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ORIGINAL PAPER

Genetic variations in rice in vitro cultures at the *EPSPs–RPS20* region

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Abstract In vitro cultures of plant cells have often been utilized to generate genetic variations, which are designated somaclonal variations. Little is known about the major genetic alterations in the cultured cells and the nature of these genetic changes. Here, we examined different lines of rice Oc cells that have been cultured for more than 20 years on agar media or in liquid media. We surveyed 35 clones obtained from PCR amplification of the 3-kb *EPSPs–RPS20* region. The sequence divergence among the Oc cells was even greater than that between Japonica and Indica rice cultivars. The divergent sequences appeared to be maintained as multiple copies in a single cell. Surprisingly, the nucleotide substitutions in the Oc cells were characterized by an extremely high frequency of transition mutations of A/T-to-G/C, a feature which is similar to that of the mutations caused by chemical mutagens such as 5-bromouracil and 2-aminopurine. Although no replacements in the exons of this region were observed among the AA-genome *Oryza* species, our results revealed that the nucleotide substitutions of the cultured cell lines occurred more frequently at replacement sites in the exons than at synonymous sites. These distinct mutation biases found in rice in vitro cultures might contribute importantly to somaclonal variations.

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Introduction

Somaclonal variation is a term that describes any variability brought about by in vitro culturing (Larkin and Scowcroft 1981). Although various types of changes in cell cultures, i.e., genetic, epigenetic and karyotypic changes, are included in somaclonal variation, a major cause of somaclonal variations has been suspected to be genetic changes (Brettell et al. 1986; Dennis et al. 1987; Larkin et al. 1984, 1989). In short-term in vitro cultures, somaclonal variations have been applied as useful mutations for breeding purposes (Larkin et al. 1984, 1989). However, the

capability of regeneration of cultured cells is gradually weakened as the period of culturing becomes longer, and consequently the ability to differentiate is eventually completely lost. When the plant hormone auxin is supplied to in vitro cultures, the plant cells are able to proliferate without differentiation. These cells, the so-called callus, are capable of living so long as a fresh medium is supplied. Such artificial environments might force the cells to undergo rapid genetic changes in order to adapt specifically for cell proliferation. In fact, a number of reports during the last decade demonstrated that transposable elements become active during in vitro culturing and promote genetic variation (Courtial et al. 2001; Hirochika 1993, 1997; Hirochika et al. 1996; Jiang et al. 2003; Komatsu et al. 2003; Ozeki et al. 1997). Although the activation of transposable elements might be a consequence of stress during in vitro culture (Phillips et al. 1994), we do not know whether the genetic variation caused by transposition of the transposable elements contributes to adaptations of cultured cells. Indeed, little is known about the details of the genetic variation that occurs in cultured cells. Key questions about the genetic variation occurring in cultured cells include: (1) what is the nature of these genetic changes? (2) what, if any, is different between these changes and natural genetic variation? If these questions could be answered, somaclonal variations could be further exploited as an alternative genetic resource.

We have studied the genomic structure around the rice *waxy* (*wx*) gene (Nagano et al. 2002) and found two housekeeping genes encoding 5-enolpyruvylshikimate-3-phosphate synthase (EPSPs) and ribosomal protein small subunit 20 (RPS20) in a region about 45 kb downstream of *wx* (Kobayashi et al. 2005; Nishi et al. 1993; Sato et al. 1999). The *EPSPs* and *RPS20* genes are separated by only a 300-bp spacer, and are directed in a tail-to-tail orientation (Kobayashi et al. 2005) (Fig. 1a). Sequence analysis of a 3-kb region across the *EPSPs–RPS20* genes revealed that the replacement sites and the 3′-untranslated region (3′-UTR) were conserved among closely related rice species, but that the introns and the spacer region had frequent nucleotide substitutions and indel mutations (Kobayashi et al. 2005). In particular, the spacer region varied markedly in size among the rice species. We suspected that the short spacer should be unstable, and that in vitro culturing would stimulate an increase in its length due to changes caused by transposable elements or replication errors. Twenty-year-old cultures of rice cells (Oc cells) were examined regarding the sequence in this region, because Oc cells are known to undergo

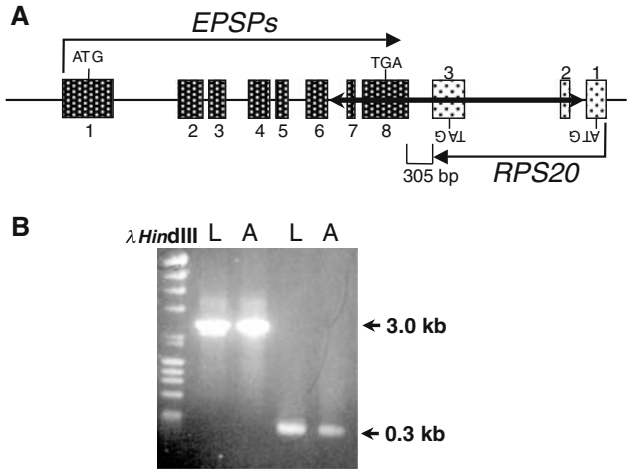

Fig. 1 The gene organization of *EPSPs* and *RPS20* and the PCR amplifications using Oc cell lines *A* and *L*. **a** The *EPSPs* and *RPS20* genes in C5924 consist of eight and three exons, respectively, and are separated by a 305-bp spacer. The *arrows* show the 5′–3′ orientation of the two genes, which are linked in a tail-to-tail orientation. In both of the genes, *ATG* represents the translation initiation site, and *TGA* indicates a stop codon. The sizes of the *EPSPs* and *RPS20* transcripts were predicted from the cDNA sequences and Northern analyses to be 2.2 and 0.75 kb, respectively (Kobayashi et al. 2005). The start and termination sites of the transcripts were predicted from the longest cDNA clone. The 2,921-bp region from *EPSPs* intron 6 to *RPS20* intron 1 indicated by the *double-headed arrow* was subjected to PCR and sequencing. **b** The 3-kb region across the *EPSPs* and *RPS20* genes and the 0.3-kb spacer region were amplified using the genomic DNAs from lines *A* and *L*. The majority of DNAs amplified for the entire region and spacer appeared at 3.0 and 0.3 kb, respectively, but a minor band was also detectable at a slightly lower position than the major band at 3.0 kb in line *A*. The *left side* shows a DNA size marker, *Hin*dIII-digested λ phage

activation of several transposable elements, such as transposon *mPing* (Jiang et al. 2003) and retrotransposons *Tos10*, *Tos17* and *Tos19* (Hirochika et al. 1996).

We surveyed the sequences in 35 clones obtained from PCR amplification of the 3-kb *EPSPs–RPS20* region. In these sequences, neither large insertions/deletions nor insertion of transposable elements was observed. Rather, most of the alterations consisted of nucleotide substitutions (159 changes), and a single-nucleotide deletion was detected at only one site. Interestingly, the major type of mutation was one-sided transition mutations, A/T-to-G/C, a type of mutation, which seems to be specifically induced by some nucleotide analogues (Freese 1959; Goodman et al. 1985). The specific induction of transition mutations might be a major cause of the somaclonal variation. In the exons of this region, mutations due to replacement in the cultured cells occurred more than eight times more frequently than mutations due to synonymous changes, and no nonsense mutations were

observed. This is the first report showing that an in vitro culture provides a particular environment that induces a specific mode of mutation, which is markedly different from that of natural variations.

Materials and methods

Cell cultures

The Oc cells used in this study were derived from *Oryza sativa* L. C5924 (Baba et al. 1986; Furukawa and Syono 1998). Callus cultures initiated from root explants were subcultured every month on Murashige–Skoog (MS) agar (1%) medium (Murashige and Skoog 1962) containing 1 mg/l 2, 4 dichlorophenoxyacetic acid (2,4-D) and 0.1 mg/l kinetin. Cell line A was established by successively subculturing the cells using this agar medium for 20 years. After the first 4 years, a portion of callus Oc was transferred to MS liquid medium containing 1 mg/l 2,4-D. Six milliliters of the suspension culture was transferred into 80 ml of the fresh liquid medium in a 300-ml Erlenmeyer flask every week. Since then, the line L has been maintained by successive subculturing in this liquid medium.

DNA isolation, PCR and sequencing

The procedure for DNA extraction was modified from the one described by Murray and Thompson (1980), i.e., to exclude RNA debris, RNase A was added and the mixture was incubated at 65°C for 20 min before chloroform extraction. The PCR amplifications were performed by using the high fidelity Taq polymerase (LA Taq: Takara Shuzo Co. Ltd, Kyoto, Japan) with the primer combination of 5′-TTG ATG TCA ACA TGA ACA ARA TGC CTG ATG T-3′ and 5′-CTC TCC TCC AAG AGC GTC AAG AAC CTC GAG AAA G-3′ (Kobayashi et al. 2005) to obtain the 3-kb *EPSPs–RPS20* segments. To amplify the spacer region between the *EPSPs* and *RPS20* genes, the primer combination of 5′-CAC AGG ATG GCC ATG GCC TTC TC-3′ and 5′-AGG TAC CAA CAC CTG GGA TCG GTT TGA GAT G-3′ was used. The amplified DNA fragments were cloned into pBluescript SK (Stratagene, La Jolla, CA). Within the 3-kb *EPSPs–RPS20* sequence, 14 primers were designed based on the corresponding sequence in Nipponbare and used for the sequencing of the cloned *EPSPs–RPS20* sequences. Double-stranded DNA fragments inserted into the vector were sequenced using a d-Rhodamine Terminator Cycle Sequencing Ready Reactidon-Sequencing Kit (PE-Applied Biosystems, Foster City, CA) and an ABI377 Automated DNA Sequencer (PE-Applied Biosystems). The sequence in the C5924 line is distinguished from that in Nipponbare by a 2-bp insertion in the spacer region and five nucleotide changes.

Comparison of nucleotide sequences

The *EPSPs–RPS20* sequences from 38 clones were aligned using the CLUSTAL W program (Thompson et al. 1994) provided by the DNA Data Bank Japan (DDBJ) and the alignments were confirmed by eye. The sequences were divided into functionally distinctive regions: replacement and synonymous sites in exons, introns, 3′-UTRs and the spacer. We calculated the average numbers of nucleotide differences per site among the clones and the numbers of all kinds of polarized mutations, assuming that the mutations occurred on the authentic sequence in C5924. This assumption is based on the fact that all but four mutations appeared only once in the 35 sequences (the remaining four appeared twice). From the sequences of 12 lines from 7 species (*O. sativa*, *O. rufipogon*, *O. glaberrima*, *O. barthii*, *O. glumaepatula*, *O. meridionalis* and *O. longistaminata* (Kobayashi et al. 2005), we also inferred the substitution pattern in the AA-genome *Oryza* species. We used only singletons to infer mutational directions. *G* test of independence with Williams's correction (Sokal and Rohlf 1995) was applied to test the frequencies of transition and transversion changes between A/T and G/C sites. We calculated the numbers of replacement and synonymous sites by using the observed transition/transversion ratio of 8.35 (142/17) and Ina's method (Ina 1995) with a small modification, in which we took nonsense mutations into account. Besides the transition/transversion bias, the AT/GC mutational bias affects the expected ratio of the numbers of synonymous and replacement changes. Assuming that the mutation rate at A and T sites is 5.6 times as large as at G and C sites, we calculated the relative frequencies of synonymous and replacement changes for each codon. These relative frequencies were multiplied by the number of appearances of the codons and summed over all the codons. The ratios of these sums represent the expected relative frequencies of synonymous and replacement changes under neutrality. We performed *G* test with Williams's correction (Sokal and Rohlf 1995) for goodness of fit for the observed frequencies of synonymous and replacement changes to the expected frequencies.

Results

Two cell lines (Oc cells), lines A and L, derived from rice strain C5924, have been maintained for 20 years since callus initiation (Baba et al. 1986). Line A has been subcultured every month on agar medium, while line L, which was derived from line A 4 years after the callus initiation, has been subcultured every week in liquid medium. PCR analyses were performed to amplify the 2,921-bp region from *EPSPs* intron 6 to *RPS20* intron 1 and the 305 bp of their spacer (Fig. 1a). For both cell lines, bands with the expected sizes of 3 and 0.3 kb were amplified, and no fragment with a large insertion was amplified from either cell line (Fig. 1b). To confirm the fidelity of the PCR and sequencing performed here, the PCR-amplified sequences of the 3-kb region from the parental C5924 genomic DNA were cloned into a plasmid prior to sequencing. No sequence alterations were observed among five independent clones of sequences amplified from the corresponding region in the parental DNA (data not shown). Of a total of 38 sequences, only three coincided with the sequences of different clones, while the remaining 35 cloned sequences, which consisted of 19 and 16 from line A and L, respectively, were different from each other (Supplementary Material). These data suggest that the cultured cells had multiple diverged sequences of this region resulting from aberrant polyploidy.

Comparison of the sequences revealed that a total of 164 alterations were distributed at 159 sites in the 3-kb *EPSPs–RPS20* sequence (Supplementary Material). Among these 164 alterations, 163 were attributable to nucleotide changes and one to a deletion. As compared with the authentic sequence, the number of nucleotide differences in a clone ranged from 0 to 14, and the means were 4.2 for line A and 5.2 for line L. The changes comprised 156 singletons and 4 nucleotide changes shared by two clones. Because these four alterations were common in lines A and L, they seem to have occurred during the first 4 years after the callus initiation (Supplementary Material). In addition, a single site had two independent mutations. Thus, 159 mutational events accounted for the nucleotide changes among the 35 different clones. The average numbers of nucleotide differences per site were estimated to be 2.9×10^{-3} for 19 sequences from line A and 3.6×10^{-3} for 16 sequences from line L (Table 1). It is noteworthy that these values were higher than the nucleotide diversity (1.7×10^{-3}) estimated between Japonica (Nipponbare) and Indica (PTB10), of which mutations have been subjected to a long period of the natural selections. Hence, the results clearly showed that both of the cell lines accumulated extremely high rates of mutations.

One striking feature was that the cultured cells showed a strong preference for transitional mutation. The ratios of transition to transversion (Ts/Tv) of lines A and L were 10.4 (73/7) and 6.9 (69/10), respectively, while the Ts/Tv ratio was estimated to be 1.7 (25/15) among AA-genome *Oryza* species (Fig. 2). This transition bias was mostly caused by A to G or T to C

Table 1 Average number of nucleotide differences among clones from lines A and L in the 2,921 bp of *EPSPs–RPS200* region

		AT content (%)		Length (bp)	A		L		A + L	
					s[a]	d[b] ($\times 10^{-3}$)	s[a]	d[b] ($\times 10^{-3}$)	s[a]	d[b] ($\times 10^{-3}$)
EPSPs	Coding region	44.5	R[c]	195.1	13	7.0	10	6.4	21[d]	6.7
			S[c]	85.9	0	0.0	2	2.9	2	1.3
	Intron	65.6		369	7	2.0	10	3.4	17	2.6
	3′-UTR	61.3		248	5	2.1	6	3.0	11	2.5
	All	57.8		898	25	2.9	28	3.9	51[d]	3.4
RPS20	Coding region	45.9	R[c]	178.4	8	4.7	4	2.8	11[d]	3.8
			S[c]	78.6	1	1.3	1	1.6	2	1.5
	Intron	64.0		1,221	38	3.3	35	3.6	72[d]	3.4
	3′-UTR	60.7		239[e]	2	0.9	6	3.1	8	1.9
	All	60.8		1,717	49	3.0	46	3.3	93[d]	3.2
Spacer		55.1		305	6	2.1	9	3.7	15	2.8
All		59.3		2,920[e]	80	2.9	83	3.6	159[d]	3.2

[a] Number of mutations

[b] Average number of nucleotide differences in 19 A clones and 16 L clones

[c] Replacement and synonymous sites were analyzed separately for coding regions

[d] Four mutations were shared by A and L clones and counted as single mutations. Therefore, the numbers do not equal the sums of the corresponding numbers of A and L

[e] The numbers are the results with 1 bp subtracted from each of the lengths (240 and 2,921 bp), because we took account of a deletion that occurred in the 3′-UTR of *RPS20* when the average numbers of nucleotide differences were calculated

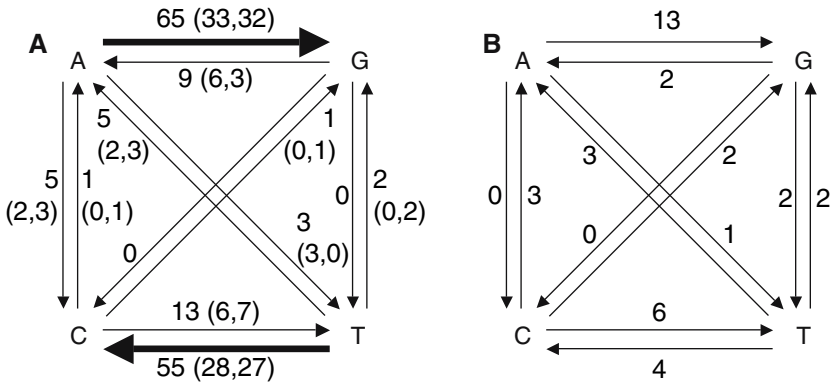

Fig. 2 Strong mutational bias in the Oc cell lines. **a** Inferred directions of the nucleotide changes observed in the *A* and *L* cell lines. The *panel* shows the numbers of independent mutational events. The numbers of the mutations that occurred in line *A* and in line *L* are provided as the left and right numbers in *parenthesis*, respectively. **b** Inferred directions of the nucleotide substitutions observed in the 12 lines from 7 AA-genome *Oryza* species (Kobayashi et al. 2005). The numbers are based on the singletons among the 12 sequences

substitutions, which were found at 120 sites, while G to A or C to T was limited to 22 sites (Fig. 2; the authentic 2,921-bp sequence consists of 964 As, 768 Ts, 668 Cs and 521 Gs). In addition to the strong transition bias at A/T sites, transitions at G/C sites also occurred much more frequently relative to transversions. Indeed, the Ts/Tv ratio did not differ between A/T and G/C sites (120/15 at A/T and 22/2 at G/C; $G' = 0.2$, $df = 1$, $P > 0.5$). These results are in sharp contrast to the nucleotide changes among the AA-genome *Oryza* species where no A/T-to-G/C mutational bias was observed (Fig. 2).

In both of the cell lines, replacement changes occurred more often than synonymous changes (Table 1). This is contrary to what is seen in the natural variation in the AA-genome *Oryza* species, in which no replacement was observed in the corresponding regions, and the nucleotide divergence at synonymous sites was highest in these regions; Kobayashi et al. (2005) estimated the average numbers of nucleotide differences per site for replacement, synonymous, intron, 3′-UTR and spacer among the AA-genome *Oryza* species as 0, 2.6×10^{-3}, 2.1×10^{-3}, 1.2×10^{-3} and 1.9×10^{-3}, respectively. Preferential occurrence of replacements was more marked in line A and the *EPSPs* gene than in line L and the *RPS20* gene, and especially notably, there was a lack of synonymous changes in *EPSPs* of line A (Table 1). The strong A/T-to-G/C mutational bias in the cultured cells could have contributed to the higher frequency of replacements relative to synonymous changes, because the AT contents in the exons of the 3-kb *EPSPs–RPS20* sequence are much higher at the first and second positions of codons (48 and 57%, respectively) than at the third position of codons (29%). Taking account of both the transition and A/T-to-G/C mutational biases, the expected ratio of replacement and synonymous changes under neutrality was calculated as 3.1, which was much larger than that under no A/T-to-G/C bias (2.3). Compared with this expected ratio, the ratio of line A (21 to 1:replacements to synonymous changes) was significantly higher ($G' = 6.2$, $df = 1$, $P < 0.025$), whereas the ratio in line L (11 to 3) was consistent with the neutral expectation ($G' = 0.1$, $df = 1$, $P > 0.5$). Therefore, the accumulation of replacements in line A implies a potential association with adaptation of the cultured cells.

Discussion

Initially, this study aimed to examine whether the 300-bp short spacer sequence between *EPSPs* and *RPS20* is elongated during long-term cell culture. Among AA-genome *Oryza* species, the spacer sequences appeared to be unstable and exhibited the accumulation of many indels, unlike the other regions within the *EPSPs* and *RPS20* (Kobayashi et al. 2005). The Oc cells used here are well known to exhibit induced-activation transposable elements such as retrotransposons *Tos10*, *17* and *19* (Hirochika et al. 1996) and the transposon *Ping* family (Jiang et al. 2003). The activation of such transposable elements is considered to greatly contribute to mutations in plant tissue culture. In our analysis, however, no insertion sequences were found in the diversified sequences of the *EPSPs–RPS20* region. The mutations observed in the Oc cell lines were mostly attributable to nucleotide changes, which were evenly

distributed throughout the examined sequence. Therefore, our results strongly suggest that nucleotide substitutions constitute a large part of mutations in the cultured cells.

In tissue culture, cells rarely maintain an authentic chromosome number (Chen and Chen 1980; Ogura 1990). Likewise, the Oc cell lines maintained by long-term culture should generally have aberrant numbers and malformations of the chromosomes (Ogura 1990). On the other hand, periodical subculturing at short intervals (every week for line L and every month for line A) should reduce the amount of between-cell variation. Thus, we think that the individual Oc cells with aberrant polyploidy harbored multiple divergent copies of genes. This multiple-copy system in a single cell might facilitate independent accumulation of mutations in each copy.

The extreme tendency of one-sided transitional mutation implies the involvement of certain mechanism(s) that enforced the substitutions of A to G and T to C. Similar transition preferences have been described for some chemical mutagens, such as 5-bromouracil (5-BU) and 2-aminopurine (2-AP) (Freese 1959; Goodman et al. 1985; Trautner et al. 1962). 5-BU (Lasken and Goodman 1984) and 2-AP (Watanabe and Goodman 1981) are analogs of thymine and adenine, and they generate specific mispairing with cytosine and guanine, respectively. The culture media used for lines A and L might both contain some agent that led to the induction of specific mispairing. The most probable responsible chemical in the culture medium used here is 2,4-dichlorophenoxyacetic acid (2,4-D), which has been described as possessing the potency to induce mutations and therefore concluded to be genotoxic in several reports (Filkowski et al. 2003; Gonzalez et al. 2005; Tripathy et al. 1993). However, little relationship has been reported between plant hormones and somaclonal variations.

The mutational bias of the Oc cells allows us to infer a contribution of mechanistically influenced mutation rather than strictly spontaneous mutation in somaclonal variation. To the best of our knowledge, detailed studies of the culture medium as a source of mutagen(s) have been limited to studies in short-term cultures, and such high frequencies of nucleotide substitutions have not been detected in in vitro cultures so far. The A/T-to-G/C mutational bias in part contributed to the preference for replacement changes in the cultured cells, but also additional explanation(s) are required to account for the preferential replacements, especially in line A as compared to line L. The replacements might be advantageous to the cultured cells with aberrant polyploidy, and the long interval between subculturing used for line A might enhance their accumulation. At the same time, the accumulation of replacements might vary depending on the genes in the Oc cells. Strong selection should be imposed on genes that are necessary to adapt to in vitro culture conditions. The genetic changes that occurred during 20 years of culturing the Oc cell lines have made it apparent that in vitro culturing of plant cells results in a particular mode of mutagenicity, which is quite distinct from the mode of the natural mutational variations.

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