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B. Szarka · I. Göntér · M. Molnár-Láng · S. Mórocz
D. Dudits

Mixing of maize and wheat genomic DNA by somatic hybridization in regenerated sterile maize plants

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Abstract Intergeneric somatic hybridization was performed between albino maize (*Zea mays* L.) protoplasts and mesophyll protoplasts of wheat (*Triticum aestivum* L.) by polyethylene glycol (PEG) treatments. None of the parental protoplasts were able to produce green plants without fusion. The maize cells regenerated only rudimentary albino plantlets of limited viability, and the wheat mesophyll protoplasts were unable to divide. PEG-mediated fusion treatments resulted in hybrid cells with mixed cytoplasm. Six months after fusion green embryogenic calli were selected as putative hybrids. The first-regenerates were discovered as aborted embryos. Regeneration of intact, green, maize-like plants needed 6 months of further subcultures on hormone-free medium. These plants were sterile, although had both male and female flowers. The cytological analysis of cells from callus tissues and root tips revealed 56 chromosomes, but intact wheat chromosomes were not observed. Using total DNA from hybrid plants, three RAPD primer combinations produced bands resembling the wheat profile. Genomic in situ hybridization (GISH) using total wheat DNA as a probe revealed the presence of wheat DNA islands in the maize chromosomal background. The increased viability and the restored green color were the most-significant new traits as compared to the original maize parent. Other intermediate morphological traits of plants with hybrid origin were not found.

Keywords Protoplast fusion · Polyethylene glycol · Somatic hybrid · RAPD analysis · In situ hybridization

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B. Szarka and I. Göntér contributed equally to this work

B. Szarka · I. Göntér · S. Mórocz
Cereal Research Non-Profit Co., H-6701 Szeged,
P.O.Box 391, Hungary

M. Molnár-Láng
Agricultural Research Institute
of Hungarian Academy of Sciences,
H-2462 Martonvásár, P.O.Box 19, Hungary

D. Dudits (✉)
Institute of Plant Biology,
Biological Research Center H.A.S,
Temesvári krt. 62, H-6726 Szeged, Hungary
e-mail: dudits@nucleus.szbk.u-szeged.hu
Fax: +36-62-433-188

Introduction

Hybridization of somatic cells has been used successfully to combine genes from a wide range of sexually incompatible species or genera (*Umbelliferaceae*: Dudits et al. 1979; *Gramineae*: Terada et al. 1987; *Cruciferaceae*: Gleba and Hoffmann 1978; Toriyama et al. 1987; *Rutaceae*: Grosser et al. 1988; *Solanaceae*: Babiychuk et al. 1992; Kim et al. 1993; *Fabaceae*: Crea et al. 1997).

The protoplast fusion has also been applied as an alternative breeding method for compatible species when the sexual cross was extremely difficult (Dudits et al. 1987; Hansen and Earle 1995; Samoylov et al. 1996). A significant number of cultivated plant species has been improved successfully by somatic hybridization-based transfer of traits of practical importance. Resistance genes were transferred against bacterial (Hansen and Earle 1995; Laferriere et al. 1999), fungal (Hansen and Earle 1997) and virus diseases (Austin et al. 1985; Gibson et al. 1988), or even nematodes (Lelivelt et al. 1993). Somatic transfer of cytoplasmic male sterility (Kyozuka et al. 1989; Akagi et al. 1995) has been achieved and improvement of drought (Begum et al. 1995) or cold tolerance (Louzanda et al. 1993) was also attempted via somatic hybridization.

Although several hundreds of hybrids and cybrids have already been produced from many different species (Earle et al. 1992; Kushnir et al. 1991; Guo and Deng 1998), only few data have been published on maize somatic hybridization (Kao and Michayluk 1974; Brar et al. 1980). One possible reason for the limited success may have resulted from difficulties in the maintenance of proper maize-protoplast culture systems (Rhodes et al. 1988; Prioli and Söndall 1989; Shilito et al. 1989;

Mórocz et al. 1990). At the same time the protoplast fusion-mediated gene transfer would have a special significance in maize improvement as an alternative approach to DNA transformation. Also, the asymmetric somatic hybrids from divergent parents can offer a unique genetic constitution for genomic projects. Therefore here, we attempted to produce maize and wheat fusion hybrids. The described culture system allowed the identification of putative fusion products. Molecular and cytological data support the conclusion that a considerable amount of nuclear DNA is present from the donor wheat genome in the chromosomes of selected hybrid plants.

Materials and methods

Plant materials

Wheat plants were grown in sterile conditions from seeds of the GK Öthalom variety (Cereal Research Non-Profit Co. Szeged, Hungary) (see Fig. 1A). Maize protoplasts were isolated from the suspension culture of the H 1160 albino maize cell line (see Fig. 1B), which was derived from anther culture of the H229 × C2-A-18 maize hybrid (Mórocz 1991).

Maize suspension culture

The maize suspension culture was maintained and subcultured weekly for protoplast isolation at the rate of 2 g/50 ml of N6M medium (Mórocz et al. 1990).

The osmolarity, pH and growth of the cell mass were monitored in order to establish a reproducible cell-culture system and try to find a specific optimum stage for fusion; 2 g/50 ml cultures were initiated and every day three samples were measured for osmolarity, pH and cell mass. After recording the data micro-calli were mixed from the three dishes for protoplast isolation and fusion.

The maize micro-calli were sedimented and the remaining liquid medium was maximally removed by an electric pipette, then the packed cell weight was recorded. In the waste culture the medium pH and the osmotic values with a cryoscopic osmometer (Osmomat 030-D) were measured before initiation of protoplast isolation.

Donor wheat plants

Prior to germination of wheat, seeds were surface-sterilized by treating with absolute ethanol (1 min), 0.1% Mercuric chloride (3 min), 50% sodium hypochlorite (15 min) then rinsed three times with sterile de-ionized water. Wheat plants were grown for 8 days in 18-cm Schott glass tubes under continuous light and 22 °C.

Protoplast isolation

Isolation of maize protoplasts was carried out according to Mórocz et al. (1990), except that we used split incubation: 14-h (overnight) at +4 °C without shaking, followed by a 2-h treatment at room temperature with gentle (15 rpm) shaking.

Prior to wheat protoplast isolation, leaves of 8-day old plants were cut off under sterile conditions and immersed into 10 ml of solution A (Sarhan and Cesar 1988). The leaves were than laid onto 2–3 drops of the same solution in the middle of a Petri dish, and the epidermis was removed from the reverse side of leaves by a fine forceps. Mesophyll protoplasts were released by floating 2 g of leaf tissue for 4 h in 10 ml of digestion solution, which was prepared according to Sarhan and Cesar (1988) without heat treatment.

Protoplast fusion and culture

Isolated maize and wheat protoplasts were mixed at 2:1 ratio and suspended in 10 ml of UM solution (Uchimaya and Murashige 1974), than centrifuged in swing bucket rotor (3 min, 1000 rpm, room temperature). A dense suspension of (1.5×10^6/400 µl UM) protoplasts was incubated for 20 min in a single droplet of 10-mm diameter on a vibration-free place. One microliter of PEG solution was added in a very slow continuous flow to the protoplasts (40 w/v% of 3500 MW Sigma PEG, dissolved in solution D according to Kao and Michayluk 1974). Careful pipetting was important to avoid flotation of the protoplasts which adhered to the bottom of a 35-mm tissue-culture quality plastic Greiner Petri dish. Elution of the PEG solution started after the first fused cells had appeared (5–10 min) and was controlled under a microscope. The fusion mixture was eluted drop-wise with 10 ml of Kao C solution (Kao and Michayluk 1974). Finally the washing solution was replaced by 1 ml of ppN6M/89 culture medium (Mórocz et al. 1990). The effectiveness of the elution was controlled at each step by taking 50-µl samples and measured by on osmometer.

Depending on the amount of the isolated protoplasts, one to six PEG-treated and three non-treated control (maize, wheat and mixed protoplasts) cultures were established in several independent experiments.

Protoplasts were cultured in liquid ppN6M/89 medium at room temperature in the dark. To facilitate the positioning of visible hybrid cells, the cultures were embedded in agarose after the first divisions had appeared (fresh 1:1 rate-mixture of double-concentrated protoplast culture medium and 2.4% low-gelling-temperature agarose).

The developing small calli of 0.5–1 mm size (2 months after fusion) were transferred onto hormone-free N6M regeneration medium. The amount of de-differentiated and embryogenic calli was regulated by the frequency of subculture, using the same medium.

The differentiated well-rooted green plants with 6–10-cm shoots were removed from the Petri dish, cleaned from the rest of medium, washed with tap water, and transferred into soil. The young plants were covered with plastic bags in order to maintain high humidity during the first 10 days of adaptation to greenhouse conditions. Plants developing ears or tassels were self- and also cross-pollinated with other maize varieties.

Identification of the hybrids

The number of hybrid cells was counted under a light microscope immediately after fusion. The positions of the visible hybrid cells were marked on the bottom of the dish.

The chromosome number of the putative hybrid calli and plants revealed by their green color was determined by the Feulgen staining method.

The total DNA for RAPD analysis was extracted with the CTAB procedure (Bousquet et al. 1990). In the PCR reaction 10 ng of DNA was used in a 20-µl reaction mix containing 5 pmol of primer (Operon Alameda), 2 µl from a 2 mM dNTP solution (Boehringer Mannheim), and 1 unit of *Taq* polymerase (Boehringer Mannheim). DNA was amplified in a Hybaid Omnigene thermocycler at 94 °C for 1 min followed by 35 cycles each with 35 °C for 30 s, 72 °C for 1 min, 94 °C for 5 s with a final cycle of 35 °C for 30 s and 72 °C for 10 min. The reaction products were separated on a 2.0% agarose MP gel (Boehringer Mannheim) that was stained for 30 min in a 0.5% ethidium-bromide bath.

In situ hybridization

The albino-maize chromosome plates were prepared for in situ hybridization according to Kao (1982), with minor modifications. The protoplast isolation was carried out according to Mórocz et

al. (1990), and the cells were stained with acetocarmine. In the case of wheat and the hybrid plants we used a squash preparation (Molnár-Láng et al. 2000).

The GISH analysis was performed according to Reader et al. (1994). Total genomic DNA was isolated from wheat tissue (GK Öthalom) and fragmented by sonication to 1000–1500 bp. The labelling procedure was as follows: 5 µl of Nick translation buffer (0.5 M Tris HCl, pH 7.8; 0.05 M $MgCl_2$; 0.5 mg/ml of Bovine Serum Albumin), 5 µl of unlabelled nucleotide mixture (0.5 mM solution of each dCTP, dGTP, dATP in 100 mM Tris HCl, pH 7.5), 3.5 µl of fluorochrome-labelled nucleotide mix [1 µl of 0.05 mM dTTP, 2.5 µl of Fluorogreen (Amersham)]; and 1 µl of 100 mM dithiotreitol and 1 µg of sonicated wheat DNA were mixed. The volume was made up to 45 µl with sterile water; 5 µl of DNA polymerase/DNase I (Gibco) was added, and incubated for 3.5 h at 15 °C. At the end of the incubation, the enzyme activity was arrested with 5 µl of 0.3 M EDTA (pH: 8.0). Unlabelled maize DNA was shared by autoclaving for 20 min and used as a competitor in 30-times the quantity of the probe-amount in order to block common sequences in the hybridization step. Then 1/10 vol of 3 M sodium acetate, and 3 vol of ice-cold ethanol were added. Mixing of the contents was followed by precipitation at –80 °C for 1 h. The supernatant was removed after spinning the tubes, and the pellet was incubated for 30 min at 4 °C with 500 µl of 70% ethanol. After centrifuge and discarding the supernatant, the pellet was dried overnight. The pellet was then dissolved in 20 µl of TE buffer.

Fifty microliters of hybridization solution containing 20 µl of 25% dextran sulphate, 5 µl of 20 × SSC, 1.25 µl of 10% sodium dodecyl sulphate and 50 ng of labelled probe, together with the competitor DNA, were loaded per slide and incubated for 2 h at 65 °C. Following appropriate washes the slides were counterstained with DAPI (4′,6-diamino-2-phenylindole, 1 µg/ml).

The chromosomes were examined with a Zeiss Axioskop 20 epifluorescence microscope equipped with Filter 10 for FITC and a triple-band filter (25) set for DAPI. The images were captured with a SPOT CCD camera using the appropriate SPOT software (Diagnostic Instruments, Inc.) and processed with Image Pro Plus software.

Results

Protoplast isolation, fusion and culture

The protoplast yield ranged from 4 to 10×10^6 protoplasts for maize and $1–2 \times 10^6$ for wheat respectively. Despite our attempts to monitor cell growth, medium pH and osmolarity to optimize protoplast yield, the quality of maize protoplasts showed a variation that determined the effectiveness of fusion treatment. The well-conditioned maize protoplasts did not suffer from the standard fusion treatment, and the percentage of the fused cells reached a maximum of 20% in the most-successful experiment. The number of hybrid cells showed high variability among the independent experiments.

Hybrid protoplasts contained the mesophyll cell-derived chloroplasts as well as cytoplasm strands from the suspension counterpart (Fig. 1C). The number of viable hybrid cells decreased below 2% before reaching the first division during the 1st week of culture.

The hybrid cells started to divide usually on the 10th day, 5–7 days later than the intact maize protoplasts (Fig. 1D). The wheat chloroplast in the maize cytoplasm remained visible for 3–4 weeks. After this period the dividing fusion products were not distinguishable from the non-fused ones.

Regeneration of green plants

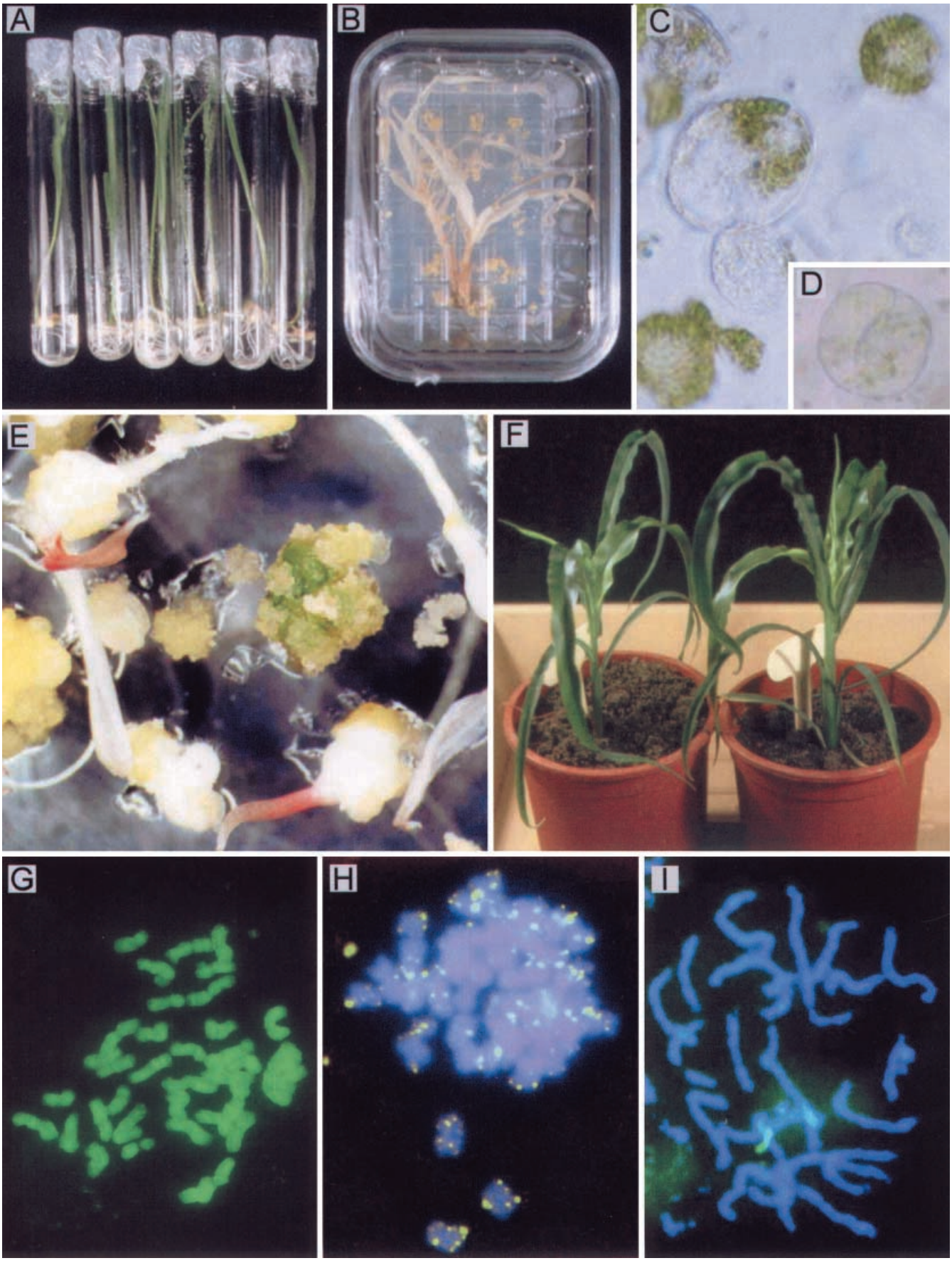
Seven embryogenic calli with green spots were selected as putative hybrids from three separate fusion experiments. The first differentiated green embryo appeared 6 months after fusion treatment but failed to develop into a plant. Increasing the time of subculture intervals from 3 to 4–5 weeks produced embryogenic structures (Fig. 1E), which were already suitable for plant regeneration. One callus-clone yielded green plants, which showed an improved morphological appearance between 6 and 12 months of the subculture, but fertility was not achieved. Green plants were obtained only from the PEG-treated mixture of parental protoplasts.

Analysis of the hybrids

The recovered green plants exhibited a maize morphology (Fig. 1F). The cytological analysis of the green callus showed a variable (47–56) chromosome number among the samples. These preparations allowed us to predict the chromosome number at the pro-metaphase stage rather than for the desired metaphase chromosomes. The root-tip cells of regenerated plants carried 56 chromosomes. The significant differences between maize and wheat chromosomes in size and morphology provided a solid basis for the conclusion that intact wheat chromosomes were not present in these plants.

The hybrid nature of the regenerated maize plants was first determined from the restoration of a green phenotype in the leaves. The DNA analysis of the various genotypes was based on RAPD analysis. Out of 30 primer combinations, OPA 07, 09 and 10 produced RAPD bands. As shown by Fig. 2A–C these primer combinations generated PCR products characteristic for both parents. In an independent analysis similar-size PCR fragments could be produced reproducibly when the total DNA was used as a template from the selected green tissues.

In a search for additional molecular evidence for the presence of wheat-specific DNA in the genome of the putative hybrid, genomic in situ hybridization was carried out on chromosomes of the regenerated green maize plants. For these studies we used total wheat-DNA labelled with fluorochrome. As shown by Fig. 1G and I, the hybridization conditions used allowed a clear discrimination of wheat and maize DNAs. Fluorescence in situ hybridization of hybrid chromosomes clearly showed the presence of wheat DNA in the maize background (Fig. 1H). Considering the number and size of signals, we postulate the integration of a significant amount of wheat DNA into the recipient maize genome. The presence of larger chromatin islands with wheat DNA reflects the fragmentation of wheat nuclei in the fused cells. Since during preparation of the hybridization probe repetitive DNAs are expected to be labelled we suggest that primarily non-coding sequences were introduced from the donor wheat genome. Figure 1F shows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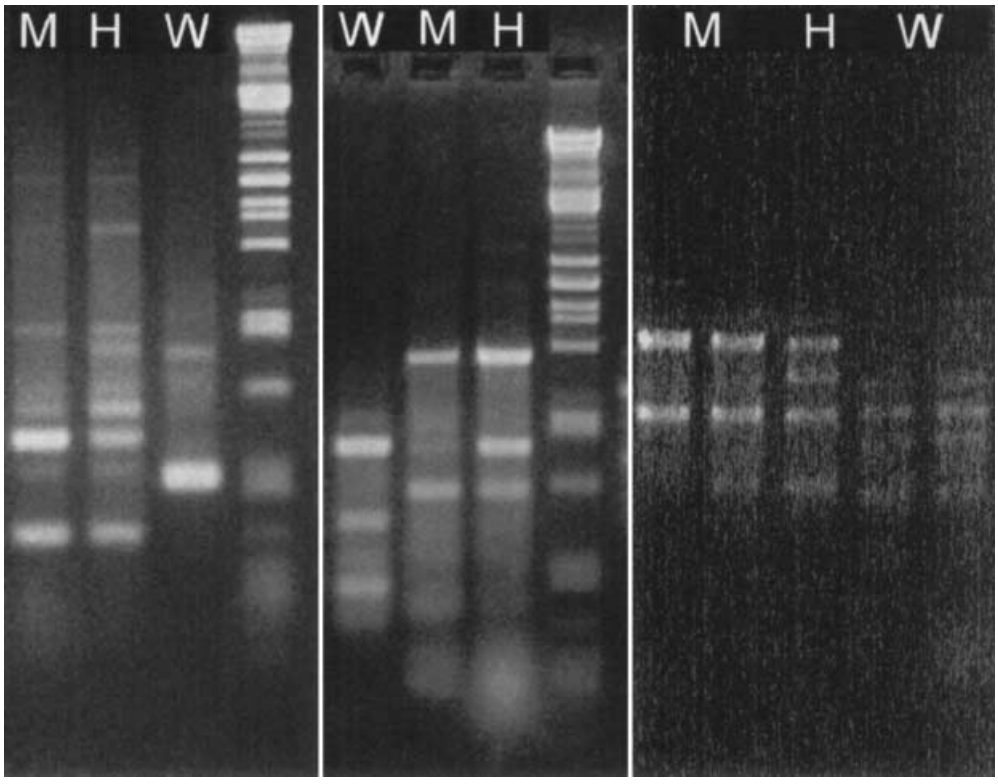

Fig. 2 RAPD patterns of the parental plants (M: maize, W: wheat) and putative hybrids (H). **Left**: OPA07 primer combination **Middle**: OPA09 primer combination **Right**: OPA10 primer combination

regenerants from the green fusion products. The overall phenotype of these plants resemble those of the maize parents. Obvious morphological traits from the wheat parent can not be recognized at the morphological level. However, we can not exclude the transfer of a functional gene; therefore, further experiments might be required by the use of a transcriptional profiling approach with DNA chip technology.

Discussion

Due to incompatibility, the production of sexual hybrids between wheat and maize resulted in limited successes (Laurie and Bennett 1988; Inagaki and Tahir 1992). In addition, in these cases maize chromosomes were eliminated after pollination of the wheat parent. Since the combination of agronomic traits from these species could have considerable significance in crop improvement we attempted the production of somatic hybrids. This technology has been successfully used for gene transfer even between phylogenetically distant species (Kisaka et al. 1997). A majority of parasexual hybridizations was reported for dicot species (Crea et al. 1997). The progress in monocot hybridization studies is much more restricted, mainly because of difficulties in the of culture of cereal protoplasts and designing an efficient selection and regeneration system.

The availability of a morphogenic albino-maize suspension culture in our laboratory stimulated fusion experiments. The genetic nature of the albino phenotype is not known.

Repeated plant regeneration attempts from this culture during the years failed to result in green revertant. Nevertheless we can not exclude completely the possibility of spontaneous reversion of albino phenotype.

The early microscopic observations clearly showed the presence of dividing fusion products with mixture of maize cytoplasm and wheat chloroplasts in the cultures after PEG treatment. Out of the few hundred fusion products we were able to recover only seven green callus tissues and among them only a single one was regenerable. These frequency data clearly show, that a very rare event can result in development of hybrid callus tissues with restored albino defect. Similar low efficiency in hybrid formation was characteristic for most cases after fusion of distantly related species (Kisaka et al. 1997).

The availability of a single hybrid makes very difficult to interpret the molecular and cytological processes

◀ **Fig. 1** Restoration of green phenotype by somatic hybridization between albino maize and wheat leave protoplasts. The genomic in situ hybridization indicates the presence of wheat DNA in the maize chromosomes. **A**: GK Öthalom seedlings, used for protoplast isolation **B**: H1160 albino plants differentiated on 2,4D-free N6M surface culture **C**: Hybrid cells that carry mixed cytoplasm, are clearly distinguishable from both types of parental protoplasts **D**: Hybrid cells that underwent division **E**: Green dedifferentiated callus developed from fused protoplast cultures and used for plant regeneration **F**: Putative hybrids grown in soil **G**: Chromosomes of wheat parent after in situ hybridization with labelled total wheat DNA **H**: Chromosomes of hybrid plant after in situ hybridization with labelled total wheat DNA **I**: Chromosomes of maize parent after in situ hybridization with labelled total wheat DNA

that can results in the formation of nuclear or cytoplasmic hybrids. The present observation that a prolonged in vitro culture period improved the viability and regeneration potential is in a good agreement with the early studies on carrot hybrids (Dudits et al. 1979, Dudits et al. 1980). This might be related to chromosomal loss or rearrangements. The extended influence of incompatible responses was evidently shown by the lack of normal function of sexual organs. Up to now we were able to propagate this genotype only in vitro cultures. Therefore we might expect a continuous alteration in genomic constitution of these plant materials. In addition to the phenotypic characterization showing maize traits, the chromosome studies were expected to provide insight to the genetic nature of the selected green tissue and plant material. Since the wheat and maize chromosomes are significantly different in size and morphology we can safely state that the selected tissues carry only maize chromosomes. The actual chromosome number was found to be 56 in the green regenerant. Fusion of more than two protoplasts was frequently observed in the mixture of different cells during PEG treatment. This observation can explain the high chromosome number in the analyzed cells. The failure of recognition of intact wheat chromosomes emphasized the need for the additional molecular tools to uncover the origin of selected genotype.

The RAPD analysis indicated the presence of wheat specific sequence elements. The size of amplified PCR fragments suggested the combination of the two parental DNA representing either nuclear and/or organelle markers.

The existence of wheat DNA in the genome of these plants was visibly shown by in situ studies. Since total wheat DNA was labelled we can suggest that repetitive sequence elements were integrated into the maize genome.

Considering the high number of signals distributed to several chromosomes we can predict an extensive rearrangement between the parental genomic DNAs. In interpretation of the origin of genomic constitution we can rely on the results of early studies on fusion between mitotic and interphase plant protoplasts (Szabados and Dudits 1980.) The premature chromosome condensation (PCC) can cause complete fragmentation of interphase nucleus. The cytological pictures showed formation chromatin droplets that can be incorporated into the nuclear DNA of the hybrid cells during the subsequent division cycles. However the S-phase related PCC is expected to occur only in very rare cases the above cytological events might provide a hypothetical explanation for the formation of DNA islands from wheat.

Despite of the fact that unique, unknown molecular and cellular events produced the described new genotype with maize and wheat DNA, the regenerated plants exhibit several potentials for applications in functional genomic and stress research. Further studies are in progress to search for expression of wheat specific genes or characters.

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