBank databases showed 23 single-base-pair substitutions (23 bp), 3 single-base-pair insertions (3 bp), and 2 single-base-pair deletions (2 bp), located on 8 chromosomes; most of them were detected by RAPD markers (Table 3). In contrast, for the 10 unchanged bands isolated from the control, we did not find any difference in sequence from their counterpart deposited in the GenBank on the basis of BlastN analysis. Thus, of the total length of both types of somaclones (9650 bp), 28 bp (0.31%) represented the genetic variation revealed by RAPD and ISSR markers in the somaclones of Nipponbare (Table 3).

Of the total of 44 primer pairs used to assess *mPing* activity in tissue culture and artificial demethylation, no copy of the element was found to jump in untreated or treated calli (Figure 5).

Discussion

Both RAPD and ISSR markers have been used to assess somaclonal variation in maize and were found to be highly efficient (Osipova et al. 2003). Moreover, Kuznetsova et al. (2005) showed their reliability in analysing DNA polymorphisms generated by long-term culture and subsequent regeneration in pea. In our study, the same molecular markers were also shown to be efficient in determining the genetic changes induced by tissue culture and by artificial DNA demethylation. At the nucleotide sequence level, mainly single-base-pair substitutions occurred (mostly transitions, while transversions were rare).

An interesting observation from this study is that although artificially induced cytosine demethylation was effective, as evidenced by the dwarf phenotype in the regenerants and more extensive cutting by methylation-sensitive enzymes in treated calli, there was only a slight increase in variation frequency, compared with untreated calli. This is contrary to the proposal that genetic and epigenetic instabilities are often interlaced. Nonetheless, we cannot rule out the possibility that increased genetic variation in the treated calli and plants occurred at certain genomic regions that escaped detection in our analysis.

It has been shown that *japonica* rice cultivars have a high copy number of *mPing*, many of which can be effectively mobilized by anther culture but not by somatic callus culture (Jiang et al. 2003; Kikuchi et al. 2003). The causes for this difference are unknown. We suspect that this might be due to differences in DNA methylation states between the gametophytic and sporophytic tissues (Janousek et al. 2000), as their genetic constitution is the same. Thus, we tested the mobility of *mPing* in 5-actydinine-treated and untreated calli. We found absolute immobility of *mPing* in both treated and untreated materials, suggesting that methylation alone does not play a decisive role in repressing the activity of *mPing*.

In conclusion, we found a moderate level of somaclonal variation even in relatively long-term somatic tissue cultures (2-year-old) of rice cv. Nipponbare. The main type of variation at the nucleotide sequence level was nucleotide substitution. Artificial demethylation by 5-azacytidine treatment did not elevate genomic variation to an appreciable degree, nor did it cause activation of *mPing*.

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