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Establishment of somatic hybrid cell lines between *Zea mays* L. (maize) and *Triticum* sect. *trititrigia* MacKey (trititrigia)

T. B. Wang [1,*], M. Niizeki [1], T. Harada [1], R. Ishikawa [1], Y. Q. Qian [2], and K. Saito [1]

[1] Plant Breeding Laboratory, Faculty of Agriculture, Hirosaki University, Hirosaki, Aomori-Ken 036, Japan
[2] Department of Bio-science and Biotechnology, Academia Sinica, Beijing 100864, China

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Summary. Somatic hybrid cell lines were constructed by the fusion of protoplasts isolated from cell suspensions of *Zea mays* L. (maize, 2n = 20) and *Triticum* sect. *trititrigia* MacKey (trititrigia, 2n = 35), a perennial hybrid of *T. durum* Desf. and *Elytrigia intermedium* (Host) Nevski. Iodoacetamide-inactivated protoplasts of maize were fused with trititrigia protoplasts, which were sensitive to the PEG/DMSO fusion treatment at high pH and high calcium. Based on physiological complementation, approximately 0.002% of the total protoplasts cultured following fusion treatment developed into cell colonies, and 79 lines of them, almost a half, were singled out and subcultured. Among the subcultured lines three were, in comparison with the parents, identified as somatic hybrids by their coupled *Xba*I restriction patterns of total DNAs probed with the ribosomal DNA of rice. Southern analysis of the digested total DNAs with a mitochondrial gene, *atp*A, from pea, or a chloroplast gene, *trn*K, from rice, revealed that all the hybrids carried only the organellar DNAs of trititrigia, which excluded the possibilities of a chimeric callus or any DNA contamination. Cytogenetically, one hybrid was mixoploid with a 2n of 46–67 in which chromosomal endoreduplication, characterized by the appearance of diplochromosomes, was occasionally observed. Its hybridity was reconfirmed by the fact that it bore the satellite chromosomes of both maize and trititrigia, which were distinguishable from each other by size. In contrast, the other two hybrids were aneuploids. The potential of gene transfer between *Zea* and *Triticum* species was thus conclusively established.

Key words: Somatic hybrid – Ribosomal DNA – Satellite chromosome – Maize – Wheatgrass

* *Permanent address:* Institute of Botany, Academia Sinica, Beijing, China
Correspondence to: T. B. Wang (at present address)

Introduction

Wide hybridization has interested both plant genetists and plant breeders for many years (Goodman et al. 1987). However, attempts to breed hybrids bridging two gramineous subfamilies, namely the Pooidea and the Panicoidea (classified by Hutchinson 1959), have been hindered by sexual incompatibilities, even though various were made to cross one parent from *Triticum, Aegilops, Hordeum* or *Secale* with another parent from maize, sorghum or pearl millet (Laurie and Bennett 1986; Laurie et al. 1990). For instance, when the hexaploid wheat variety, Chinese Spring, was crossed with the maize variety, Seneca 60, the fertilized eggs eliminated all the maize chromosomes within first three cell division cycles during the development of the embryo. Clearly, it is not yet possible to mutually incorporate agronomically important characters, such as the high photosynthetic efficiency of maize or the drought and cold hardiness tolerance of wheat, into cultivated wheat or maize due to their sexual isolation.

Beyond the limits of the traditional method, somatic hybridization in a wide variety of higher plants has shown the potential to extend wide crosses across genetic gaps between incompatible species. Using this approach, nuclear and/or cytoplasmic traits have been successfully transferred from one species to another and novel plants have been synthesized from two different species (Gleba and Sytnik 1984). Nevertheless, in spite of a few successful reports (Tabaeizadeh et al. 1986; Terada et al. 1987; Hayashi et al. 1988), attempts at cell fusion between gramineous species, including many important crops, are still limited by the recalcitrance of in-vitro protoplast manipulation and difficulties in selecting hybrids. Recently, plants have been regenerated from protoplasts isolated from embryogenic suspension cells of trititrigia,

a perennial hybrid of durum wheat and intermediate wheatgrass (Wang et al. 1990), and a plant regeneration procedure of maize protoplasts was described earlier by Cai et al. (1987). Wheatgrass strains (*Elytrigia,* Syn: *Agropyron*) have contributed much to wheat breeding, including the transfer of drought tolerance, cold hardiness and salinity tolerance (Fedak 1985), and of disease resistant traits to virus (Brettell et al. 1988), fungi (Mathre et al. 1990) and insect pests (Tremblay et al. 1988). Maize is a worldwide cultivated crop. The present study describes the establishment of somatic hybrid cell lines between maize and tititrigia.

Materials and methods

Cell suspension cultures

Cell suspensions of tititrigia, a hybrid of *Triticum durum* Desf. (2n = 28) and *Elytrigia intermedium* (Host) Nevski (2n = 42), were described previously (Wang et al. 1990), and have been successfully subcultured every 4 days in modified MS medium (Murashige and Skoog 1962), i.e., S4 medium supplemented with 100 mg/l inositol, 150 mg/l aspartic acid, 200 mg/l glutamine, 300 mg/l casein hydrolysate, 3% sucrose and 2.5 mg/l 2,4-D at pH 5.8. A 1.5 year-old maize embryogenic callus induced from F_1 premature embryos of cv Hsiaopatang × cv Shuipai was provided by Prof. C. S. Kuo (Institute of Botany, Academia Sinica, Beijing, China). It was then suspended in S4 medium and subcultured for 6 months in the same way as for tititrigia and until the isolation of protoplasts.

Isolation and fusion of protoplasts

Settled suspension cells obtained 2 days after renewing the medium were used to isolate protoplasts by enzyme treatment. The enzyme solution consisted of 1.5% Cellulase Onozuka RS, 0.075% Pectolyase Y-23 for tititrigia, and 2% Cellulase Onozuka RS, 1% Rhozyme HP-150 and 0.2% Pectolyase Y-23 modified from Zhang's formula (Zhang et al. 1990) for maize, dissolved in a wash solution (WS) containing 0.5 m*M* KH_2PO_4, 10 m*M* $CaCl_2$, 2 m*M* $MgSO_4 \cdot 7H_2O$ and 0.6 M mannitol at pH 5.8. Tititrigia cells were digested for 3 h, and maize cells for 4 h, in a water bath at 30 °C on a shaker at 30 rpm.

The digested mixture of each cell line was filtered through eight layers of gauze, and the isolated protoplasts were harvested by centrifugation at 80 *g*. Maize protoplasts were tested for sensitivity to iodoacetamide by treatment with 0.25, 0.5, 1.0, 2.0, 5.0 and 10 m*M* iodoacetamide dissolved in WS at pH 5.7 for 15 min at room temperature. Seven weeks after culturing the treated protoplasts, a few cell colonies were formed from the cultures pretreated with the lowest concentration of 0.25 m*M* iodoacetamide; no cell colonies were observed in the other cultures. Therefore, 1 m*M* iodoacetamide was used in the fusion experiments.

The inactivated maize protoplasts were washed twice with WS, and mixed with tititrigia protoplasts at a ratio of approximately 1:1. The mixture was packed by centrifugation and gently resuspended in W_5 solution containing 154 m*M* NaCl, 125 m*M* $CaCl_2$, 5 m*M* KCl and 5 m*M* glucose at pH 5.6 (Menczel et al. 1981). Protoplast density was adjusted to $4-6 \times 10^6$ cells/ml before fusion treatment. The fusion solution contained 20% polyethylene glycol (PEG) 4000 (average MW 3000, Wako Pure Chemical Industries Ltd., Japan), 0.4 M glucose, 10% dimethyl sulfoxide (DMSO), 0.1 M $CaCl_2$, and buffered to pH 10.0 with 3 m*M* 3-cyclohexylaminopropanesulfonic acid (CAPS) and NaOH. The fusion procedure, modified from Kao's method (Kao and Michayluk 1974; Kao et al. 1974), was as follows: about 200 µl of a protoplast mixture in W_5 solution was pipetted into a 6-cm Falcon petri dish. After the protoplasts had settled for 10 min at room temperature, 300 µl of PEG solution was gently added around the protoplast drop. The mixture was incubated for 10 min, and two aliquots of an elution solution containing 0.4 M glucose and 50 m*M* $CaCl_2$ at pH 5.6 were added to the protoplast mixture at 5 min intervals. Finally, the fused protoplasts were diluted with 3 ml of the Kp medium described previously by Wang et al. (1990), and then incubated for 30 min at room temperature. The protoplasts were harvested by centrifugation at 80 *g* and washed once with Kp medium. They were cultured in Kp medium solidified with 1.2% Seaplaque agarose at a density of $2-3 \times 10^5$ cells/ml in Petri dishes at 25 °C in the dark.

The cell colonies which regenerated 7 weeks later from the fused cells were transferred onto NK medium consisting of the major elements of N6 (Chu et al. 1975), the minor elements of B5 (Gamborg et al. 1968), the vitamins of Kp, 100 mg/ml inositol, 100 mg/l glutamine, 100 mg/l casein hydrolysate, 0.5% coconut milk, 1 mg/l 2,4-D, 3% sucrose, and solidified with 0.7% agar at pH 5.8. Then, 2–4 weeks later, they were used to isolate total DNA.

Southern analysis

Total DNA was isolated from parental cell suspensions and 9–11 week-old fusant cell lines, respectively, by precipitation with cetyltrimethylammonium bromide (CTAB) as described by Murray and Thompson (1980). About 0.5–1 µg of total DNA from each cell line was digested in 25 µl of reaction solution containing 12 units of *Xba*I under the conditions given by the manufacturer (Takara Shuzou Co. Ltd., Japan). After fractionation in a 0.8% agarose gel with 0.5 µg/ml of ethidium bromide, DNA was blotted onto a Hybond N^+ nylon membrane (Amersham). The hybridization and the detection of restriction patterns were conducted according to the ECL gene detection system (Amersham). Genomic DNA was probed with a ribosomal DNA (rDNA) fragment including the 5.8*s*, 17*s* and 25*s* rDNA of rice excised from plasmid pRR217, while mitochondrial and plastid DNAs were probed with the *atp*A of pea and with rice *trn*K in a cpDNA clone, E-10, respectively.

Cytological observations

Approximately 4-month-old hybrid cell lines were fixed in a 3/1 ethanol/acetate acid solution for 4–24 h after 24 h pretreatment in ice water. The fixed cells were squashed after soaking for 2 days in 1% carmine in 45% acetate acid.

Results

Maize protoplasts were completely inactivated by treatment with 1 m*M* iodoacetamide for 15 min at room temperature. No cell division or colony formation were observed in cultures of the treated protoplasts in at least three replications. After fusion treatment tititrigia protoplasts died within 1 week of culture according to examination with Evans blue. By contrast, fusion cultures, involving nearly 5×10^6 iodoacetamide-inactivated maize protoplasts and an equal number of tititrigia protoplasts, produced about 180 fusant colonies which were

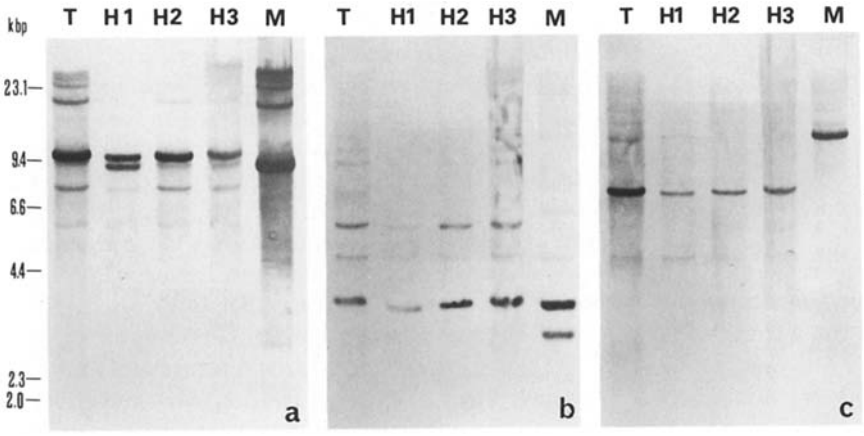

Fig. 1a–c. Southern-blot analysis of *Xba*I-digested total DNAs from trititrigia (*T*), maize (*M*) and their somatic hybrids (*H1, H2*, and *H3*). Blots were hybridized with genomic ribosomal DNA (**a**), chloroplast *trn*K DNA (**b**) and mitochondrial *atp*A DNA (**c**), respectively. Fragment size is given in kilobase pairs (kbp)

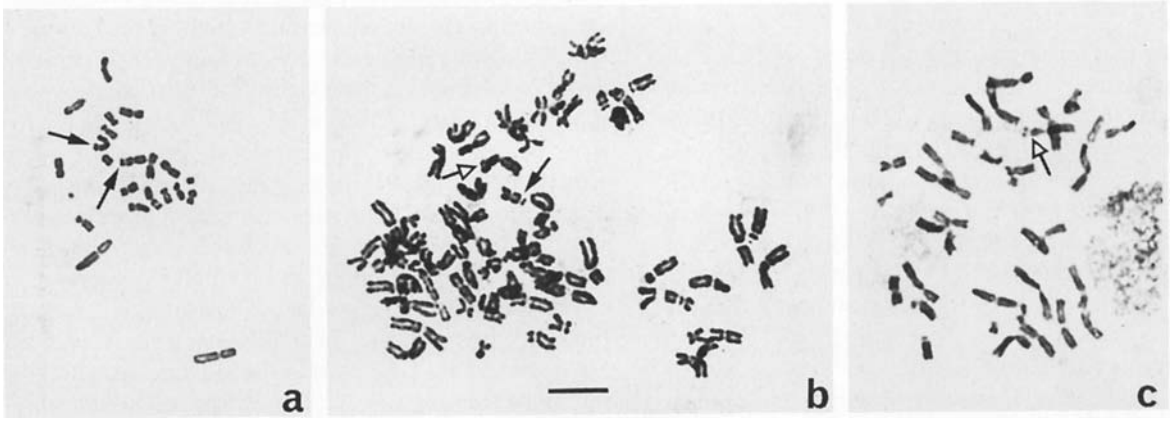

Fig. 2a–d. Metaphase chromosomes of cultured parental cells and somatic hybrid cells. **a** maize, 2n = 20; **b** H1 hybrid, 2n is about 65; **c** trititrigia, 2n = 35; **d** satellite chromosomes of maize (*M*), H1 hybrid (*H*) and trititrigia (*T*) from Fig. **a**, **b** and **c**. The *solid arrows* indicate maize satellite chromosomes, or that from maize in the hybrids, while the *open arrows* indicate satellite chromosomes of trititrigia, or that from trititrigia in the hybrid. Scale bar represents 10 μm

visible to the naked eye within 7 weeks. From these, 79 larger colonies able to be singled out for subculture were transferred onto NK medium for further analyses. All the fusant colonies were either yellow or white just like the calli of maize or trititrigia; therefore, a chimeric callus could be readily distinguished.

The large scale screening through Southern hybridization of the *Xba*I-digested total DNAs, isolated from the fusants and their parental suspensions, with an rDNA probe exposed three genomic hybrid cell lines (designated as H1, H2 and H3). These were characterized by their coupled RFLP (restriction fragment length polymorphism) patterns from both parents (Fig. 1a), which were all white. Additional probing tests of the digested total DNAs with the organellar DNA probes confirmed that all the hybrids carried only the extrachromosomal DNAs from trititrigia (Fig. 1b, c). The Southern diagnoses also verified that there were no cybrids among the other cell lines recovered from the mass culture of the fused protoplasts.

Cytological observation showed that most of the maize chromosomes were smaller than those of trititrigia, and their satellite chromosomes were unequivocally distinguishable from each other by size (Fig. 2). H1 was a mixoploid with a 2n chromosome number ranging from 46 to 67, in which the coexistence of the larger satellite chromosome of trititrigia and the smaller one of maize was evident (Fig. 2b, d). Endoreduplication, characterized by the appearance of diplochromosomes (4-chromatids), was also observed in H1 hybrid cells (data not shown). H2 and H3 were aneuploids with 2n = 38 plus one fragment and 2n = 39, respectively.

Discussion

Intersubfamilial somatic hybrids of maize and trititrigia were efficiently constructed with a selective protocol which predominantly favoured the development of heterokaryons from the fusion due to physiological complementation. The sensitivity of trititrigia protoplasts to the fusion treatment with PEG solution at high pH and high calcium abolished their competence to regenerate into cell colonies. No cell division or colony formation as observed in the control cultures despite the fact that they divided regularly at a frequency of 12% (Wang et al. 1990). On the other hand, treatment with iodoacetamide solution completely inactivated maize protoplasts unless they were fused with untreated ones. For over a decade this chemical approach has been broadly employed to eliminate one fusion partner in somatic hybridization (Medgyesy et al. 1980; Sidorov et al. 1981). A similar selection strategy for somatic hybrids based on the inability of one partner to survive the PEG/DMSO fusion treatment has also been described (Handley et al. 1986). Recently, selection for somatic hybrids by dualantibiotic resistance has proven more efficient than the other methods (Thomas et al. 1990; Sproule et al. 1991). However, fusion partners with expressing selection markers are not always available. Moreover, the introduction of a marker into a gramineous species by transformation still remains difficult to achieve.

Considering that parent escapees could be recovered frequently from the pretreated protoplasts employed in the fusion, due to the effect of co-culturation, Southern analyses of *Xba*I-digested total DNAs with genomic or organellar DNA probes were carried out. The hybridization with a ribosomal DNA probe provided evidence for the genomic hybridity of the fusants, while that with the organellar DNA probes, especially the cpDNA probe, confirmed the hybridity by excluding the possibility of a chimeric callus or any DNA contamination. Because the chloroplast genotype in most regenerated somatic hybrids has been found to be one or the other of the two parental types (Maliga and Menczel 1986), only hybrids with genomic DNA from both parents and chloroplasts DNA from a single parent could display DNA restriction patterns like those described in the present study. Others have pointed out the significance of detecting novel hybrid isozymes, which can not be formed in a mixture of parental extracts, as evidence for a hybrid structure (Evans et al. 1980; O'Connell and Hanson 1985; Tabaeizadeh et al. 1986). However, in somatic hybrids between two subfamilies or two families, sub-units from different parents have failed to form heterodimers in vivo (Wetter 1977; Chien et al. 1982; Terada et al. 1987). On the other hand, tests with a large number of isozymes are usually necessary to confirm hybridity since most isozymes from either parent disappear at the time of identification, thus paralleling the loss of chromosomes in a somatic hybrid between incompatible species. Therefore, it is difficult to certify the hybridity of wide somatic hybrids by isozyme analysis.

One interesting observation was that the ribosomal DNA band from maize in the H2 and H3 hybrids was much less evident than the main band from trititrigia, whereas they showed the same intensity in the H1 hybrid. Considering the fact that the 5.8*s*, 18*s* and 25*s* rDNA species, which constitute the rDNA probe used, are all located on only one pair of maize chromosomes (Coe et al. 1990), one might postulate that most cells of H2 or H3 may have lost the satellited maize chromosomes before the analysis. The result also demonstrated the tendency of the hybrids to eliminate maize chromosomes.

The cytological characterization of the species-specific satellite chromosomes convincingly confirmed the nature of the somatic hybrid obtained in this study. Morphological differences between the parental chromosomes has been used as evidence for *Nicotiana*-soybean hybrids (Kao 1977; Chien et al. 1982) as well as for hybrids between *Rauwolfia serpentina* and *Vinca minor* (Kostenyuk et al. 1991). By contrast, no convincing cytological proof other than chromosome number was obtained for somatic hybrids of *Oryza sativa* and *Echinochloa oryzicola* (Terada et al. 1987), *Lycopersicon esculentum* and *Solanum muricatum* (Sakamoto and Taguchi 1991), or *Nicotiana tabacum* and *N. debneyi* (Sproule et al. 1991) because of the indistinguishability of parental chromosomes. Moreover, species-specific chromosomes can not always be observed in a population of hybrid cells due to chromosome elimination, which is a frequent event in a somatic hybrid between genetically incompatible species. As compared with the sum of 2n chromosomes from the two parents, the H1 mixoploid hybrid was composed of cells with various 2n numbers ranging from hypoaneuploid to hyperaneuploid. Hypoaneuploid cells probably results from chromosome elimination, whereas the hyperaneuploid cells might result from an initial polyploidization of the hypoaneuploid followed by chromosome elimination from the doubled cells. Thus, diplochromosomes, indicative of chromosomal endoreduplication, were observed in the hypoaneuploid cells, and all cells bore fewer chromosomes than the doubled 2n number of the endoreduplicated cells. The aneuploid hybrids, H2 or H3, might also derive directly from a fused cell by chromosome elimination.

The somatic hybrids were, in a sense, more stable than the wheat-maize hybrid zygotes derived from sexual hybridization, in which maize-originated chromosomes were completely eliminated within the first three cell division cycles (Laurie et al. 1990). The heteroplasmic status of freshly fused cells, i.e., heterokaryocytes, might favour the reduplication of chromosomes from both maize and trititrigia at the initiation of hybrid cell division. Addi-

tionally, one fusion partner, trititrigia, is itself a stable intergeneric hybrid, which might make the hybrids more tolerant to genetic incompatibility. Even so, most of the maize-originated chromosomes of the hybrid were eliminated eventually as confirmed by DNA analysis using the AP-PCR method (data not shown).

The three hybrids screened out by the rDNA probe were probably not the only hybrids deriving from the fusion. Thus, some possible hybrids with uniparental rDNA might not have been detected because of chromosome elimination.

In conclusion, our results demonstrate the feasibility of utilizing somatic hybridization for the genetic modifications of maize and wheat, or their relatives, with each other. Chromosome elimination, as expected, was the major problem in hybrid development. However, it should be possible for some genes, including active transposable elements or highly efficient photosynthetic genes of maize, to be incorporated into C_3 plants like wheat.

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