Intermediate fertile *Triticum aestivum* (+) *Agropyron elongatum* somatic hybrids are generated by low doses of UV irradiation

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ABSTRACT

We report the production and characterization of somatic hybrids between *Triticum aestivum L*. and *Agropyron elongatum* (Host) Nevishi (the synonym is *Thinopyrum ponticum*). Asymmetric protoplast fusion was performed between *Agropyron elongatum* protoplasts irradiated with a low UV dose and protoplasts of wheat taken from nonregenerable suspension cultures. More than 40 green plantlets were obtained from 15 regenerated clones and one of them produced seeds. The phenotypes of the hybrid plants and seeds were intermediate between wheat and *Agropyron elongatum*. All of the regenerated calli and plants were verified as intergeneric hybrids on the basis of morphological observation and analysis of isozyme, cytological, 5SrDNA spacer sequences and random amplified polymorphic DNA (RAPD). RFLP analysis of the mitochondrial genome revealed evidence of random segregation and recombination of mtDNA.

Keywords: *Triticum aestivum*, *Agropyron elongatum*, somatic hybridization, mitochondrial genome.

INTRODUCTION

To keep pace with the continuing increase in the world population, a sufficient food supply can only be achieved by various improvements in the productivity of major cereal crops, including wheat. Somatic hybridization has the potential to be used as a plant breeding technique due to the possibility that it affords to combine sexually incompatible species, and to transfer both nuclear and cytoplasmic gene(s) simultaneously. However, only limited success has been achieved to date in Gramineae[1-5] and especially in wheat [6-8]. *Agropyron elongatum* is a perennial grass that expresses certain traits that would improve wheat for protein content, disease resistance and particularly salinity tolerance. The hybrids of *Triticum aestivum* and *A. elongatum* produced by sexual crossing have been previously reported[9-10]. But the traditional work is tedious and time consuming. This disadvantage may be overcome by asymmetric somatic hybridization. Additionally, in contrast to sexual hybrids, somatic hybrids can include cytoplasmic genomes of the fusion parents and this has the potential to derive novel variability[2, 8]. Two sorts of somatic hybrid progenies have been obtained from asymmetric and symmetric fusions of wheat (+) *A. elongatum*. One (from symmetric somatic hybridization[11]) resembled *A. elongatum*, and the other (from asymmetric somatic hybridization[8]) resembled wheat. In the present work,

we performed asymmetric protoplast fusion with a lower dose of UV radiation of the donor protoplasts. It is interesting that the phenotype of the regenerated hybrids obtained is intermediate between wheat and *A. elongatum* with chromosomes ranged from 58-64. The chromosome number of the hybrids obtained from the asymmetric hybridization using higher UV dosage ranged from 40-44, and most of them had a normal wheat chromosome number (2n=42)[12], which gave evidence on the effect of UV dosage on the donor chromosome elimination in somatic hybridization. In addition, we confirmed the hybrid status of the regenerated calli and plants with biochemical and molecular markers. This type of hybrid can't be obtained by sexual progress and provide a new germplasm for grass and wheat breeding.

MATERIALS AND METHODS

Common wheat cv. Jinan 177 (2n=6x=42) and *A. elongatum* (2n=10x=70) were used as fusion parents. Suspension cultures of wheat were prepared from an embryogenic callus line that has been maintained in long-term culture, as previously described[13]. The induction, sub-culture and selection of embryo-derived calli of *A. elongatum* have been described elsewhere[11]. Protoplasts were isolated from non-regenerable suspension cultures of wheat and embryogenic calli of *A. elongatum* as previously described[11,13]. After isolation and purification, monolayer protoplasts of *A. elongatum* were placed on 3 cm petri dishes, and UV-irradiated at an intensity of 260 $\mu W/cm^2$ for 30s. Protoplast fusion and culture were carried out as described by Xia et al[7].

Chromosome numbers in metaphase cells of the fusion parents

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and regenerated calli were counted in chromosome spreads. For this, freshly regenerated calli of the parents and putative hybrids were excised and pre-treated in water (0ºC) for 24 h, and then fixed in ethanol: glacial acetic acid (3:1) for at least 12 h. After rinsing in water for 20 min, they were incubated in an enzyme mixture (1% cellulase Onozuka RS, 0.3% pectolyase Y-23, 0.6M mannitol, 5 mM CaCl$_2$, pH 5.8) at 37ºC for 45min, rinsed in water gently for 30 min and then fixed in 70% ethanol. The material was transferred to a clean slide and squashed to spread the chromosomes. The chromosome spreads were stained by standard aceto carmine methods.

Post-fusion products were genotyped by analysis of profiles of isozymes, RAPDs, specific PCR (5SrDNA spacer) and organellar RFLPs. Esterase and peroxidase profiles were generated from PAGE in 4% -10% gels, following the procedures of Xia *et al* [7]. For PCR and RFLP, DNA was extracted from calli or leaves of putative hybrids and parents according to the modified CTAB method. RAPD reaction mixtures consisted of 10×PCR buffer; 20 µM dNTPs; 2.5 mM MgCl$_2$; 20 ng primer (Operon Technology, USA); Ampli Taq polymerse 1U and 50 ng of genomic DNA in a total volume of 20 µl. PCR amplification was carried out in a programmable thermal controller (PTC-100™): denaturation - 3 min at 94 ºC, followed by 43 cycles of 10 s at 94 ºC, 30 s at 36 ºC, and 50 s at 72ºC. Finally, the samples were kept at 72 ºC for 7 min. For 5S rDNA spacer sequence analysis, the two primers: 5'-GAGAGTAGTACATCGATGGG and 5'-GGAGTTCTGAC-GGGATCCGG were employed. The 25 µl reaction mixtures contained 100 ng of genomic DNA, 40 pM of each primer, 200 µM dNTPs, 2.5 mM MgCl$_2$, 1U Ampli Taq polymerse, 2.5 µl 10×PCR buffer. PCR amplification was carried out by a denaturation step of 3 min at 94ºC, followed by 35 cycles of 94ºC for 1min, 60ºC for 1min, 72ºC for 2 min. A 72ºC incubation for 10 min was included as a final extension step. Amplification products were electrophoresed on agarose gels (RAPDs: 1.5%, 5S rDNA spacer: 2.5%) and visualized with UV light after staining with ethidium bromide.

To analyze RFLP in the mitochondrial genome, 20 µg of DNA was digested with *Hind* III or *Bam* HI.The digested DNA was separated on a 0.7% agarose gel in TAE buffer and blotted onto a nylon membrane (Hybond N$^+$, Amersham) using 5×SSC. Mt DNA-specific probes *atp* 6 and *cox* I were kindly provided by Dr Spangenberg (Institute for Plant science, Swiss Federal Institute of Technology). Probe labeling, hybridization and washing were carried out with the ECL Direct nucleic acid labeling and detection system (ECL RPN3004), according to the manufacturer's instructions.

RESULTS

### Protoplast culture and plant regeneration

After 35 days of culture, fifteen colonies of 1.5-2 mm in size formed and were transferred onto proliferation medium and later differentiation medium. Shoot formation was observed after two months sub-culture. Shoots were maintained on the same medium until they had reached sufficient size, and were subsequently transferred to NAA-containing medium for root induction. After four months, more than 40 green plantlets were regenerated from 12 colonies. These were considered to be putatively hybrid, because the protoplasts of *A. elongatum* could not

divide under the conditions of this experiment and the suspension cultures of wheat had lost their differentiation ability in the course of sub-culture. The appearance of the plants was intermediate between the two parents. For example, leaves of *A. elongatum* are narrow and prostrate, and the stem emerge from ground level (Fig 1a); but the stems of the regenerated plants are upright and the leaves on the stem are broad and short, as in wheat. In contrast, the morphology of the spikelet resembled that of *A. elongatum* (Fig 1b). Regenerated plants growing in soil were healthy, and three remained green throughout two winters, indicating that they are perennial, which is an important distinguishing feature of *A. elongatum*. Seeds with intermediate morphology between the parents have been obtained from the regenerated plant produced from clone 2 (Fig 1c).

Hybrid identity of regenerated calli and plants

As a result of long time sub-culture, the chromosome number of the wheat material had fallen to 21-29, while that of *A. elongatum* mostly ranged between 42 and 50. Hybrid chromosome numbers were determined in cells of lines 1, 5 and 14, and these were mostly in the range of 58-64 (Tab 3 and Fig 2). Esterase patterns demonstrated that the profiles of clones 2, 3, 5 and 14 included band (s) characteristic of both parents, along with additional band (s) (Fig 3a). Similarly, the peroxidase profiles showed that these same clones had band(s) specific to both parents (Fig 3b). Nuclear DNA analysis with primers specific for the 5S rDNA spacer showed that calli 1 and 14 amplified characteristic products of both fusion parents (Fig 4a) and plants 2, 3 and 14 had bands characteristic of both fusion parents as well as a new band. In contrast, plant 8 had a pattern identical to *A. elongatum*. (Fig 4b). Ten RAPD primers identified polymorphic products (Tab 1). The selected three calli (Tab 2 and Fig 5) and plants regenerated from them (Fig 6) were confirmed to be intergeneric hybrids between *T. aestivum* and *A. elongatum*, as their profiles included product(s) derived from each parent. From above results, some new bands appeared in different analysis, which maybe rose from the recombination of the parent genome.

The RFLP analysis of the mtDNA (Fig 7) showed that clone 14 carried *Hind* III fragments homologous with *atp* 6 derived from both parents, along with a novel fragment not present in either parent; while clone 2 had one novel fragment along with only wheat-specific fragments. The other two hybrids had a restriction fragment pattern identical to that of wheat. When the *cox* I probe was hybridized to *Bam* HI-digested DNA, all the hybrids had the wheat profile.
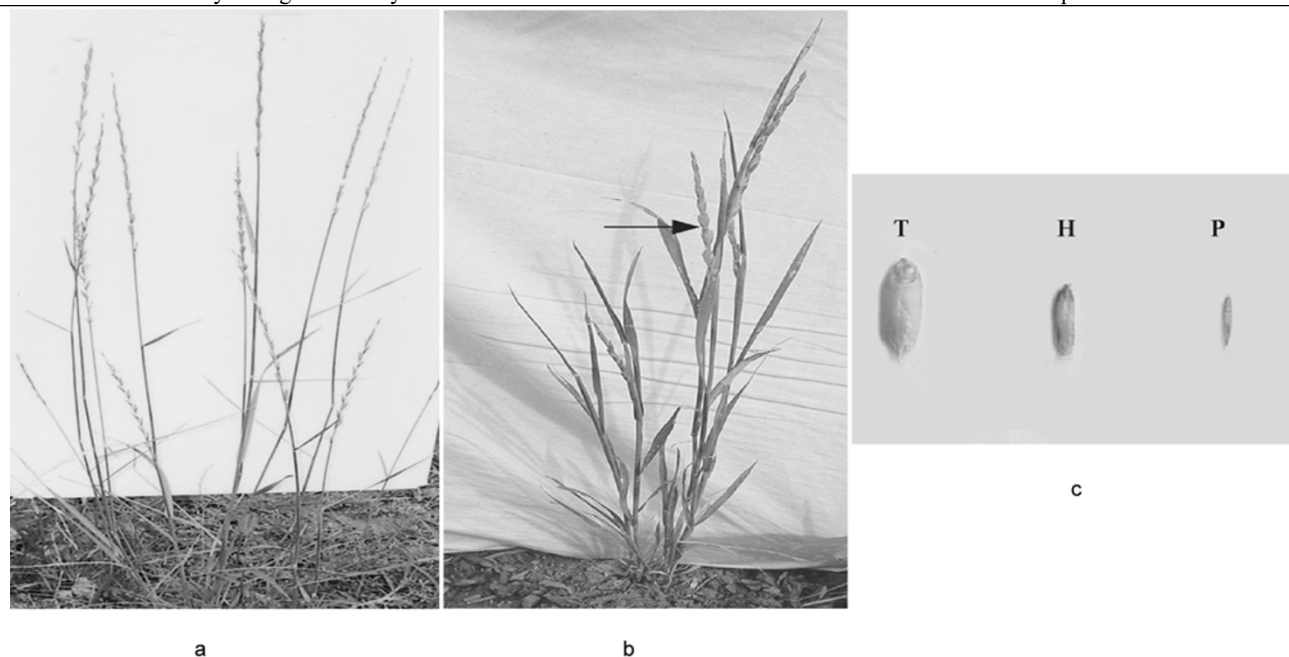
Fig 1. *A. elongatum* (**a**); the perennial hybrid (**b**),   →: Spike; Seeds of hybrid and parents (**c**), T: *T. aestivum*, H: somatic hybrid, P: *A. elongatum*.

DISCUSSION

In a previous investigation, highly asymmetric somatic hybrid plants that resembled the wheat parent were obtained, using a higher dose of UV to treat the *A. elongatum* proto-plasts. In this material, GISH showed the presence of only one or two *A. elongatum* chromosome(s) and /or only chromosome fragment(s) in the hybrid plants[8]. In contrast, the hybrids produced in the present study are intermediate between the two parents. The asymmetric level of the somatic hybrids is influenced by a number of possible internal and external factors, in particular, the dose of irradiation used, the phylogenetic distance between the parental species, the chromosome number of each parent, and the cell cycle of the protoplasts. The most commonly utilized method to produce highly asymmetric hybrids has been to damage the donor protoplasts, pre-fusion, using irradiation (UV-light, γ-ray). Some experiments showed that γ-ray had dose dependent effect on the elimination of the donor chromosome in the asymmetric somatic hy-bridization[14-15], but others showed contrary results[16-17]. As for UV light, we reported the chromosome elimi-nation and fragmentation of oat caused by UV irradiation in the hybrid of wheat with oat[18]. But in that experiment, most of oat chromosome was excluded and the chromo-some numbers of hybrids from different UV dosage were similar. The increase in dosage mainly resulted in more small donor chromosome fragment production and introgression. So, we suggested that both UV and phylo-genetic distance play an important role in the donor chro-mosome elimination of wheat remote hybrid. In compari-

Tab 1. DNA sequence of RAPD 10-mer primer

Primer	Sequence	Primer	Sequence
OPA1	CAGGCCCTTC	OPH1	GGTCGGAGAA
OPA4	AATCGGGCTG	OPH4	GGAAGTCGCC
OPA17	GGTCCCTGAC	OPH11	CTTCCGCAGT
OPA19	CAAACGTCGG	OPF12	ACGGTACCAG
OPJ20	AAGCGGCCTC		

Tab 2. RAPD analysis of regenerated calli

| Primer	OPA1	A4	A17	A19	H1	H4	H11	F12	J20
Clone 2	T.P	P	N.T.P	N.T.P	T.P	P	T.P	N.T.P	T.P
Clone 5	T.P	P	N.T.P	T.P	T.P	P	T.P	N.T.P	T.P
Clone 14	T.P	P	N.T.P	T.P	T.P	P	T.P	N.T.P	T.P

T: wheat, P: *A. elongatum*, N: new bands.

Tab 3. The chromosome number[*] and the isozyme of the regenerated clones

| Hybrid number	Chromosome number	Esterase	Peroxidase
1	58-64	–	–
2	–	T+P+N	T+ P
3	–	T+ P+N	T+ P
5	59-61	T+ P+N	T+ P
14	58-63	T+P	T+ P
Wheat	21-29	T	T

T: wheat, P: *A. elongatum,* N: new bands. [*]100 cells were counted
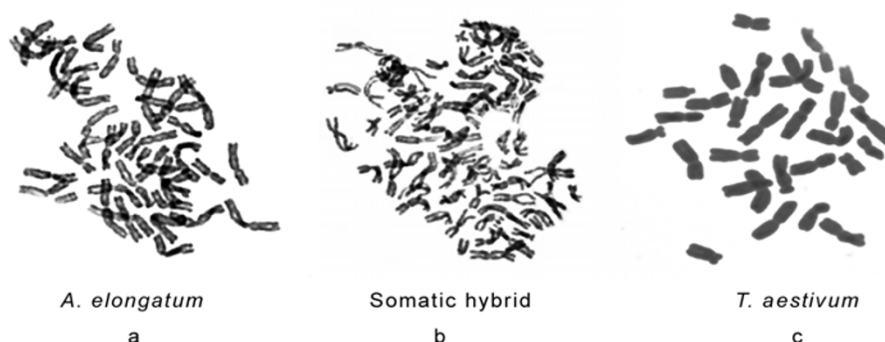
Fig 2. Chromosomes of hybrid line 1 and the parents. (**a**) *A. elongatum,* (**b**) hybrid, (**c**) parent wheat.
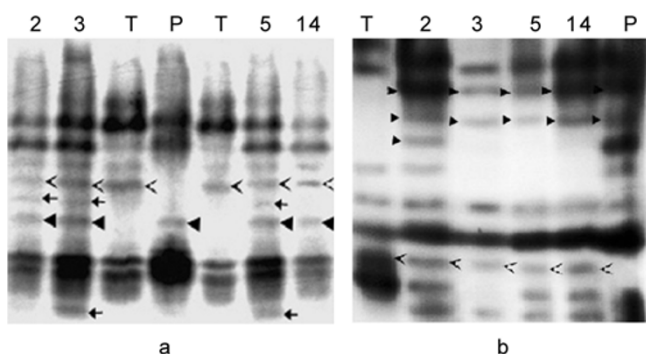

Fig 3. Profiles of esterases (**a**) and peroxidases (**b**). T: *T. aestivum* P: *A. elongatum* 2; 3; 5; 14: somatic hybrids. ◀ band characteristic of wheat; ◀ band characteristic of *A. elongatum*; ← band not present in either parent.
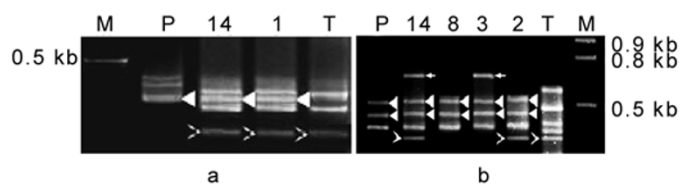

Fig 4. 5S rDNA analysis of calli (a) and the leaf (b) of the parents and the regenerated somatic hybrids. T: wheat, P: A. elongatum, 1; 2; 3; 8; 14: somatic hybrids. M: λDNA/*Hind* III+*Eco*RI molecular marker.◀ band characteristic of wheat; ◀ band characteristic of *A. elongatum*;← band not present in either parent.
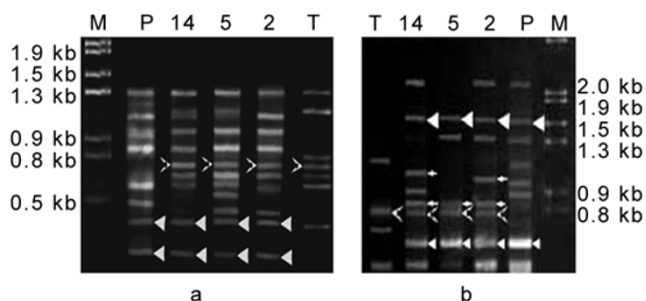
Fig 5. RAPD profiles of putative hybrids and their parents amplified from DNA of calli from (**a**) primer OPA17; (**b**) primer OPF12. T: wheat, P: *A. elongatum* 2; 5; 14: putative hybrids. M: λDNA digested by *Hind* III+*EcoR* I. ◀ band characteristic of wheat; ◀ band characteristic of *A. elongatum*; ← band not present in either parent.
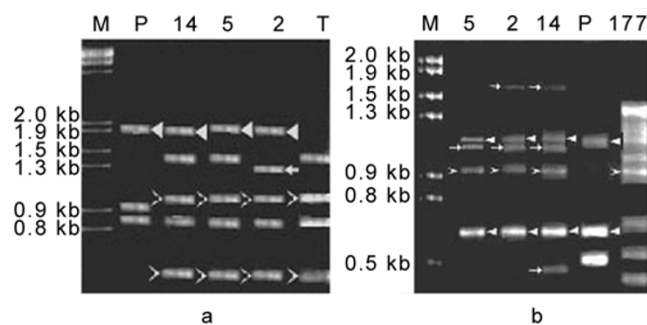
Fig 6. RAPD patterns amplified from leaf DNA of putative hybrids and their parents from (**a**) primer OPA19; (**b**) primer OPG14. T: wheat, P: *A. elongatum* 2; 5; 14: putative somatic hybrids.M: λDNA/ *Hind* III+*Eco*RI molecular marker.◀ band characteristic of wheat; ◀ band characteristic of *A. elongatum*;← band not present in either parent.
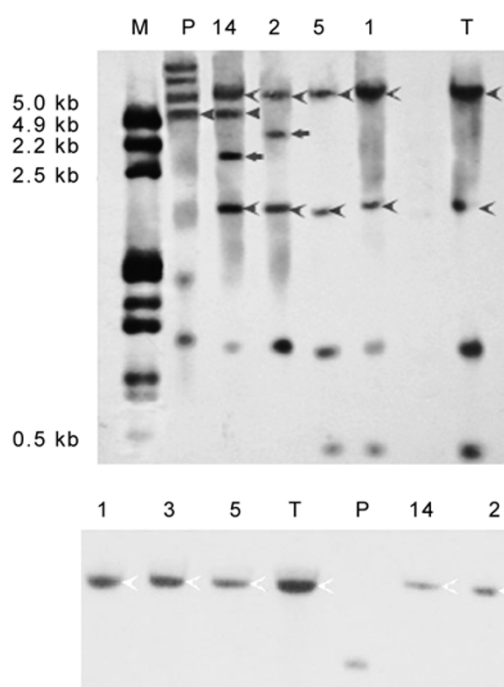
Fig 7. Southern hybridization patterns from *Hind*III-digested DNA probed with mtDNA probe *atp* 6 (left) and the *Bam*HI-digested DNA probed with the mtDNA probe *cox* I (right). T: wheat, P: *A. elongatum* 1; 2; 3; 5; 14: putative somatic hybrids. M: λDNA/*Hind* III+*Eco*RI molecular marker. ◄ band characteristic of wheat; ◄ band characteristic of *A. elongatum*; ← band not present in either parent.

son with the highly asymmetric hybrid of wheat and *A. elongatum*[8], we can conclude that hybrids with intermediate phenotype and chromosome number were formed due to the lower dose of UV applied. These hybrids were verified in nature by isozyme, RAPD and RFLP analysis. The result indicated that we could control the quantity of alien genetic material of *A. elongatum* transferred into wheat by using different UV dosage. This result will be useful for the further work to obtain different germplasm by asymmetric somatic hybridization in the related somatic hybridization. So, it is very important both to theory and practice research in asymmetric somatic hybridization induced by UV.

Random segregation and recombination of mtDNA during somatic hybridization has been reported in many plants [19, 20]. The most likely explanation for mtDNA to undergo genetic rearrangements is its size and its ability to exist in several genomic forms[21]. Conversely, a lack of recombination within the mitochondrial genome has also been reported in Brassica. Moreover preferential amplification of one parental mtDNA was documented in somatic hybrids of the Gramineae[22]. So, the composition patterns of hybrid mt DNA are very different. In the present

study, of the *Hind* III -digested total DNA fragments that hybridized with *atp* 6, although one clone (No. 14) had specific bands of both parents, the other three clones had only wheat fragments. The *A. elongatum cox* I sequence was not inherited by any of the clones analyzed. Thus, these somatic hybrids had a predominance of wheat mt DNA, at least in and around the *atp* 6 and cox I region. This is only partially consistent with the situation found in symmetric somatic hybrids of wheat with *Haynaldia villosa* [20], where mitochondria coexisted or recombined. We speculate that the absence of *A. elongatum* mt DNA (as opposed to nuclear DNA) relates to the greater susceptibility of mt DNA to fragmentation as a result of UV treat-ment. The direct impact of UV irradiation on mtDNA has been described in sugar-beet (*Beta vulgaris L.*) protoplasts by Hall et al[23], who found that UV radiation generated many DNA fragments, with mtDNA appearing to be more sensitive to damage than nuclear DNA.

In conclusion, intermediate hybrid fertile plants have been successfully obtained from the asymmetric somatic hybridization between wheat and *A. elongatum*, so this procedure is a model experiment for the future, indicating how intergeneric hybrids could be produced from wheat with other related species.

ACKNOWLEDGEMENTS

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