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Genomic changes at the early stage of somatic hybridization

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ABSTRACT. A broad spectrum of genetic and epigenetic changes is induced by wide hybridization and subsequent polyploidization, but the timing of these events remains obscure because early hybrid cells are very difficult to harvest and analyze. Here, we used both cytological and genetic marker approaches to analyze the constitution of very young somatic hybrid cells between *japonica* rice (*Oryza sativa* L. subsp *japonica*) and *indica* rice (*Oryza sativa* L. subsp *indica*) and between *japonica* rice and bread wheat (*Triticum aestivum* L.). Chromatin elimination, simple sequence repeats, and retrotransposon profile deletions were already apparent within six days of the fusion event. The evidence we have presented suggests that genomic changes induced by genomic shock occur soon after the formation of hybrid cells.

Key words: Genomic changes; Young somatic hybrid cells; Genomic shock; Simple sequence repeat; Retrotransposon; Chromatin elimination

INTRODUCTION

The concept of genomic shock (McClintock, 1984) refers to the genomic perturbation, which is expressed as a spectrum of genetic and epigenetic changes, experienced when unrelated genomes are brought together in wide hybrids (Feldman et al., 1997; Liu et al., 1998; Ozkan et al., 2001; Kraitshtein et al., 2010). A relatively well-documented early cellular event following the formation of a hybrid zygote is the partial or complete elimination of one of the parental set of chromosomes (Gernand et al., 2005). At the DNA sequence level, it has been suggested that directed loss can occur during or soon after the formation of the hybrid zygote (Ozkan et al., 2001), and that the re-activation of retrotransposons, a class of sequences that comprises a significant proportion of most plant genomes, is induced at an early stage of hybrid zygote development (Kraitshtein et al., 2010). The detection of genomic alterations induced by genomic shock has been attempted using a number of methods. At the cytological level, a common method is genomic *in situ* hybridization (GISH), a technique that is able to identify alien chromosomes and chromatin, as well as chromosomal rearrangements, especially those resulting from the formation of mosaic chromosomes (Gernand et al., 2005). At the DNA sequence level, various marker platforms have proven informative, especially those targeting retrotransposons (Bento et al., 2008; Kraitshtein et al., 2010). Several genomic variations induced by genomic shock from F1 to successful generations have been discovered (Feldman et al., 1997; Liu et al., 1998; Ozkan et al., 2001); however, the specific timing of the elimination is hard to establish in sexual hybrids because of the technical difficulty of collecting sufficient DNA for analytical purposes from very young zygotes.

In addition to the formation of sexual wide hybrids, unrelated genomes can also be merged via somatic hybridization, a procedure that has been successfully applied to a number of combinations of graminaceous species in our research group (Xia et al., 1996; Zhou et al., 2001; Xu et al., 2003). The occurrence of both genetic and epigenetic changes in these hybrid plants and progenies has been repeatedly demonstrated (Zhou et al., 2001; Xia et al., 2003; Xu et al., 2003, Liu SW, Li F, Sun Y, Chen SY, et al., unpublished data) in hybrid progenies, and the assumption is that, as for sexual wide hybrids, the primary cause of these events is genomic shock. Somatic hybrids experience whole and/or partial chromosome elimination, in a way very similar to the behavior of sexual hybrids, and the evidence shows that most of this elimination occurs soon after the fusion event, which is the earliest time the elimination could be verified with GISH, which was achieved in our laboratory for the first time (Cui HF, Sun Y, Deng JY, Wang MQ, et al., unpublished data). However, the DNA sequence variation has not yet been detected.

Here, we report a genetic analysis of young somatic hybrid cells involving *indica* and *japonica* rice, the cultivated species' two major subspecies, as well as those derived from the fusion of *japonica* rice and bread wheat. We have focused on the identification of genetic changes at the level of chromosome elimination and DNA sequence in six day-old fusion nuclei.

MATERIAL AND METHODS

Cell lines and protoplast fusion

Calli of *indica* and *japonica* rice were induced from mature grains by culturing on N_6D medium following Toki (1997), and were then maintained for over one year on NB_2

medium. A suspension cell line of the bread wheat cultivar Jinan177 was established and maintained on MB_2 liquid medium (Xu et al., 2003). Protoplasts were prepared and fused as described elsewhere (Cui et al., 2009) in a 3.5 cm culture dish. Somatic hybrids were induced both between *japonica* and *indica* rice, and between *japonica* rice and bread wheat. In both cases, the *japonica* rice protoplasts were treated as the recipient, and both symmetric and asymmetric somatic hybrids were induced. For the former, the donor protoplasts were irradiated pre-fusion with UV light (Xia et al., 2003), the *indica* rice protoplasts were irradiated for either 0.5 or 2 min, and the wheat protoplasts were irradiated for 2 min (Table 1). In the case of the symmetric hybrids, no UV pre-treatment was performed. The fusion procedure followed protocols detailed elsewhere (Xia et al., 2003; Cui et al., 2009), in which the products could be cultured for six months until they formed calli. The parent self-fusion controls were carried out and cultured in the same conditions to eliminate the effects of somaclonal variation, protoplast isolation, different parental chromosome numbers, ploidy, and genome size on the results. Each cell fusion experiment was repeated three times.

Table 1. Parental combinations used to create somatic hybrids.

Receptor	Donors	UV treatment of donor		
japonica rice	*indica* rice	0 min	0.5 min	2 min
	Wheat	0 min	-	2 min

DNA preparation

Six days after fusion, the liquid medium was discarded, and the cells were fixed by holding in 3:1 ethanol:glacial acetic acid for 1-2 h. After replacing the fixation medium with absolute ethanol, the cells were pelleted by centrifugation (12,000 rpm for 5 min), the pellet was air dried for 3-5 min, and finally ground in liquid nitrogen in preparation for CTAB-based DNA extraction. The samples of cells present in each extraction were obtained from approximately 100 3-cm Petri dishes with a population of approximately 2 x 10^7 cells.

Marker analysis

For the analysis of the *japonica*/*indica* somatic hybrids, a set of 24 microsatellite simple sequence repeat (SSR) primer pairs, detecting loci distributed across all 12 rice chromosomes, was selected from the Gramene database (http://www.gramene.org/db/markers/marker_view) (Table 2). For the *japonica*/wheat material, 8 wheat genomic SSR and 15 expressed sequence tag-SSR (Table 3) loci were assayed. Protocols for the detection of amplicons followed those described in Chen et al. (1997). Inter-retrotransposon amplified polymorphism (IRAP) and retrotransposon-microsatellite amplified polymorphism (REMAP) assays were performed following Kalendar and Schulman (2006). For the *japonica*/*indica* somatic hybrids, LTR primers were used for IRAP assays, while for REMAP, these same primers were combined with the primers RM50R, RM202R, or RM202F (Tables 4 and 5). For the *japonica*/wheat somatic hybrids, the IRAP assays described by Bento et al. (2008) were used. All polymerase chain reaction (PCR) products were resolved by electrophoresis on either 6% denaturing polyacrylamide or on 2% agarose gels.

Table 2. Primer sequences used for the SSR genotyping of *japonica*/*indica* somatic hybrids.

Primer pairs	Sequences (5'-3')	Chromosomal localization
RM23	CATTGGAGTGGAGGCTGG GTCAGGCTTCTGCCATTCTC	1S
RM259	TGGAGTTTGAGAGGAGGG CTTGTTGCATGGTGCCATGT	1S
RM240	CCTTAATGGGTAGTGTGCAC TGTAACCATTCCTTCCATCC	2L
RM250	GGTTCAAACCAAGCTGATCA GATGAAGGCCTTCCACGCAG	2L
RM85	CCAAAGATGAAACCTGGATTG GCACAAGGTGAGCAGTCC	3L
RM282	CTGTGTCGAAAGGCTGCAC CAGTCCTGTGTTGCAGCAAG	3S
RM241	GAGCCAAATAAGATCGCTGA TGCAAGCAGCAGATTTAGTG	4L
RM303	GCATGGCCAAATATTAAAGG GGTTGGAAATAGAAGTTCGGT	4S
RM289	TTCCATGGCACACAAGCC CTGTGCACGAACTTCCAAAG	5L
RM421	AGCTCAGGTGAAACATCCAC ATCCAGAATCCATTGACCCC	5S
RM30	GGTTAGGCATCGTCACGG TCACCTCACCACACGACACG	6L
RM50	ACTGTACCGGTCGAAGACG AAATTCCACGTCAGCCTCC	6S
RM234	ACAGTATCCAAGGCCCTGG CACGTGAGACAAAGACGGAG	7L
RM214	CTGATGATAGAAACCTCTTCTC AAGAACAGCTGACTTCACAA	7S
RM80	TTGAAGGCGCTGAAGGAG CATCAACCTCGTCTTCACCG	8L
RM310	CCAAAACATTTAAAATATCATG GCTTGTTGGTCATTACCATTC	8S
RM201	CTCGTTTATTACCTACAGTACC CTACCTCCTTTCTAGACCGATA	9L
RM215	CAAAATGGAGCAGCAAGAGC TGAGCACCTCCTTCTCTGTAG	9L
RM271	TCAGATCTACAATTCCATCC TCGGTGAGACCTAGAGAGCC	10L
RM184	ATCCCATTCGCCAAAACCGGCC TGACACTTGGAGAGCGGTGTGG	10L
RM206	CCCATGCGTTTAACTATTCT CGTTCCATCGATCCGTATGG	11L
RM224	ATCGATCGATCTTCACGAGG TGCTATAAAAGGCATTCGGG	11L
RM235	AGAAGCTAGGGCTAACGAAC TCACCTGGTCAGCCTCTTTC	12L
RM247	TAGTGCCGATCGATGTAACG CATATGGTTTTGACAAAGCG	12S

In situ hybridization analysis

Prior to the GISH procedure, the *japonica* rice/wheat hybrid cells were fixed as described above, washed three times in ddH_2O, and squashed in a drop of 45% glacial acetic acid. The squashes were then processed according to Cui et al. (2009). The probe was represented by nick-translated rice genomic DNA (labeled with DIG-Nick Mix, Roche), while unlabeled wheat genomic DNA was used for blocking, employing a probe:block ratio of 1:50. The fusion frequency was calculated using the ratio of the number of parent fused cells to that of other fusion products.

Table 3. Primer sequences used for the SSR genotyping of *japonica* rice/wheat somatic hybrids.

Primer pairs	Sequence (5'-3')
xgwm302	GCAAGAAGCAACAGCAGTAAC
	CAGATGCTCTTCTCTGCTGG
xgwm311	TCACGTGGAAGACGCTCC
	CTACGTGCACCACCATTTTG
xgwm428	CGAGGCAGCGAGGATTT
	TTCTCCACTAGCCCCGC
xgwm533	AAGGCGAATCAAACGGAATA
	GTTGCTTTAGGGGAAAAGCC
xgwm111	TCTGTAGGCTCTCTCCGACTG
	ACCTGATCAGATCCCACTCG
xgwm126	CACACGCTCCACCATGAC
	GTTGAGTTGATGCGGGAGG
xgwm608	ACATTGTGTGTGCGGCC
	GATCCCTCTCCGCTAGAAGC
xgwm635	TTCCTCACTGTAAGGGCGTT
	CAGCCTTAGCCTTGGCG
ksum46	CAGCATCTACCTGTTTGCGA
	TTGGGTTTGAAATTGTTGCC
ksum47	AGCTGAGGATGGACGAACAC
	CAAAACCTACAGCTCCCTCG
ksum74	CCACCCCCATCCATCCATCC
	AGCTCCCCCATGTCGAAGAG
ksum88	GCAAGCTCACAAGTATCGCA
	GCTGAGGCTGCTGGTAGGAG
ksum117	CAGCAGCTCCAGTGCGCAGC
	CTTGGTGGTGGAGCACTCAG
ksum123	ACTACCATATTCACAGCCAC
	GCCTGGCACTGCGACTGCTC
ksum124	CTAGGGCAGCAACAACCATT
	TTCCATGCGCTATGTTGTGT
cnl64	AATCAACAACAACGCCACAC
	CATCAGCCAGAACAAGCTCA
cnl65	GCGAATCTCAAGTCAAGACG
	CTCCGACAACACTCCATCTG
cnl134	CTTGCCACGAAGTGATTGC
	TGAGCTGGTTGTTGAGGTTG
cnl145	ATCTCCTCTCCTCCGTCCTC
	TCACCGACATAGGCATCCTT
cnl152	ACAAAGGCTCACCGTGGAA
	GTCGGAGGCGATGAACTCT
cnl70	CCGGCCCGAGGAAAAGCT
	GGCCGAAGAGGCGGTAGATCTT
cnl79	TTGGTTGGCAGAGTGAAGC
	GGCCTGGAACTCAAACTTCT
cnl66	ACGTTGGGCATTAGATTTGC
	TGTGGCATACAGACATCACAA

Table 4. Primer combinations used for the IRAP and REMAP genotyping.

Fusion combinations	Analysis items	Primer pairs
japonica rice (+) *indica* rice	IRAP	LTR1+LTR1; LTR1+LTR2; LTR1+LTR3; LTR1+LTR4; LTR2+LTR2; LTR2+LTR3; LTR2+LTR4; LTR3+LTR3; LTR3+LTR4;
	REMAP	LTR1+RM202F; LTR1+RM202R; LTR2+RM202F; LTR2+RM202R; LTR3+RM202R; LTR3+RM50;
japonica rice (+) wheat	IRAP	Nikita+Sukkwla; Sabrina+Sukkwla; Sukkwla+Stowaway

IRAP = inter-retrotransposon amplified polymorphism; REMAP = retrotransposon-microsatellite amplified polymorphisms.

Table 5. Primer sequences used for the IRAP and REMAP genotyping.

Primer pairs	Sequence (5'-3')
LTR1	TTGGATCTTGTATCTTGTATATAC
LTR2	GCTAATACTATTGTTAGGTTGCAA
LTR3	CCAATGGACTGGACATCCGATGGG
LTR4	CTGGACATGGGCCAACTATACAGT
Nikita	CGCTCCAGCGGTACTGCC
Sabrina	GCAAGCTTCCGTTTCCGC
Sukkula	GATAGGGTCGCATCTTGGGCGTGAC
Stowaway	CTTATATTTAGGAACGGAGGGAGT

RESULTS

Chromatin elimination in response to somatic hybridization

By analyzing the fusion products with GISH at different times, we found that the highest fusion frequencies of up to 40% appeared at day six after fusion, which is similar to our previous results in other fusion experiments (Cui HF, Sun Y, Deng JY, Wang MQ, et al., unpublished data). Thus, the fusion products obtained at this time point were used in the assays. In the six day-old *japonica*/wheat somatic hybrid cells, the wheat DNA appeared to lie in the center of the nuclei, while the *japonica* DNA was dispersed around the outside in the form of either brightly (Figure 1a-c) or rather weakly (Figure 1d) fluorescing micronuclei. The former probably represented one or more intact or nearly intact rice chromosomes, while the latter most likely represented fragmented rice chromatin. A similar zoning of parental chromatin has been noted in somatic hybrids between wheat and both tall wheatgrass (*Thinopyrum ponticum*) and maize (Cui HF, Sun Y, Deng JY, Wang MQ, et al., unpublished data). Such an arrangement of chromatin is consistent with the selective elimination of rice DNA, as it closely resembles the behavior of pearl millet chromatin in sexual hybrids with bread wheat (Gernand et al., 2005).

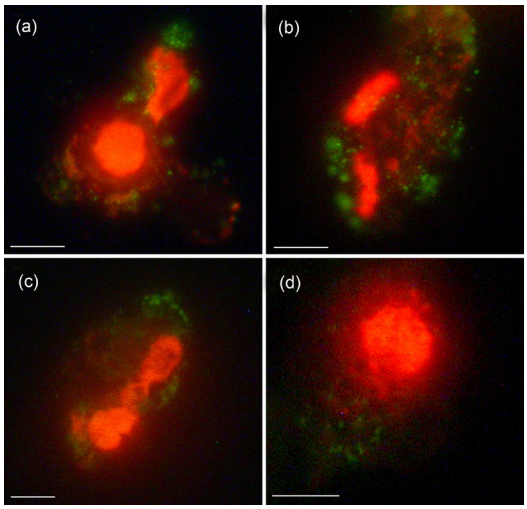

Figure 1. Chromatin elimination from six day-old *japonica* rice/wheat somatic hybrid cells. **a. b.** asymmetric hybrid cells, **c. d.** symmetric hybrid cells. Rice genomic DNA fluoresces green, while wheat DNA appears red. Rice chromatin appears as intensely fluorescing micronuclei (**a-c**) or more weakly fluorescing small fragments (**d**). Bar = 10 µm.

Polymorphism at SSR loci in response to somatic hybridization

Among the 24 rice SSR loci (Table 2) assayed in the *japonica*/*indica* symmetric and asymmetric hybrids, 23 displayed the presence of both parental alleles (e.g., RM224); the exception was RM50, where only the *indica* allele was present in all hybrids (Figure 2a). The difference between the two alleles lays in the number of CTAT and CT repeat units (Figure 2b). Profile deletion occurred in 4.2% (1/24) of the loci.

Of the 23 SSR loci (Table 3) analyzed among the *japonica* rice/wheat hybrids, most displayed the alleles from both (e.g., *Xgwm111*), while the absence of the *japonica* rice allele was noted at only two loci (*Xgwm126* and *Xcnl70*) (Figure 2c). Overall, the deletion occurred in 8.7% (2/23) of the loci tested.

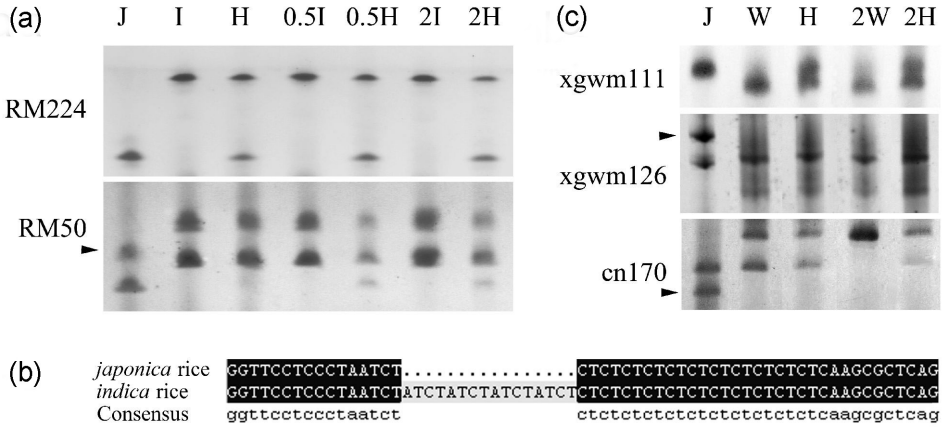

Figure 2. Alteration in SSR profiles among somatic hybrids. **a.** Most loci displayed both parental alleles in the *japonica*/*indica* hybrid cells (e.g., RM224), but for RM50, the *japonica* allele was absent from all the hybrids. I, J = self-fusion *indica* and *japonica* non-irradiated cells; 0.5I, 2I = self-fusion *indica* rice cells UV irradiated pre-fusion for 0.5 and 2 min, respectively, H, 0.5H, 2H = somatic hybrids in which the donor protoplasts were UV irradiated pre-fusion for 0, 0.5 and 2 min, respectively. **b.** Most loci displayed both parental alleles in the *japonica*/wheat hybrids (e.g., *Xgwm111*), but *japonica* alleles were absent in the hybrids' *Xgwm126* and *cnl70* profiles. Arrowheads indicate deleted fragments. **c.** DNA sequence of the deleted RM50 fragment. J, W = self-fusion *japonica* and wheat non-irradiated cells; H, 2H = symmetric somatic hybrids UV irradiated pre-fusion for 0 and 2 min, respectively, 2W = self-fusion wheat cells UV irradiated pre-fusion for 2 min.

IRAP and REMAP polymorphism induced by somatic hybridization

The 15 primer pairs (nine IRAP, six REMAP) (Table 4) deployed to analyze the *japonica*/*indica* material amplified 44 fragments from the two parents. Some of these fragments were absent from all hybrid materials, in the same way as observed for the RM50 SSR. The amplification profile produced by the IRAP primer pair LTR3+LTR3 included two *indica* fragments that were absent from both the symmetric and asymmetric hybrids (Figure 3a), while the REMAP primer pair LTR3+RM202R profile similarly included a deleted *japonica* rice fragment (Figure 3b). One REMAP fragment, amplified by LTR2+RM202F, was eliminated in some of the hybrids (Figure 3c).

The parents of the *japonica* rice/wheat hybrids produced 40 distinct fragments from the three primer pairs deployed (Table 4). One of these (Nikita+Sukkula) identified two *japonica* rice fragments, which were not present in some of the hybrids (Figure 3d). In all, eight fragments were variably present, and all of these originated from the *japonica* rice parent genome. Two wheat-specific fragments (amplified by Nikita+Sukkula) were lost specifically in the asymmetric hybrids (Figure 3d).

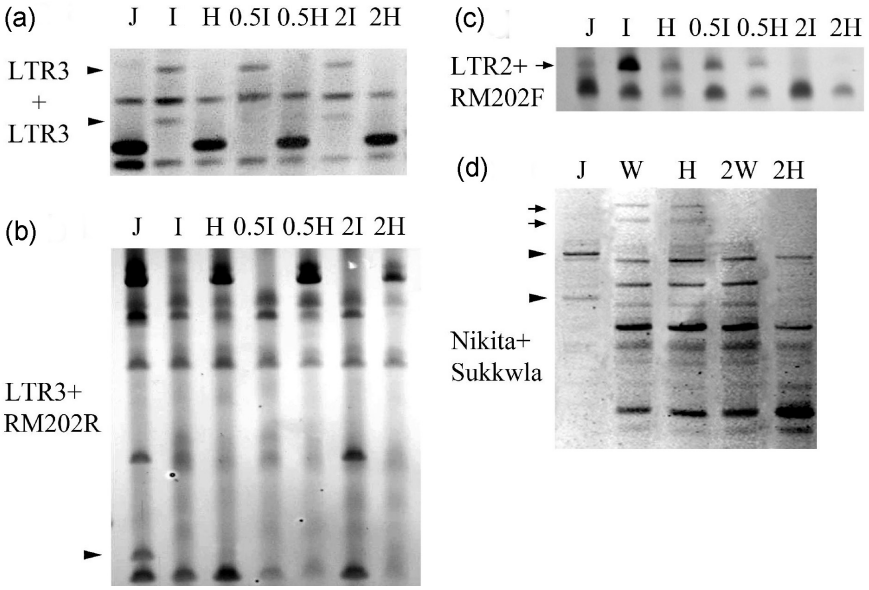

Figure 3. IRAP and REMAP profiles of somatic hybrids. **a.** IRAP LTR3+LTR3 profile of *japonica*/*indica* somatic hybrids; arrowheads indicate deleted *indica* rice fragments. **b.** REMAP LTR3+RM202R profile of *japonica*/*indica* somatic hybrids; arrowheads indicate deleted *japonica* rice fragments. **c. d.** Nikita+Sukkula profile of *japonica*/wheat somatic hybrids; arrowheads indicate deleted *japonica* rice fragments, while arrows show fragments only deleted in 2H asymmetric hybrids. For abbreviations, see legend to Figure 2.

DISCUSSION

Despite the lack of circumstantial data, the assumption generally made is that the genetic events associated with genomic shock take place during or soon after the formation of the hybrid zygote (Ozkan et al., 2001; Kraitshtein et al., 2010). Here, we exploited the more easily accessible somatic hybrid cells (compared to sexual hybrid cells) to establish the specific timing of genomic shock events. We found that the majority of the fusion products were found to be hybrids (up to 40% based on GISH) (Cui HF, Sun Y, Deng JY, Wang MQ, et al., unpublished data), although minor fusing products other than real hybrids might also exist, such as unfused cells or those of the self-fusion of two or more cells. Using controls of fusion of the same species, the influence of the latter products could be excluded, and the effect of genomic shock during somatic hybridization could be studied. The GISH analysis of six day-old somatic rice/wheat cells showed that chromatin elimination was most likely already underway at this stage; surprisingly, even in the asymmetric hybrids, it was the donor (wheat)

rather than the recipient (rice) chromatin that was selectively retained (Figure 1). A similar selective elimination of donor chromatin has also been observed in somatic hybrids involving wheat as the receptor (Zhou et al., 2001).

Genomic shock-induced genetic changes occur early after cell fusion

The process of diploidization represents an important mechanism allowing allopolyploids to limit the expansion of their genome and to regulate their gene expression. Sequence deletion has been suggested as a component of diploidization (Feldman et al., 1997), and sequence elimination has been experimentally established in *de novo* wide hybrids (Gernand et al., 2005). Here, we showed evidence for the loss of parental SSR alleles both in the *japonica*/*indica* rice and in the *japonica* rice/wheat somatic hybrids. The results were consistent with previous reports demonstrating that fragment deletion is common in F1 hybrids. For instance, sequence loss appears to be the most common genomic change occurring in various *Aegilops* sp x *Triticum* sp hybrids (Ozkan et al., 2001), while in the case of resynthesized *Brassica* allopolyploids, the majority of induced changes in cDNA-AFLP profiles were due to fragment loss in the earliest generation (Gaeta et al., 2007). In the present study, obvious genomic changes were detected in young somatic hybrid cells, although only a few kinds of molecular markers were inspected.

Deletions induced by genomic shock have also been detected in genomic regions surrounding retrotransposons, indicative of their re-activation, as, for instance, in *de novo* allopolyploids involving wheat (Kraitshtein et al., 2010). Allopolyploidization comprises first the hybridization event followed by the polyploidization event, whereas the somatic hybridization process does not involve the latter. Therefore, the observation that genomic shock affects somatic hybrids implies that the genomic shock phenomenon is probably not associated with the chromosome doubling process.

Extent of induced variation depends on the genetic distance between the fusion parents

The frequency of both SSR and IRAP/REMAP fragment loss was higher in the *japonica* rice/wheat somatic hybrids than in the *japonica*/*indica* hybrids. This difference suggests that chromatin is more readily eliminated the more distant the genetic relationship between the two hybrid parents. A similar trend has also been noted both for somatic hybrids within Brassicaceae (Sundberg and Glimelius, 1991), as well as for sexual crosses (Kalloo and Chowdhury, 1992).

Extent of DNA loss following somatic hybridization is related to the fertility of the hybrid

Of the 131 fragments evaluated (24 SSR and 44 IRAP/REMAP fragments in the *japonica*/*indica* hybrids, and 23 SSR and 40 IRAP fragments in the *japonica* rice/wheat hybrids), 17 were lost in some or all of the hybrids. Among these deleted fragments, the RM50 SSR locus is linked to *S5*, a gene that controls sterility in *japonica* x *indica* sexual hybrids (Singh et al., 2006), and that is located within a recombination hotspot. Given that the extent of chromatin elimination is higher in the more exotic somatic hybrids, such pressure could affect a number of genomic regions containing genes responsible for wide hybrid sterility, which merits further investigation.

UV irradiation increases the frequency of genetic alterations at the early stage of somatic hybridization

UV irradiation has been previously studied as a type of genomic shock (Molinier et al., 2006). In the present study, the frequency of genetic alterations was slightly higher among the young asymmetric somatic hybrids than among the symmetric ones, since there were three donor fragments that were lost only from the former group. This result indicates that genetic alterations affected by UV irradiation already occur at the early stage.

In conclusion, we revealed genomic variation in hybrid cells via somatic genomic shock for the first time. Although the DNA yield was very limited for the fusion products at the early period, we could nonetheless obtain a partial picture of genomic change through GISH and PCR-based marker analysis. The results showed the possibility to reveal genomic shock through the somatic hybridization method. Further improvements in the young hybrid cell yield would enable more experiments. For example, parental and hybrid DNA could be compared probed by Southern hybridization with some genomic repeats as probes, as well as gene expression profile and epigenetic analyses.

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