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Asymmetric somatic hybridization between wheat (*Triticum aestivum* L.) and *Agropyron elongatum* (Host) Nevishi

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Abstract Suspension-derived protoplasts of *Agropyron elongatum* irradiated by ultra-violet light (UV) were fused with the suspension-derived protoplasts of *Triticum astivum* using PEG. Fertile intergeneric somatic hybrid plants were produced and various hybrid lines have been selected and propagated in successive generations. Their hybrid nature was confirmed by analysis of profiles of isozymes, RAPDs, and 5S rDNA spacer sequences, and via GISH analysis. By the procedure described, the phenotype and chromosome number of wheat could be maintained besides transfer of a few chromosomes and chromosomal fragments from the donor *A. elongatum*. The results above indicated that highly asymmetric fertile hybrid plants and hybrid progenies of wheat were produced via somatic hybridization.

Keywords *Triticum aestivum* · *Agropyron elongatum* · Ultra-violet light · Fertile somatic hybrid plants and progenies · GISH

Introduction

Somatic hybridization offers the possibility of circumventing barriers to sexual reproduction and allows for the improvement of a crop species by the transfer of agronomically relevant traits (Glimelius et al. 1991). As technical improvements have been made in hybrid induction, more and more interspecific and intergeneric fertile hybrids have been created (Waara and Glimelius 1995; Li et al. 1999), but it remains difficult to produce fertile hybrids involving widely separated Gramineae species. Thus, for example, in the combination *Saccharum officinarum* (Sugarcane) + *Pennisetum americanum* (pearl millet), only somatic embryos could be produced (Tabaeigadeh et al. 1986); while for *Oryza sativa* (rice) + *Echinochloa* (barnyard grass), only short-lived seedlings (1 cm in height) were recovered (Terada et al. 1987). Flowering festulolium (*Festuca arundinacea* + *Lolium multiflorum*) somatic hybrid plants represent the first example of mature hybrids within the *Gramineae* (Spangenberg et al. 1995). These plants were able to set seed following artificial pollination with *L. multiflorum*. Recently, fertile hybrids have also been obtained from the combination rice + barley (Kisaka et al. 1998), and rice + *Zizania latifolia* (Griseb) (Liu et al. 1999). For wheat, work carried out in our laboratory has generated intergeneric hybrid plants (Xia and Chen 1996; Zhou et al. 1996, 2001b) which could flower, but could not set seeds.

Agropyron elongatum (2n = 70) is a perennial forage grass characterized by interesting levels of tolerance to abiotic stress, resistance to some important diseases of wheat and a high seed protein content. Derivatives of sexual hybrids between wheat and *A. elongatum* have been used to generate high quality wheat cultivars (Li 1995). However the necessary chromosome engineering techniques are tedious and time consuming. The present report describes a somatic hybrid route to introgression, which will not only be more efficient than sexual crossing, but will also allow the exploitation of non-nuclear genes from both parents, which is not possible by the sexual route.

Materials and methods

Origin of wheat protoplasts

Protoplasts of common wheat (cv Jinan 177, 2n = 42) were prepared from cell suspensions which divided at a high frequency but were not capable of regenerating into whole plants. The methods for establishing suspensions and preparing protoplasts have been described previously (Xia et al. 1995; Xia and Chen 1996). The chromosome number of 90% cells varied from 2n = 30 to 2n = 38.

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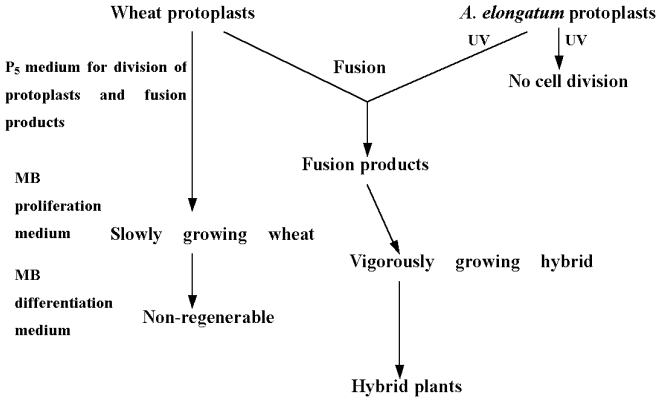

Fig. 1 Protoplast fusing and selecting procedure

Origin and UV treatment of A. elongatum protoplasts

Young embryos of *A. elongatum* (2n = 70) were used for the establishment of calli. After 2 years of subculture, yellow/white, loose, fast-growing structures with a chromosome number of 50–60 were used as a source of protoplasts. The methods for callus and suspension establishment, and for protoplast preparation, were the same as for wheat. The calli and suspensions were non-regenerable, and the protoplasts did not divide under the conditions used in this experiment.

Newly prepared protoplasts were placed in small Petri dishes and care was taken to ensure that cell density was adjusted to permit the formation of a monolayer on the bottom of the Petri dish. These protoplasts were exposed to ultraviolet light at an intensity of 380 μW cm^{-2} for 0.5, 1, 3 and 5 min respectively, and washed twice with 0.6 M mannitol +0.5 M $CaCl_2$.

Selection strategy

The procedure for hybrid production and selection was as follows (Fig. 1).

Fusion and culture procedure

For fusion, both the wheat and UV-irradiated *A. elongatum* protoplasts were adjusted to a density of 1×10^6 ml^{-1}, mixed thoroughly in a ratio of 2:1 and fused using the PEG method (Lindsey 1991). The following fusion combinations were carried out: wheat protoplasts (T) + *A. elongatum* protoplasts (A), the latter receiving UV irradiation of 380 μW cm^{-2} for 0.5 min (I), 1 min (II), 3 min (III) and 5 min (IV). For each combination, the following controls were cultivated: T, A, T (+) A (mixture), T (+) T and A (+)A. The fusion products were cultured with P_5 and then MB medium was developed for wheat protoplast culture (Xia et al. 1995). The former contains 1 mg·l^{-1} of 2,4-D, and the latter 1 mg·l^{-1} of 2,4-D and 0.5 mg·l^{-1} of zeatin. Fast-growing clones were transferred on to regeneration medium (MB containing 0.5 mg·l^{-1} each of indo-3-acetic acid and zeatin). To encourage rooting 2–3-cm-high plantlets were transferred to MB medium containing 1–3 mg·l^{-1} of pacclobutrozol and 0.5 mg·l^{-1} of naphthalene acetic acid, and cultured under fluorescent light at 2000 lx for 12 h per day at 4–12 °C before being potted into soil about 3–4 weeks later.

Culture of hybrid ovaries

Ovaries derived from hybrid plants were induced to form calli on MB medium containing 2 mg·l^{-1} of 2,4-D followed by subculture every 15–20 days. When the calli turned light yellow and granular about 2 months later, they were transferred to differentiation medium to regenerate plants (F_0) and then transplanted into soil as described above.

Identification of putative hybrid calli and plants

Isozyme analysis

The soluble proteins of the regenerated calli and the leaves of hybrid plants and parents were extracted and separated on polyacrylamide gels. Esterase, peroxidase and malic dehydrogenase isozymes were analyzed as described elsewhere (Xia and Chen 1996).

RAPD analysis

Genomic DNA was isolated from young leaves of putative hybrid plants and parents according to Doyle and Doyle (1990). Informative RAPD primers were OPJ-04 (CCGAACACCG), OPJ-05 (CTCCATGGGG), OPJ-09 (TGAGCCTCAC), OPJ-11 (ACTCCTGCGA), OPJ-12 (GTCCCGTGGT), OPJ-13 (CCACACTACC), OPJ-14 (CACCCGGATG), OPB-08 (TGGACCGGTG) and OPB-09 (CTCACCGTCC). Methods for the production of RAPD profiles followed Xia et al. (1998).

5S rDNA spacer sequence analysis

Total DNA of F_1 lines was isolated as above. Two pairs of primers (p^I + p^{II}; p^{III} + p^{IV}) were used to amplify the spacer regions, following the methods of Zhou et al. (2001a). The sequences of these primers were: p^I. 5'-GGTAGTACATCGATGGG; p^{II}. 5'-GGAGTTCTGACGGGATCCGG; p^{III}. 5'-GGATGGGTGACCTCCCGGGAAGTCC and p^{IV}. 5'-CGCTTAACTGCGGAGTTCTGATGGG.

Cytogenetical analysis of the hybrids and progenies

Chromosome analysis of the calli was performed according to Xia and Chen (1996). Root tips were excised, pretreated in water (4 °C) for 24 h, fixed in 3:1 ethanol:glacial acetic acid for 2 h at room temperature, rinsed in water for 15 min and incubated in an enzyme mixture [1.5% cellulase (Onozuka R-10), 0.1% Pectolyase Y-23] in 0.6 M mannitol, 5 mM $CaCl_2$, pH 5.8 at 25 °C for 0.5–1 h. The partially digested root tips were gently rinsed in water for 15–30 min, fixed in 3:1 ethanol:glacial acetic acid for 15–30 min and then pressed for chromosome spreading on a glass slide. In situ hybridization was performed following the methods described by Zhou et al. (2001a).

Results

Fusion of wheat and *A. elongatum* protoplasts and culture of the fusion products

Wheat protoplasts appear transparent with dense cytoplasm, while *A. elongatum* ones contain abundant starch grains (Fig. 2a). About 4 h after fusion some hybrid cells could be recognized from their spherical shape (Fig. 2b). The cultures of combination I generally entered first division after 4–5 days. After 2 weeks in culture, some embryo-like structures (absent in the other combinations and controls) and many compact cell clumps were found, and some of these reached a size of 1–1.5 mm after 1 month of culture. Fourteen such clumps were transferred to a solid medium in the first lot, but only three

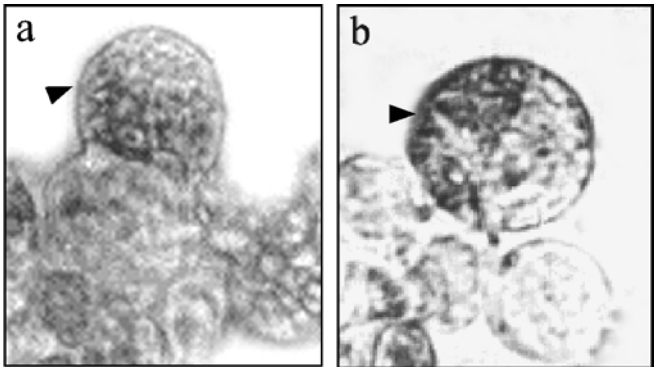

Fig. 2a, b Protoplast fusion. **a** Fusing protoplasts of wheat and *A. elongatum*. ▲: *A. elongatum* protoplast. **b** A fused cell between wheat and *A. elongatum*. ▲: cytoplasm of *A. elongatum*

regenerated normal green plantlets. Two clones produced albinos, one gave rise to short-lived seedlings, while others showed only green sports. Later lots of clones from combination I and the clones from combinations II and III grew slowly, and were all non-regenerable. No clones were observed from combination IV.

In all combinations, the control protoplasts showed either no sign of cell division or produced only small calli from which no plants differentiated. A total of 29 regenerated green plants were obtained in this experiment. Five of these survived and grew to the flowering stage, although more slowly than normal wheat. Stems and ears were wheat-like, but none were self-fertile. All dissected flowers had the normal complement of three stamens and a normal-looking pistil. It is possible that the sterility of these hybrids was due to the unfavorable climatic factors for flowering, rather than to any physiological or genetic defect.

Identification of hybridity of regenerated plants

Morphology

All regenerated plants were wheat-like, but showed traces of *A. elongatum* characteristics. The wheat ligule is long and broad, while that of *A. elongatum* is short with hairs on the top; the hybrid had no detectable ligule. The auricle of wheat is long with scattered hairs, while that of *A. elongatum* is small and sharp with thick hairs; the hybrid auricle is short, somewhat like *A. elongatum* but hair-less (Fig. 3). These characters were especially visible at the seedling stage.

Isozyme patterns

The hybrid nature of all the early formed clones (I1–I6) were identified by their esterase profiles, and the regenerated plants in addition by both malic acid dehydrogenase and peroxidase profiles (Fig. 4a, b). Only about 30% of the slowly growing calli showed esterase isozyme

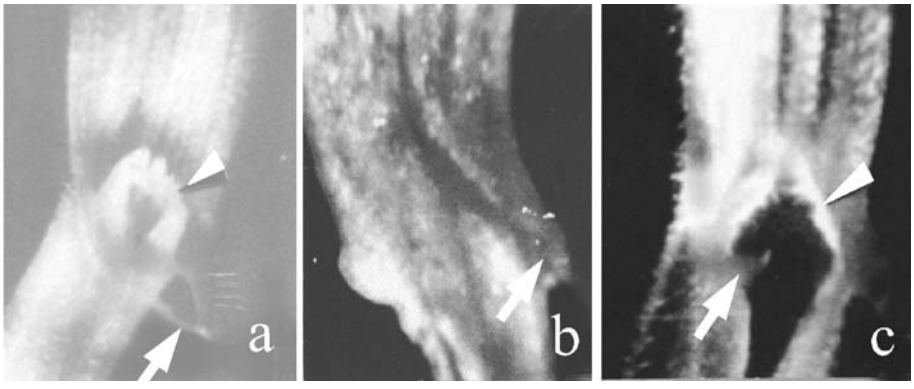

Fig. 3a–c Appearance of ligules and auricles on hybrid plants and parents. **a** Wheat. **b** Middle: hybrid. **c** *A. elongatum*. ▲: ligule, ↑: auricle

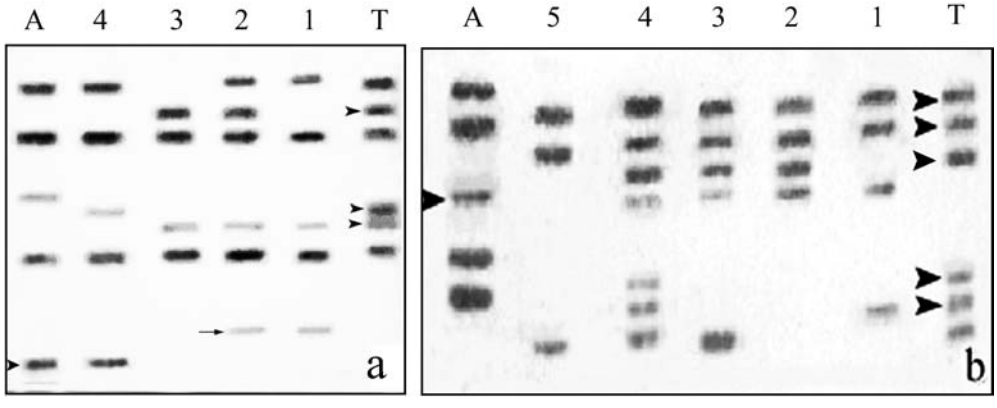

Fig. 4a, b Isozyme patterns of peroxidase and malic dehydrogenase in hybrid plants and the parents. *T*: wheat; *A*: *A. elongatum*; *1,2,3,4,5*: hybrid plants from the No. I1–I5 clone. ▲: specific band of parents. ↑: new band. **a** Peroxidase pattern. **b** Malic dehydrogenase pattern

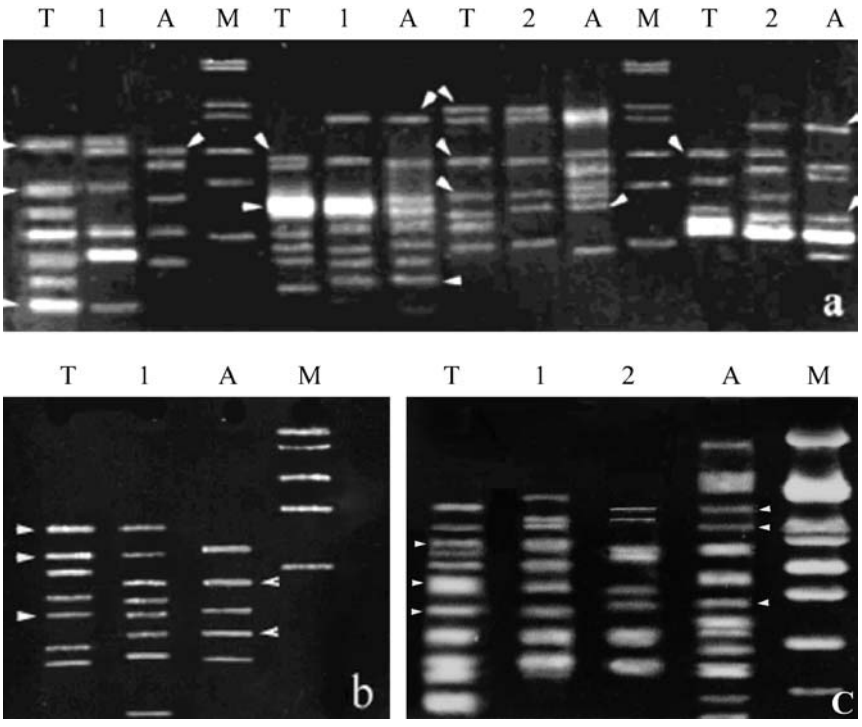

Fig. 5a–c RAPD profiles of hybrid plants and the parents. *T*: wheat; *A*: *A. elongatum*; 1, 2: hybrid plants. *M*: DNA molecular-weight marker. ▲: parental fragment. **a** Primer OPJ-11~OPJ-14. **b** Primer OPB-08. **c** Primer OPJ-04

band(s) of both parents, but all of these were non-regenerable. This analysis indicated that only vigorous growing calli were hybrid in nature and had the capacity to regenerate whole plants.

RAPD analysis

Figure 5a, b, c show the RAPD profiles using the primers OPB-08, OPJ-04, -11, -12, -13, -14 for wheat, *A. elongatum* and hybrid plants. The results confirm that the hybrid plants carried DNA from both parents.

Chromosome counting

Over 90% of the chromosome numbers of the calli derived from wheat protoplasts were in the range of 30–38, while that in the root tips of the hybrids were 40–46 and chromosome fragments or small chromosomes could be observed in most of the hybrid cells.

From the above observations, and given that neither of the parental protoplasts were regenerable, we concluded that the plantlets obtained were asymmetric hybrids.

Hybrid progeny production and their characteristics

Fertile plant regeneration from hybrid ovaries

None of the regenerated plants set selfed seeds, although their flowers appeared to be morphologically normal. In order to obtain further generations, ovary culture was employed to induce plant regeneration. A total of 23 F_0 seedlings from ovary derived clones were obtained, of which six grew to maturity. The plants grew slowly under natural spring conditions and were stunted in height (about 20 cm tall). Their phenotypes were largely wheat-like (Fig. 6a). Two plants (9%), originating from independent clones, set several seeds later than did the parent wheat. Hybrid seeds were smaller than those of common wheat, but were normal in shape and plumpness (Fig. 6b). These seeds gave rise to healthy mature F_1 plants of varying phenotype.

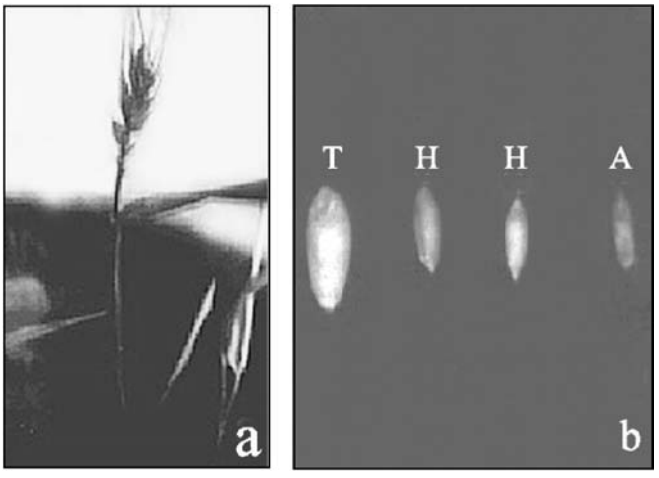

Fig. 6a, b Plant and seed phenotype of hybrid plants. **a** One F_0 mature plant. **b** Seeds of F_0 hybrid (*H*), wheat (*T*) and *A. elongatum* (*A*)

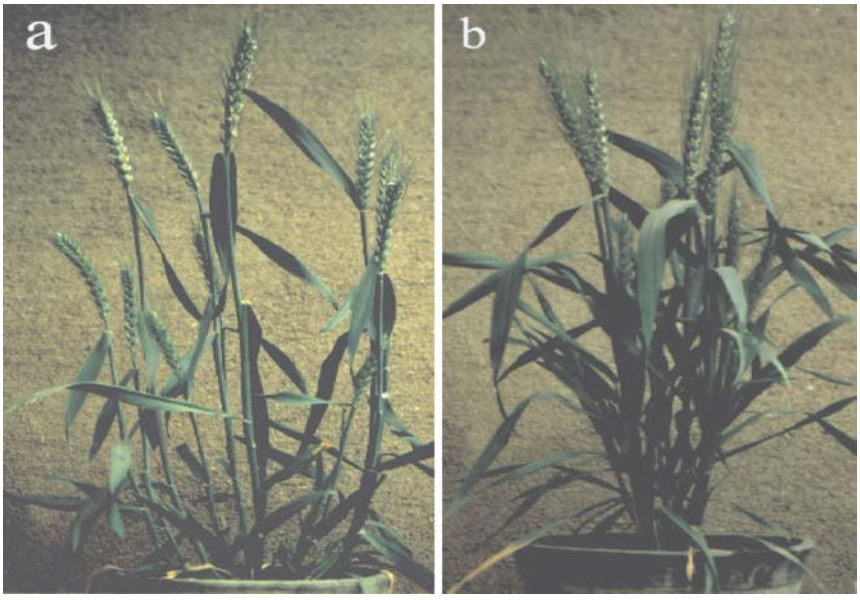

Fig. 7a, b F_1 hybrid mature plant from the small seed. **a** Hybrid I-1. **b** Hybrid II-1

Morphology of hybrid progeny

Two distinct phenotypes were observed among these progenies: type I had taller stems (75–85 cm) with large ears and grains (Fig. 7a), while type II had short stems (average 55 cm) with a strong tillering ability, smaller ears and grains (Fig. 7b). Through propagation and selection from F_2 to F_5, both hybrid lines were stabilized for morphology.

5S rDNA analysis

Variation in 5S rDNA spacer length was used to analyze nuclear DNA of the hybrid progenies at two specific loci. Hybrid DNA profiles consist of different combinations of fragments from each parent (Fig. 8a, b), demonstrating the hybridity of the progenies, which demonstrates the existence of parental DNA in the hybrid progenies.

GISH analysis of F_2 and F_5 hybrid plants

From GISH karyotypes of the different hybrid lines, variation in somatic chromosome number was in the range of 38–44, about 70% of which showed 2n = 42. This included 0–2 chromosomes or chromosomal fragments of *A. elongatum,* either as entire *A. elongatum* chromosomes and/or as translocated (intercalary or terminal) wheat/*A. elongatum* chromosomes (Fig. 9a, b).

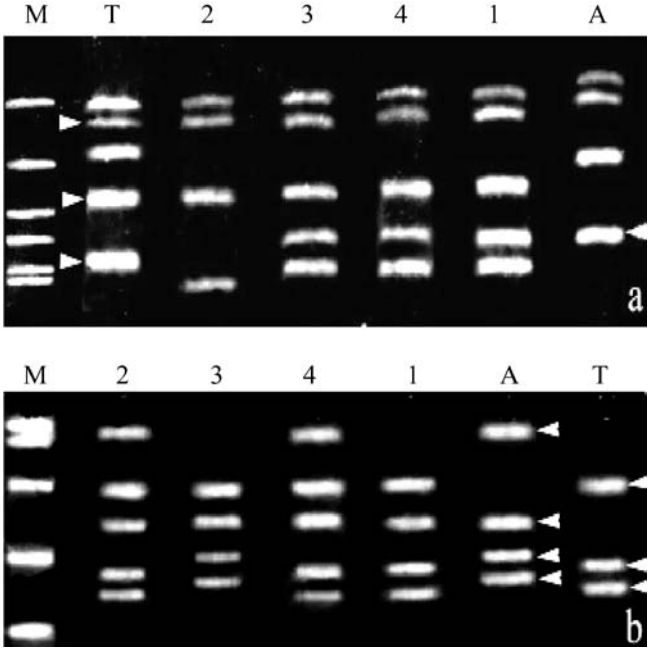

Fig. 8a, b 5S rDNA profiles of hybrid plants and the parents. *T*: common wheat cv Jinan 177, *A*: *A. elongatum*, *1–4*: hybrid plants. ▲: specific band of parent. **a** Primer $p^{I} + p^{II}$. **b** Primer $p^{III} + p^{IV}$

Discussion

Selection and regeneration of asymmetric somatic hybrids of wheat × *A. elongatum*

The commonly used method for selection of asymmetric somatic hybrids involves irradiation (χ-, γ-ray or UV light) of the donor (Imamura et al. 1987; Famelaer et al. 1989; Vlahova et al. 1997; Forsberg et al. 1998) and chemical treatment (such as the metabolic inhibitor IOA)

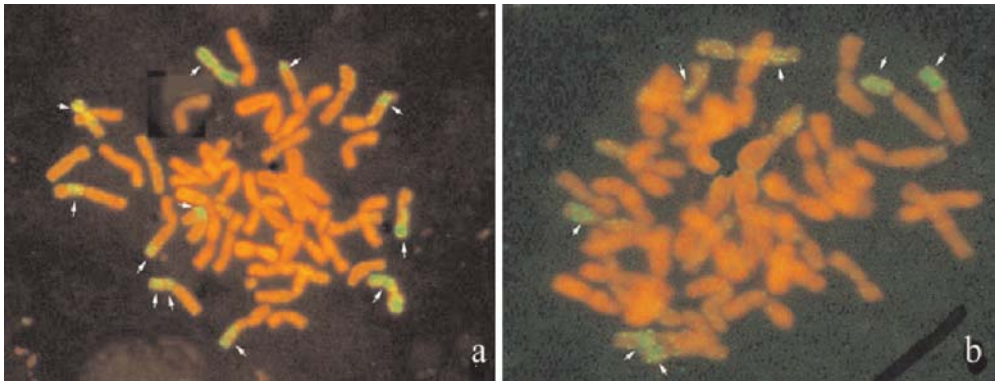

Fig. 9a, b GISH karyotypes of two hybrid progenies. **a** F_2 II-1–4 hybrid plant showing two *A. elongatum* chromosomes and some translocations and insertions of chromosomal fragments of *A. elongatum*. **b** F_5 I-1–5 showing translocations between parent chromosomes. ↑: chromosome or chromosomal fragment/arm of *A. elongatum*. (*green–yellow colour* indicative of *A. elongatum* origin)

of the acceptor. However, such treatments inevitably damage the growth and development of some resulting hybrid cells (Hansen and Earle 1997; Zhou et al. 1996, 2001b; Xia and Chen 1996). An ideal selection system should involve minimum harm to hybrid cells. The restoration of morphogenetic potential in fusion products was a good selection system, which was demonstrated in *Arabidopsis thaliana*/*Brassica campestris* fusions (Gleba et al. 1984), and was also found in our wheat/*A. elongatum* hybrids. Somatic hybridization in Gramineae species has proven to be problematic, partly because of the difficulty in establishing embryogenic calli or cell suspensions as a source of protoplasts for fusion, and partly because it is hard to maintain embryogenic potential during cell subculture. Thus the strategy of using complementation of regeneration for the hybrid cells can also overcome a major block in hybrid plant production in wheat + grass combinations.

Effect of UV on chromosome fragmentation and elimination

Production of highly asymmetric somatic hybrids with a few chromosomes, or chromosomal fragments of the donor species, simultaneously overcomes both hybrid sterility (Dudits et al. 1987; Hinnisdales et al. 1991) and the introgression of too much exotic genetic material into the crop species. Beside the ionizing radiation, UV has also been used to induce production of asymmetric somatic hybrids. Fertile asymmetric hybrid plants were produced between *Nicotiana plumbaginifolia* and UV irradiated *Lycopersicon esculentum* (Vlahova et al. 1997). Forsberg et al. (1998) obtained highly asymmetric fertile hybrid plants between *Brassica napus* and UV-treated *A. thaliana*, and confirmed that DNA elimination was UV dose-dependent. Using GISH analysis in our experiments, it was clear that UV induced both donor chromosome elimination and fragmentation, and so could generate novel chromosomes, including intercalary translocations (Fig. 9a). Such chromotypes are particularly interesting in the context of alien introgression, since they can incorporate a minimal amount of alien chromatin. In contrast, introgression based on recombination or meiotic chromosome breakage tends to generate only single-breakpoint chromotypes, and a round of intercrossing overlapping introgression lines is required to generate intercalary introgressions.

Somatic hybridization in wheat breading

Many hybrids between common wheat and a number of its closely related grass species have been obtained by sexual hybridization, and some of these have been used as a basis for cultivar development (Prem and Ravindra 1999). However, poor fertility in early generations due to unbalanced chromosome number, and the absence of recombination between wheat and non-wheat chromosomes, makes this a cumbersome route for introgression. In contrast, somatic hybridization offers a quicker strategy for the introduction of novel traits (Yue et al. 2001). A further advantage of the somatic hybridization strategy is that it widens the range of donor species beyond those which are sexually compatible; as an example, we have reported the production of asymmetric hybrid plants between wheat and oat (*Avena sativa L.*) showing only one or two oat chromosomes, as well as lines only carrying oat chromosome fragments (Xiang et al. 2002). Following somatic hybridization, many hybrid plants with different genetic compositions can be obtained from one fusion experiment. Additionally, in contrast to sexual hybrids, somatic hybrids will include elements of both the nuclear and cytoplasmic genomes of the fusion parents, and this has the potential to derive novel variability. Some important traits are controlled by cytoplasmic genes, in particular cytoplasmic male sterility (CMS), and asymmetric somatic hybridization has been used successfully to transfer a wild abortive CMS gene into rice (Bijoya et al. 1999). Mixed-origin mitochondrial genes have been verified in the somatic hybrids of wheat + *Haynaldia*

villosa (Zhou et al. 2001a) and other combinations of wheat + grass (unpublished data).

Phenotyping of the hybrid lines is underway, and preliminary data indicate that some lines show positive effects on flour quality and stress tolerance. For example, type I and type II hybrid lines we have described above show elevated seed protein content (about 17–22%) compared to that of the parent wheat (about 14%). The type II line appears to be of high quality for bread making (according to tests conducted by the Quality and Resource Test Centre of the Agricultural Science Academy of China), and type I has potential for enhanced yield and salt tolerance.

In conclusion, we have obtained highly asymmetric somatic hybrid plants and wheat genotypes with some specific traits via asymmetric hybridization, and we propose that asymmetric protoplast fusion between wheat and intergeneric grasses could be a promising tool for wheat introgression.

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