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Production of a highly asymmetric somatic hybrid between rice and *Zizania latifolia* (Griseb): evidence for inter-genomic exchange

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Abstract A highly asymmetric and fertile somatic hybrid plant was obtained via protoplast fusion in an intergenric combination. Gamma-ray-irradiated *Zizania latifolia* (Griseb). Turcz. ex Stapf mesophyll protoplasts were electrofused with idoacetamide-inactivated rice protoplasts derived from a 2-month-old suspension cell culture. Two of the six putative hybrid calli regenerated plants. Cytological observation showed that the somatic chromosome numbers of both plants were the same as the rice parent ($2n = 24$). Nevertheless, the hybrid nature and inter-genomic exchange events of one of the plants, i.e. SH6 (SH for somatic hybrid), were confirmed by Southern analysis using both total genomic DNA and moderate-copy, *Z. latifolia*-abundant DNA sequences as probes; in both cases, parental specific and/or new intergenomic recombinant hybridization fragments were detected. In both plant and seed morphology, the hybrid (SH6) was distinct from its rice parental cultivar, as well as from the wild donor species, *Z. latifolia*.

Key words Rice · *Zizania Latifolia* (Griseb). Turcz. ex Stapf · Somatic hybridization · Introgression

Introduction

Modern plant breeding practice has dramatically increased the productivity of major crops. This success has been achieved, however, at the expense of diminishing their genetic variability which is the basis for any further genetic improvement. One of the ways to increase the genetic variability of a given crop is to introduce genes of interest from its related wild species, with which, however, the crop plant is usually sexually incompatible. In such cases, if the gene(s) has been cloned, genetic transformation would be the method of choice; otherwise, asymmetric somatic hybridization mediated by protoplast fusion offers an alternative approach for the introgression of the gene(s) from the donor genome to that of the recipient (Dudits et al. 1987). The most commonly used method for achieving asymmetry is to subject cells of the donor plant to high-dose ionizing radiation prior to protoplast fusion. By this technique, numerous asymmetric nuclear hybrids have been produced, though highly asymmetric ones are rare (Dudits et al. 1987; Hinnisdaels et al. 1991).

Rice provides a staple food for more than 50% of the world's population. Since conventional sexual hybridization can be performed, to the greatest extent, at the interspecific level, it would be of value if somatic hybridization could be applied to this crop to widen its genetic basis. In fact, fertile interspecific somatic hybrids between rice and wild rice have been produced (Hayashi et al. 1988). Intergenric somatic hybridization between rice and barnyardgrass (*Echinochloa oryzicola* Vasing) was also attempted but only plantlets were obtained (Terada et al. 1987). Recently, it was reported that even interfamily somatic hybrids between rice and carrot can be produced (Kisaka et al. 1994). *Zizania latifolia* (Griseb). Turcz. ex Stapf is a wild perennial grass belonging to the tribe *Oryzeae*, thus related to rice (*Oryza sativa* L.), but the two species can not be sexually crossed. *Z. latifolia* posses numerous traits valuable for rice breeding, such as disease and insect resistance, cold and flooding tolerance, and high grain quality. We report here on the production of a highly asymmetric somatic hybrid between rice and *Z. latifolia* by using

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a novel procedure to achieve a high level of asymmetry; namely, irradiating mesophyll cells of *Z. latifolia* prior to their fusion with idoacetamide-inactivated rice protoplasts isolated from embryogenic suspension cell cultures.

Materials and methods

Plant material, protoplast isolation and pre-treatments

Seeds of a *japonica* rice variety, Zhonghua 8, and a local accession *Z. latifolia* were respectively provided by the Chinese Academy of Sciences (Beijng, China) and the Tonghua Academy of Agriculture (Jilin, China). Callus was induced from germinating seeds of rice on solidified (7 g/l agar) MS medium plus 2 mg/l of 2,4-D and 0.2 mg/l of zeatin. Friable and embryogenic callus was selected and subjected to liquid culture in the same medium minus agar, and then subcultured weekly. Highly embryogenic and fast-growing cell suspension cultures were established within 6 weeks by the procedure described by Tao et al. (1997). Protoplasts were isolated from the rice suspension cell cultures according to the method of Kyozuka et al. (1987), except that the concentration of cellulase 'Onozuka' RS (Yakult Honsha, Tokyo, Japan) was reduced to 2%. The rice protoplasts were then treated with different concentrations of idoacetamide (IOA, Sigma) for various durations, further washed, purified by 0.6 M sucrose solution, density adjusted and kept in ice-water. Mesophyll protoplasts of *Z. latifolia* were isolated from leaf bases of 14-day old aseptic seedlings essentially according to the protocol for rice mesophyll protoplast isolation (Gupta and Pattanayak 1993). The protoplasts while in enzyme solution were irradiated by ^{60}Co γ-rays at a dose rate of 50 Gy/min. The protoplasts were then washed, purified, density adjusted and kept in ice-water.

Protoplast fusion, culture, and putative hybrid plant regeneration

Parental protoplasts were mixed in equal proportions to give a total population density of approximately 5×10^5/ml, then further washed twice by a fusion solution (0.5 M mannitol, 0.2 mM $CaCl_2 \cdot H_2O$). Protoplast electrofusion was performed as described (Liu and Liu 1995; Liu et al. 1995). Both treated and untreated protoplasts were cultured in a modified MS medium (Wu et al. 1993) in a nitrocellulose-filter-supported nurse culture (Kyozuka et al. 1987; Liu et al. 1995), in which the rice suspension cells were used as nurse cells. Samples were taken regularly for microscopic observation. Colonies visible to the naked eye were transferred along with the filter to a new medium with reduced osmosity and without nurse cells (Liu et al. 1995). When colonies were 2–3 cm in size, they were transferred to N6 medium (Chu 1976) with 6% sucrose, various concentrations of zeatin (0–6 mg/l) and 2,4-D (0–1 mg/l) or indole-3-acetic acid (0–4 mg/l) for shoot and root formation. Developed plantlets were transferred to soil.

Genomic DNA isolation, cloning of DNA probes and Southern analysis

Total genomic DNA was prepared from protoplast-regenerated plants of rice (cv Zhonghua 8), in vitro cultured seedlings of *Z. latifolia* and putative somatic hybrid plants by the CTAB method (Kidwell and Osborn 1993). The DNAs were then digested with the restriction enzymes *Eco*RI, *Dra*I and *Hin*dIII (New England Biolabs), separated on 1% agarose gels and blotted onto a Hybond-N + nylon membrane (Amersham) by the alkaline method. Two types of Southern analysis were performed. Total genomic probing by using the genomic DNA of *Z. latifolia* as a probe was essentially according to a procedure reported by Li et al. (1993), with the following modifications: (1) the blocking rice DNA was fragmented to about 500 bp by autoclaving at 121°C for 6 min; (2) 2-h after pre-hybridization, all the blocking DNA (a120 × excess of the probe DNA) was added. The pre-hybridization and hybridization were carried out as described by Li et al. (1993). The final washing stringency was 0.1 × SSC and 0.1% SDS at 65°C. Conventional Southern analysis was performed by using 11 moderate-copy DNA sequences cloned from *Sau*3AI-digested genomic DNA of *Z. latifolia*. Briefly, genomic DNA of *Z. latifolia* was digested with *Sau*3AI and separated with 1% low-melting agarose gel. DNA fragments in the range of 0.5–2.0 kb were eluted, purified and ligated to *Bam*HI-cleaved PUC19 plasmid using T4 ligase. *Escherichia coli* JM103 competent cells were transformed with the ligation mixture, and ampicillin-resistant white colonies were picked up at random. Plasmid DNA was prepared by the alkaline method and dot-blotted onto Hybond-N + membrane. Total genomic DNA of both *Z. latifolia* and rice were used as probes to estimate the relative copy numbers of the sequences in the two species. Eleven clones were chosen because they were significantly more abundant in *Z. latifolia* than in the rice genome. The hybridization and washing conditions were as described earlier (Liu et al. 1997). In both types of Southern analysis, probes were labelled by the random primer method (Feinberg and Vogelstein 1983). The hybridized blots were exposed to X-ray film at −70°C for 1–3 days.

Cytology

Rapidly growing hybrid callus and roots of regenerated plantlets were fixed in acetic-ethanol (absolute ethanol : glacial acetic acid 3 : 1) for 10–24 h at 4°C, enzyme-macerated, stained with 2% Giemsa and examined under a light microscope. In each case the chromosomes of at least ten well-spread metaphase cells were counted.

Results

Pre-fusion treatment, electrofusion and recovery of putative hybrid callus

Effects of IOA treatment on the cell division of rice (cv. Zhonghua 8) suspension cell protoplasts were studied. Treatment with 10 mM for 15 min at room temperature was found sufficient to prevent any sustained cell division under nurse culture conditions; no colony was recovered after 4-weeks of culture. The unirradiated mesophyll protoplasts of *Z. latifolia* under nurse culture conditions could undergo a few cell divisions, but none of them was observed to form a colony. In order to induce chromosome fragmentation, the mesophyll protoplasts of *Z. latifolia* were irradiated by γ-rays at a dosage of 1000 Gy, which was chosen based on previous results in naked oat (*Avena nuda* L.) (Liu and Liu 1995). The double inactivated parental protoplasts were then electrofused under conditions described by Liu and Liu (1995) and Liu et al. (1995). Both the fusion-treated and the non-treated protoplast mixtures were cultured. Since initial fusion products were readily distinguishable, it was estimated that about a 5% binary heterofusion frequency was obtained.

After a 4-week culture, cell colonies were found only in fusion-treated protoplasts. From a total of 3×10^6 fusion-treated parental protoplasts, 24 individual colonies were identified; but only six of them grew further upon transfer to new medium and developed to calli.

Plant regeneration from the putative hybrid calli

Green plantlets were regenerated from two (SH2 and SH6) of the six calli cultured on N6 regeneration medium within 2 months. The embryogenic appearance of the callus and the frequent simultaneous development of shoots and roots in the same medium suggested that the pathway to plantlet regeneration was via somatic embryogenesis. Plantlets of both calli survived after transplantation into pots. Several well-developed seeds were obtained from each of the plants. Morphologically, plants that regenerated from callus SH2 were almost identical to the rice parent. Plants that regenerated from callus SH6 were, however, distinctly different from its rice parental variety in several morphological traits: plant height was shorter, their leaves were broader, and they ripened earlier (see Fig. 2 A). While the seed morphology of the SH2 plants did not show any apparent difference from the rice parental variety, those of the SH6 plants were significantly smaller in size and darker in color (a trait characteristic of de-husked seeds of *Z. latifolia*) than their rice parent (Fig. 1 B).

Characterization of the "rice + *Z. latifolia*" somatic hybrid plants

Total genomic probing by using genomic DNA of *Z. latifolia* as a probe and autoclaved genomic DNA of rice as a blocker was performed. After testing different proportions of probe/blocker DNAs, a ratio of 1 : 120 was found to give the best results. Under this probe/blocker ratio and the hybridization/washing stringency, the *Z. latifolia* lane was almost a complete smear, but the common sequences of the two species which had not been suppressed by blocking could be resolved as discrete bands on the rice as well as on the two putative hybrid lanes after an overnight exposure (Fig. 2 A). Both of the putative hybrid plants gave different hybridization patterns from that of the rice parent as well as from each other. But the difference between SH2 and rice was slight; with only size changes and/or loss of copy numbers of the 7-kb hybridization fragment, while the number of fragments remained the same. The SH6 plants, on the other hand, differed from rice conspicuously, with an apparent loss of rice parental fragments/copy numbers (marked by circles) and the appearance of at least nine new fragments (arrowed). In particular, four fragments (i.e. 9.0, 5.5, 3.8 and 3.2 kb) that were prominent in the *Z. latifolia* lane, in spite of the overall smearing, seemed to be present in SH6. Since the total genomic probing signals were obtained after an overnight exposure, they represented highly repetitive DNA sequences. Eleven moderate-copy genomic DNA clones from *Z. latifolia* that were

Fig. 1A, B Production of a fertile and highly asymmetric somatic hybrid between rice and *Z. latifolia*. **A** Morphology of regenerated plants from callus cultures of *Z. latifolia* (***Z***) and of rice, cv. Zhonghua 8 (***R***), as well as of a somatic hybrid plant of SH6 (***SH6***). Note that hybrid plant is short, with broader leaves and earlier ripening (already with spikes). **B** Seed morphology of the donor species *Z. latifolia* (***Z***), the somatic hybrid (***SH6***), and the parental rice, cv Zhonghua 8 (***R***). Note that seeds of the somatic hybrid are significantly smaller and darker than those of the rice parent

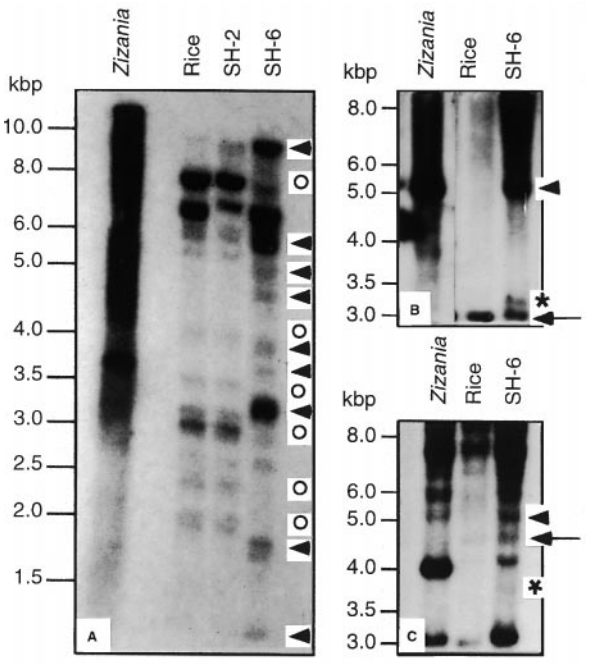

Fig. 2A–C Southern-blot hybridization of *Hin*dIII-restricted genomic DNAs of the two parental species and of the two putative somatic hybrids probed with total *Z. latifolia* genomic DNA **A**, and with two moderate-copy and *Z. latifolia*- abundant genomic DNA sequences, Zg3 **B** and Zg81 **C**. *Arrow heads and circles* in (**A**) respectively refer to fragments which appear in the somatic hybrid (SH6) but do not exist in the rice parent and the apparent loss of fragments/copy numbers in the somatic hybrid. *Arrow heads and long arrows* in (**B**) and (**C**) respectively refer to *Z. latifolia* and rice species-specific hybridization fragments, while *asterisks* indicate novel fragments. Sizes in kbp are indicated according to the 1-kb DNA-ladder size marker

significantly more abundant in the genome of *Z. latifolia* than in that of rice were also used as probes for Southern analysis. For SH2 plants, all probes produced hybridization patterns identical to those of the rice parent in all three enzyme digests. For SH6 plants, nine probes also produced identical hybridization patterns to those of the rice parent, but two probes, namely, Zg3 and Zg81, produced hybridization patterns with species-specific fragment(s) of *Z. latifolia* and/or new fragment(s) which existed in neither of the parents, in all three enzyme digests (e.g. Fig. 2 B and C). To rule out the slight possibility that the changed hybridization patterns in SH6 were due to somaclonal variation of the rice "escapers", 16 randomly chosen plants derived from protoplasts of the rice parental cultivar (Zhonghua 8) were also subjected to analysis. Though some of the plants exhibited copy number deviations from the parental rice patterns, none gave the strong hybridization signals characteristic of the donor species *Z. latifolia*, as shown by the SH6 plants with two of the sequences (Fig. 2 B, C). The SH2 plant was probably either a rice "escaper" or a cytoplasmic hybrid.

Cytological analysis of root-tip cells of SH2 and SH6 showed an unexpected result in that both plants had a consistent chromosome number of $2n = 24$ in all the cells examined, although chromosome variations, in both number and structure, were occasionally observed in SH2 and SH6 callus cells.

Discussion

In the present study, we demonstrated the occurrence of inter-genomic exchange between rice and *Z. latifolia* via asymmetric somatic hybridization based on several lines of evidence. First, total genomic probing indicated the existence of species-specific, highly repetitive DNA sequences of *Z. latifolia* in SH6 plants. Second, Southern analysis, using two moderate-copy and *Z. latifolia*-abundant genomic DNA sequences cloned from *Z. latifolia* genomic DNA as probes, showed that the SH6 plant possesed hybridization fragments respectively specific to either of the parents as well as unique fragments, implying that the hybrid contained DNA sequences homologous to the two probes from both parents and that intergenomic recombination within or flanking these regions had probably occurred. Third, the hybrid plant had the same somatic chromosome number as the rice parent, suggesting that intergenomic recombination and integration had taken place prior to the complete elimination of the *Z. latifolia* chromosomes.

Most of the published work on somatic hybridization indicated that although asymmetric somatic hybrids could be produced, highly asymmetric ones were difficult to obtain (Dudits et al. 1987; Hinnisdaels et al. 1989; Melzer and O'Connell 1992). Moreover, it was evident that the degree of asymmetry was mainly determined by the phylogenetic relatedness and hence the extent of somatic compatibility of the fusion partners, rather than by the dose of irradiation applied to the donor parent (Melzer and O'Connell 1992). Therefore, highly asymmetric somatic hybrids were more likely to occur in remote combinations (Dudits et al. 1987). In order to produce highly asymmetric somatic hybrids in relatively close combinations, treatments other than irradiation are probably required. It was also reported that the fusion of protoplasts from rapidly dividing suspension cell cultures with mitotically inactive mesophyll cells would generate fragmentation of the chromosomes of mesophyll protoplasts due to the phenomenon of the premature chromosome condensation (PCC) (Dudits et al. 1982; Liu et al. 1994). We assumed that the combination of PCC with high-dose irradiation may enable chromosomes of the donor parent to be eliminated more efficiently and drastically, while the transient co-existence of two genomes in the initial few divisions and of one intact genome with numerous fractionated chromosome fragments several

divisions later on would probably provide enough interactions for introgression events to occur. Although more data is required, the results of the present study seemed to support this idea. The advantages of somatic hybrids with an intact genome from one parent and only a few introgressed segments from the other, i.e. highly asymmetric hybrids, are several: (1) high fertility can be expected; (2) unwanted traits are greatly reduced; (3) genetic recombination has already been accomplished, which may otherwise be difficult during sexual reproduction due to strict control of chromosome pairing at meiosis in many higher plants.

Intergeneric somatic hybrids between rice and barnyard grass have been produced previously, but these hybrids were not able to develop beyond the plantlet stage due to necrotic death (Terada et al. 1987). Recently, an interfamily hybrid between rice and carrot was reported, and the morphology of the hybrid plants was similar to that of the carrot parent (Kisaka et al. 1994). Although this wide hybrid could be of value for basic studies, it was apparently not suitable for breeding purposes. The somatic hybrid plants between rice and *Z. latifolia* produced in this study could be easily transplanted to soil and produced seeds. But only those plants regenerated from a single regenerable callus, i.e. SH6, have been found to be hybrid; thus, the chances of desirable traits of *Z. latifolia* being incorporated into rice are reduced. The available evidence has not supported the hybrid nature of the other regenerable callus, SH2; it could well be either a rice "escaper" or more likely a "cytoplasmic hybrid". The latter case means that the nucleus of the plant is solely (or predominantly) from rice, but that it contains some cytoplasmic organelles derived from the donor species, *Z. latifolia*, which could fully restore the otherwise inactivated metabolic state in rice cells due to IOA treatment (Galun and Aviv 1983). Currently, the culture and fusion conditions are being further improved, so that more somatic hybrids between rice and *Z. latifolia* can be obtained, which should provide valuable material for rice improvement.

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