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High UV-tolerance with introgression hybrid formation of *Bupleurum scorzonerifolium* Willd

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Abstract

The protoplasts of *Arabidopsis thaliana* (L.) Heynh. treated with 7.5 mM of IOA (iodoacetoamide) were fused with those of *Bupleurum scorzonerifolium* Willd, using PEG-mediated method. The protoplasts of *B. scorzonerifolium* were irradiated with ultraviolet light (UV) at an intensity of 380 $\mu W/cm^2$ for 30, 60, 90, and 120 s, respectively, before fusion. In cell clones produced in all the fusion combinations, only those from Combination VI (UV 120 s) differentiated to plantlets. The cell lines and plantlets were identified as somatic hybrids by analysis of chromosome size and number, RAPD, 5S rDNA spacer sequence respectively. Genomic in situ hybridization (GISH) revealed that there was a complete set of chromosomes of donor (*B. scorzonerifolium*) in all hybrids with a few intact chromosomes and chromosome segment(s) of recipient (*A. thaliana*) in the hybrid cell lines from Combinations I–V or only chromosomal segment(s) in the regenerated cell lines from Combination VI. The numbers of chromosomal fragments from *A. thaliana* translocated or inserted to donor chromosomes increased with UV dose enhanced. The UV-tolerance of *B. scorzonerifolium* and indirect effect of UV on the exclusion and introgression of receptor (*A. thaliana*) chromosomes in the hybrid cells was discussed.

Keywords: *Arabidopsis thaliana*; *Bupleurum scorzonerifolium*; Asymmetric somatic hybridization; UV-tolerance of donor; Chromosome elimination and introgression of recipient

1. Introduction

Genes characterized so far have primarily been those involved in basic genetic regulation and in various fundamental processes related to plant growth and development [1]. Because of its small genome, *Arabidopsis thaliana* has already proved to be a very valuable source in the isolation of genes [2]. *A. thaliana* can also be used as a model species in the study of plant structural and functional genomes of other plants, due to the completion of its whole genome sequencing recently. Somatic hybridization offers interesting possibilities for transferring valuable genes from other plants into *A. thaliana,* which can aid in interpreting the genetic composition and targeting introgression genes after protoplast fusions. Since the first somatic hybrids of *Brassica* with the genome of *A. thaliana* were obtained in 1980 [3]. *A. thaliana* has been fused with many other plants of different genera [1,2,4–7]. *Bupleurum scorzonerifolium* is a Chinese medicinal herb contained more than 70 useful chemical components, including myrcene, rutin, narclssin, camphene, 4-methyl-hexanol, etc. [8]. It was used as a remedy for various ailments and was beneficial for several antibacterial properties. Because of the sexual incompatibility of *B. scorzonerifolium* with *A. thaliana,* somatic hybridization provides a novel way for transferring the medicinal important genes of *B. scorzonerifolium* to *A. thaliana*. In recent years, asymmetric somatic hybridization was used for the introgression of small chromosome segments from grasses and cereals to wheat (*Triticum aestivum* L.) in our lab [9–11]. The same method was used in the somatic hybridization of *A. thaliana* with *B. scorzonerifolium* in this experiment. Our initial goal was to transfer chromosome fragments or partial genome related to the medicine contents of *B. scorzonerifolium* into *A. thaliana*, so as to characterize the secondary metabolic pathway and

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pertinent genes. In this paper, however, we described an interesting contrary result: somatic hybrids obtained contained whole complete chromosomes of *B. scorzonerifolium* and a few chromosomes and/or small chromosome fragments of *A. thaliana*.

2. Materials and methods

2.1. Isolation and preparation of parental protoplasts

Seedling-derived calli of *A. thaliana* ($2n = 10$) were subcultured on MB [12] medium containing 1 mg/l 2,4-D (MB2) at 25 °C. After half year of subculture, the strumae calli with light-yellow color were selected and suspended in liquid MB medium containing 1 mg/l 2,4-D. *B. scorzonerifolium* embryogenic calli from stem nodes were subcultured on MB2 media for more than 12 years. The methods used for establishing suspensions of *B. scorzonerifolium* have been mentioned previously [13]. Both suspension lines were incubated in enzyme solution (0.6 M mannitol, 5 mM $CaCl_2$, 1.5% cellulase Onozyka RS and 0.3% pectolyase Y-23) for 1.5–2 h, respectively, until large amounts of protoplasts were isolated. Prior to fusion, protoplasts from the *A. thaliana* were treated with 7.5 mM of IOA (iodoacetoamide) for 10 min at room temperature. Meanwhile, monolayer protoplasts of *B. scorzonerifolium* were spreaded on 3 cm petri dishes and irradiated with UV at an intensity of 380 $\mu W/cm^2$ for 30, 60, 90 or 120 s, respectively.

2.2. Fusion combinations

The donor (*B. scorzonerifolium*) protoplasts were fused with that of the recipient (*A. thaliana*) using PEG method [14]. The following combinations of fusions and controls were designed:

(I) *A. thaliana* (IOA 7.5 mM);
(II) *B. scorzonerifolium* (UV 30, 60, 90 and 120 s);
(III) I + II (UV 30 s);
(IV) I + II (UV 60 s);
(V) I + II (UV 90 s);
(VI) I + II (UV 120 s).

Procedures of cell fusion and fusion products culture were undertaken as described by Xia and Chen [12].

2.3. PCR analysis

Total genomic DNA was extracted from regenerated calli or leaves by a modified CTAB procedure [15]. Ten RAPD primers (Operon Technology, USA) were used. PCR amplification was performed following Xia et al. [16]. 5S rDNA spacer sequence was PCR amplified as described by Zhou et al. [17]. The 20-mer primers were used to amplify the 5S rDNA spacer sequence: PI (5′-GAGAGTAGTACATC-GATGGG-3′), PII (5′-GGAGTTCTGACGGGATCCGG-3′) [17]. The PCR products were electrophoresed in 1.5% (RAPDs) and 2.5% (5S rDNA) agarose gels, analyzed with Syngene gel imaging system (Syngene, USA) after staining with ethidium bromide.

2.4. Chromosome counting and GISH analysis

Fresh cell lines of putative hybrids and the parents were pretreated for chromosome counting or the genomic in situ hybridization (GISH) followed Xiang et al. [10]. Total genomic DNA of *A. thaliana* was labeled as a probe and the GISH procedure was performed as described by Xiang et al. [10]. The hybridization mixture of 20 μl (per slide) contained 20 ng probe DNA and 200 ng blocking DNA of *B. scorzonerifolium*. Images were generated on a Nikon Eclipse E600 fluorescence microscope and captured with a Nikon Coolpix 990 digital camera.

3. Results

3.1. Development of fusion products and controls

A summary of the development of fusion products from the different combinations/controls in four independent experiments was listed in Table 1.

The phenotype of *A. thaliana* calli was strumae in structure and light-yellow in color (Fig. 1A). Whereas, the *B. scorzonerifolium* consisted of loose clusters with green-yellow color (Fig. 1B). Both calli lost the regeneration ability because of long-term subculture. The Controls I and II could not divide or only form small cell clusters in different experiments (Table 1).

About 65 days of culture after fusion, the putative hybrid clones of 1.5–2 mm in size were transferred to proliferation medium and then to differentiation medium. Most calli from the different fusion combinations showed *B. scorzonerifolium* type (B type); but a few had strumae structure and green-yellow color (M type) (Table 2). In the differentiation medium, many cell lines in Combinations III–VI could form abnormal green leaves or shoots but could not regenerate plantlets (Fig. 1C). Out of a total number of 211 cell lines,

Table 1
Calli formation and differentiation in the combinations/controls

Combinations/controls	Number of calli	Regeneration	Frequency (%)
I	None	None	0
II	None	None	0
III	54	Shoot	7.4
IV	62	Shoot	8.1
V	56	Shoot	5.4
VI	39	Shoot/plantlet	20.5

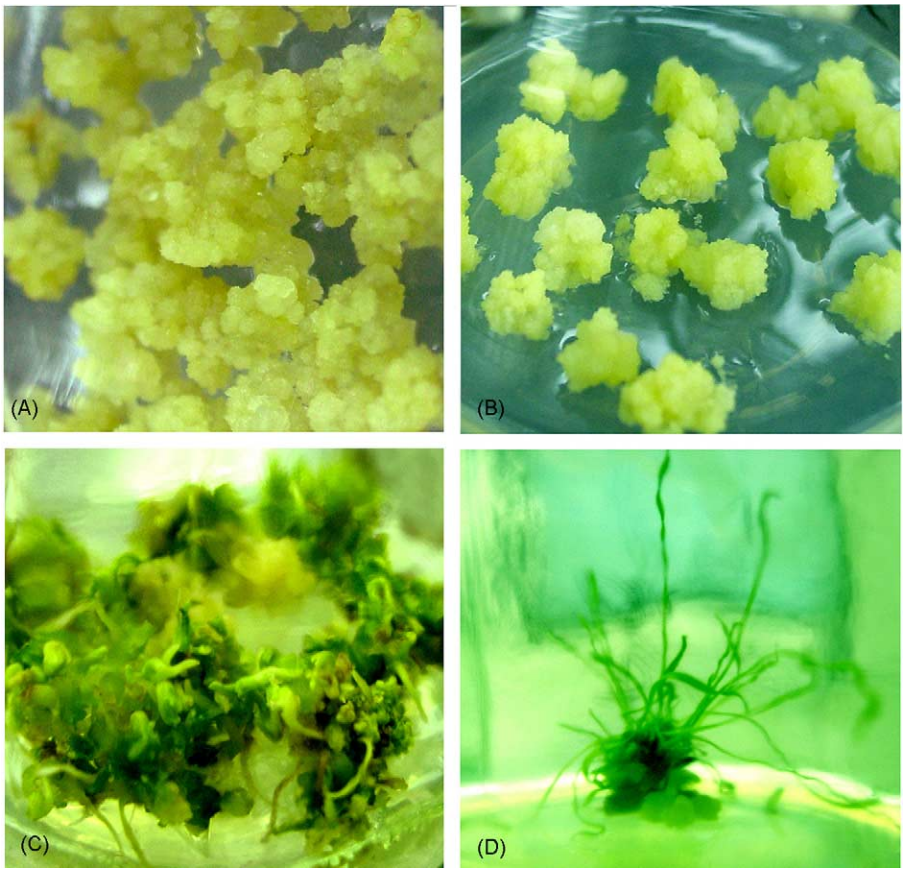

Fig. 1. Morphology of the biparental calli and hybrid clones. Calli of: (A) *A. thaliana* and (B) *B. scorzonerifolium.* (C) Abnormal green leaves and shoots from Combination III. (D) A hybrid plantlet regenerated from no. 47 in Combination VI.

Table 2
Morphology, chromosome number, RAPD, 5S-rDNA and differentiation ability of the hybrid cell lines from different fusion combinations

Fusion combination	No. of cell clone	Morphology of calli	Range of chromosomes	No. of fragment/small chromosomes	5S-rDNA pattern	RAPD pattern	Differentiation
III	3	B	12–15	0–3	P	P	Shoot
	15	B	12	–	P	P	–
	32	B	13–15	1–3	P	P	–
	52	B	13–16	1–4	P	P	Shoot
	73	B	13–15	1–3	P	P	–
IV	11	B	12	–	P	P	Shoot
	57	B	12–14	0–2	P	P	Shoot
	92	B	13–15	1–3	P	P.N	Shoot
V	23	M	13–14	1–2	P	P	–
	108	B	13–16	1–4	P	P	Shoot
	125	B	13–17	1–5	P	P	Shoot
VI	29	B	12	–	P.N	P	Shoot/plantlet
	35	B	12	–	P.N	P	Shoot/plantlet
	47	B	12	–	P.N	P.N	Shoot/plantlet
	69	B	12	–	P	P.N	Shoot/plantlet
	106	B	12	–	P	P	Shoot/plantlet
	132	M	12	–	P	P	Shoot/Plantlet

M: middle type (strumae with green-yellow color); B: *B. scorzonerifolium* type (loose structure with green-yellow color); P: containing partial bands of both parents; N: containing new band(s). (–) Not examined or no differentiation.

Table 3
Primers resulted in RAPD markers in the analysis of calli and plantlets from *A. thaliana* (+) *B. scorzonerifolium*

Primer	5′–3′ sequence	Polymorphic location	Primer	5′–3′ sequence	Polymorphic location
G10	ACAACGCGAG	2–7	A1	CAGGCCCTTC	3–5
H4	GGAAGTCGCC	4–5	A8	GTGACGTAGG	3–4
H20	GGGAGACATC	2–6	F5	CCGAATTCCC	5–6
A19	CAAACGTCGG	3–4	J19	GGACACCACT	4–6

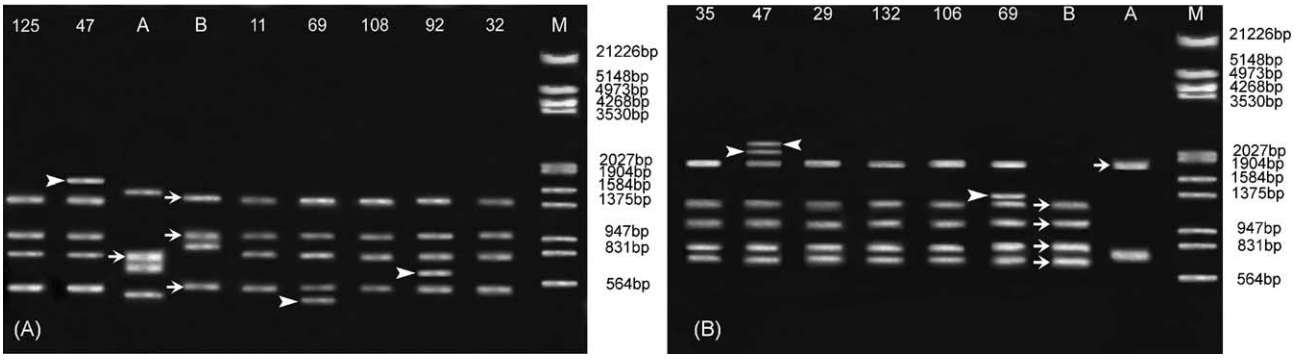

Fig. 2. RAPD analysis of regenerated clones and the parents. Electrophoresis pattern of RAPD using: (A) OPH-4 and (B) OPG-10. A, *A. thaliana*; B, *B. scorzonerifolium*; M, the lambda DNA size marker cut with *Eco*RI + *Hin*dIII. No. 11, 32, 92, 108, 125 hybrid calli; no. 29, 35, 47, 69, 106, 132 regenerated clones. (↑) The specific bands of both parents; (◀) the new band.

only six derived from Combination VI regenerated normal green plantlets (Fig. 1D). Interestingly, the morphology of the regenerated leaves, shoots and plantlets all resembled that of *B. scorzonerifolium*, which were verified as hybrids in the following analysis.

3.2. RAPD analysis

Eight of the 10 primer pairs used could provide evidences for hybridity (Table 3). Although most of the cell lines were similar to *B. scorzonerifolium*, 39 out of 211 cell lines had the specific RAPD bands of each parent, and some of them had novel bands (Fig. 2A; Table 2). RAPD profile using OPG-10 (Fig. 2B) showed that the regenerated plantlets from Combination VI contained specific segments presented in parents and new segment(s).

3.3. 5S rDNA spacer sequence analysis

5S rDNA spacer sequences of the 39 hybrid clones from RAPD assays were analyzed. The profiles showed partial specific bands of both parents and a few new band(s) in the hybrids (Fig. 3; Table 2).

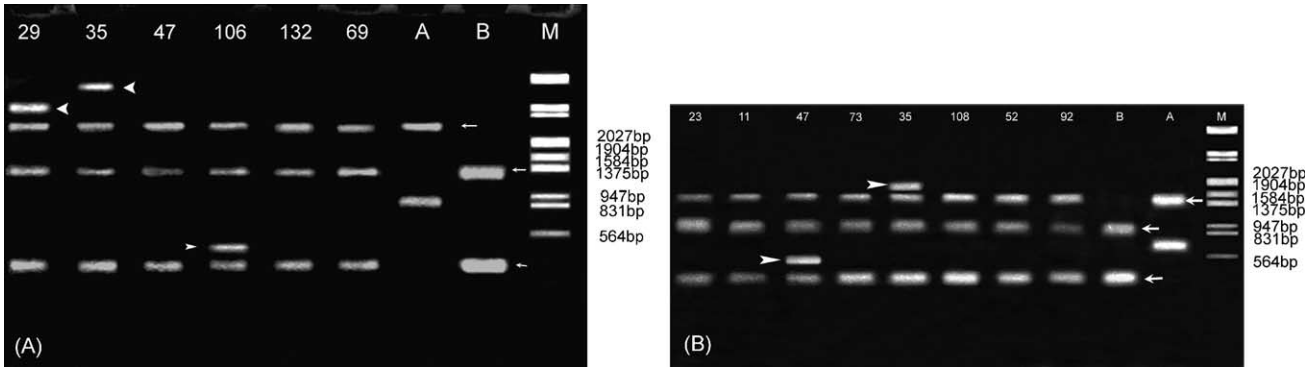

Fig. 3. 5S rDNA electrophoresis pattern of PCR products using 20 mer primer pairs. (A) 5S rDNA spacer sequence pattern of regenerated hybrid calli from Combination VI. (B) 5S rDNA spacer sequence pattern of hybrid calli from cell lines in Combinations III–VI. A, *A. thaliana*; B, *B. scorzonerifolium*; M, the lambda DNA size marker cut with *Eco*RI + *Hin*dIII. No. 11, 23, 52, 73, 92, 108 hybrid calli; no. 29, 35, 47, 69, 106, 132 regenerated calli. (↑) The specific bands of both parents; (◀) The new band.

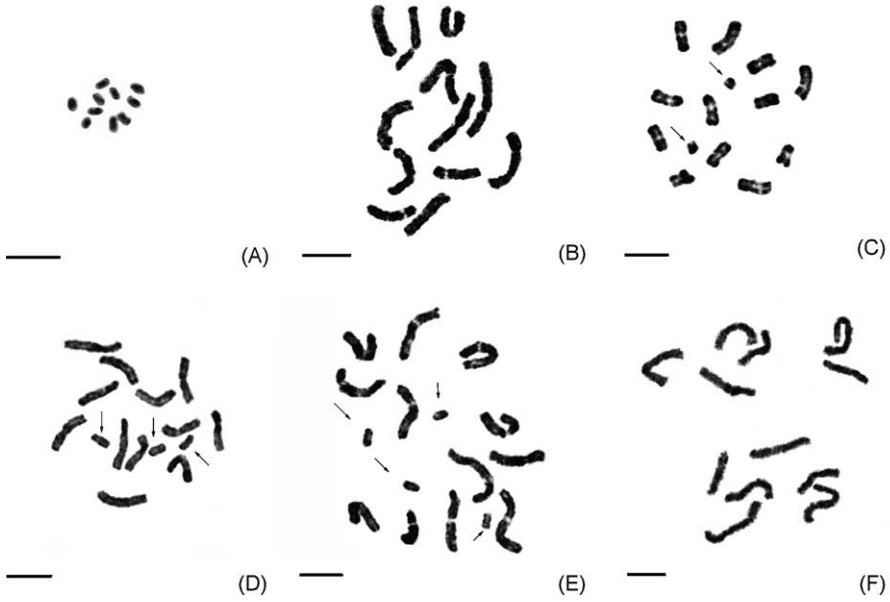

Fig. 4. Chromosomes of the parents and hybrid cell lines. (A) Chromosomes of *A. thaliana* calli, $2n = 10$. (B) Chromosomes of *B. scorzonerifolium* calli, $2n = 12$. (C) Chromosomes of cell line no. 32 in Combination III, $2n = 14$ (containing 12 chromosomes of *B. scorzonerifolium* and two small chromosomes from *A. thaliana*); (D) no. 92 in Combination IV, $2n = 15$ (containing 12 chromosomes of *B. scorzonerifolium* and three small chromosomes from *A. thaliana*); (E) no. 108 in Combination V, $2n = 16$ (containing 12 chromosomes of *B. scorzonerifolium* and four small chromosomes from *A. thaliana*). (F) Chromosomes of regenerated cell line no. 47 formed in Combination VI, $2n = 12$. (↑) Chromosomes of *A. thaliana*; bars = 0.5 μm.

Both RAPD and 5S rDNA molecular markers revealed that 39 clones and six regenerated plantlets were somatic hybrids.

3.4. Chromosome counting

Although parent *B. scorzonerifolium* calli were subcultured for about 12 years, the chromosome numbers of over 90% cells still maintained $2n = 12$, the complete karyotype. While those of *A. thaliana* were between 10 and 30, over 75% cells remained $2n = 10$ (Fig. 4A and B). The chromosome numbers of the hybrids in different combinations were diversity, ranging from 12 to 17 with 1–5 small chromosome in Combinations III–V (Fig. 4C–E; Table 2), and 12 without small chromosome in Combination VI (Fig. 4F; Table 2).

3.5. GISH analysis

The chromosomes of *A. thaliana* (green) and *B. scorzonerifolium* (red) were showed in Fig. 5A and B. In Combinations III–V, several hybrid cell lines produced green leaves and shoots had complete chromosomes of *B. scorzonerifolium* and 1–5 intact chromosomes of *A. thaliana* (Fig. 5C). The cell lines from Combination VI with ability of regenerating plantlets contained complete chromosomes of *B. scorzonerifolium* and several translocating and inserting fragments of *A. thaliana*, absenting any intact *A. thaliana* chromosomes (Fig. 5D–F). The results indicated that the exclusion and introgression of *A. thaliana* chromosomes was indirectly enhanced by UV dose.

4. Discussion

4.1. Hybrid calli selection

Hybrids were obtained after inactivation of the recipient and donor parental protoplasts by IOA-treatment and UV-irradiation, respectively, although biparents treated were either unable to regenerate. Similar system for hybrid selection via complementation was reported in the fusion between *Brassica oleracea* and *B. campestris* [18]; *A. thaliana* and *Brassica* spp. [6] and *A. thaliana* and *Brassica napus* [7]. Terada et al. [18] found that protoplasts treated with IOA (inhibitor of mitochondrial oxidative phosphorylation) of much higher concentration than that required for inactivation could survive and regenerate after all fusion treatment. In this study, the IOA concentration (7.5 mM) was much higher than those used for the *Brassica* and *A. thaliana* inactivation [6,7]. There were 211 cell lines mostly with phenotypes of *B. scorzonerifolium*-like and a few middle types from all combinations. None of the *A. thaliana* protoplasts survived in this experiment. Meanwhile, various doses (380 $\mu W/cm^2$ for 30, 60, 90 and 120 s) of UV could inactivate the protoplasts of *B. scorzonerifolium*. So, selection of the somatic hybrids was successful through the metabolic inactivation of *A. thaliana* protoplasts in combination with the nucleus-damaged *B. scorzonerifolium* protoplasts. However, only 18.5% (39 out of 211) of the cell lines were identified as nuclear hybrids. The remaining cell lines with morphological characters of *B. scorzonerifolium* derived from its escaped protoplasts. The formation of escaped cell lines was also reported in other experiments

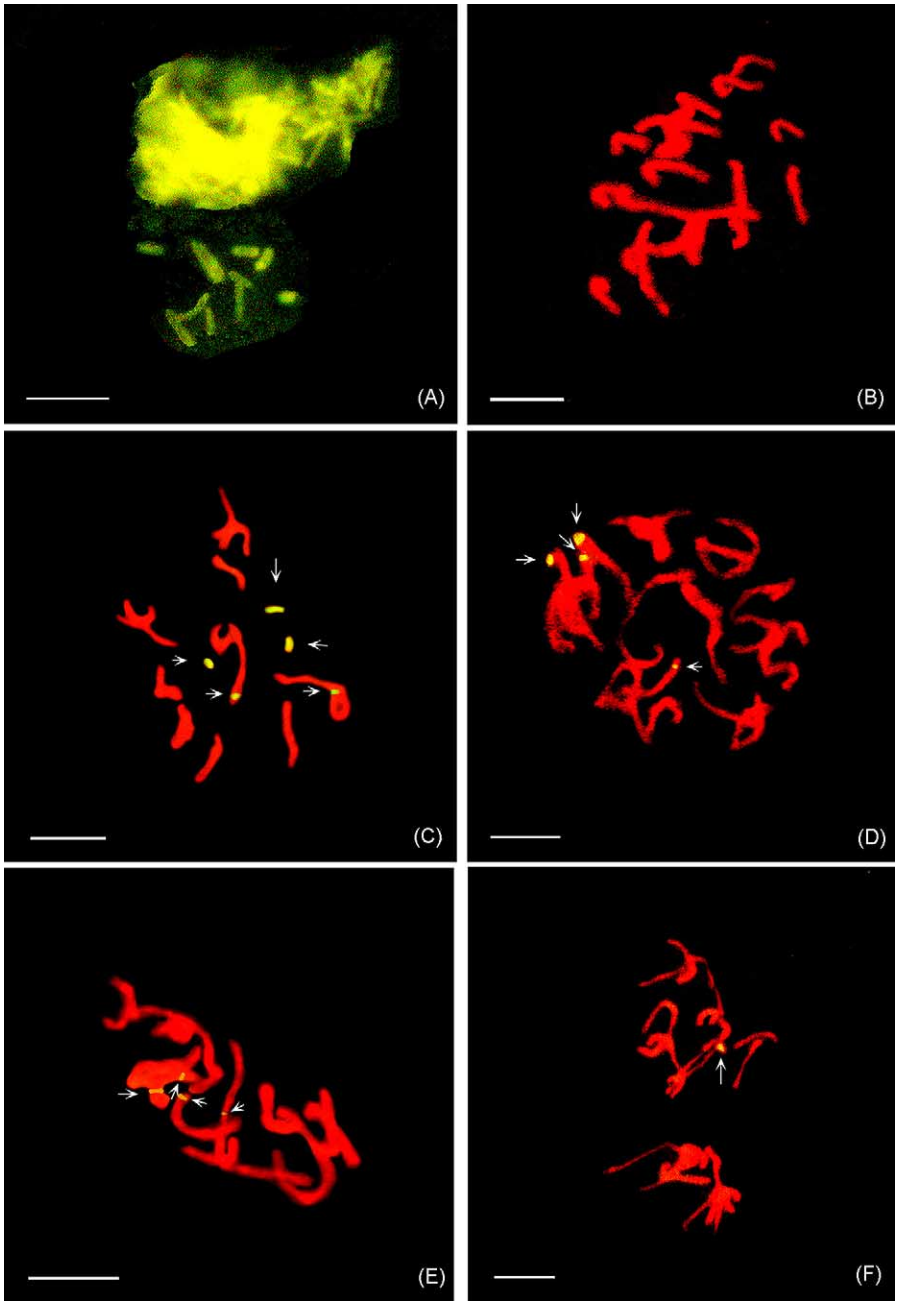

Fig. 5. GISH results of the cells of calli in some combination (green-yellow color indicated chromosomes and fragments of *A. thaliana*). Total genomic DNA of *A. thaliana* was labeled as probe. (A) *A. thaliana*; 2*n* = 10. (B) *B. scorzonerifolium*; 2n = 12. (C) Chromosomes of cell line no. 52 in Combination III, showing 12 chromosomes of *B. scorzonerifolium*, three intact *A. thaliana* chromosomes and two chromosome segments translocated. (D)–(F) Chromosomes from cell lines no. 47, 69 and 35 in Combination VI showing 12 chromosomes of *B. scorzonerifolium* and one to several chromosome segments integrated. (↑) *A. thaliana* chromosome or chromosome segment; bars = 0.5 μm.

[7,19]. Whether this kind of escape involves the transfer of *A. thaliana* mitochondria, which was not checked in the experiment, needs to be investigated.

4.2. UV-tolerance of *B. scorzonerifolium*

Szarka et al. [20] suggested that a prolonged period in vitro culture altered the viability and regeneration ability. The variation of chromosome number and structure often occurred in tissue cultures [21]. *B. scorzonerifolium* calli used in this experiment has lost the regeneration ability for more than 12 years of subculture, but remained complete 12 chromosomes in most cells. The same chromosome numbers of *B. scorzonerifolium* also retained in the hybrid cell lines between *A. thaliana* and *B. scorzonerifolium* treated with different doses of UV. The results implied that the chromosomes of *B. scorzonerifolium* are stable in tissue culture and in the hybridization via UV treatment, especially

with the high tolerance to UV radiation. The normal plantlets could regenerate from the combination of *A. thaliana* with high dose UV-treated *B. scorzonerifolium*.

The same UV doses were used to treat donors (e.g., grass and cereal) in our previous fusion test. But only lower dose, 380 μW/cm^2/UV 30 s, could induce production of vigorous plant [9–11]. In the combination of common wheat and Russian ryegrass, the plants derived from the treatment with lowest dosage UV (380 μW/cm^2/30 s) had a higher regeneration frequency and grew more vigorously than those from the combination treated with a higher UV dose (380 μW/cm^2/1 min). This demonstrated that the UV treatment given to the donor influenced the growth and development of the fused protoplasts [22].

UV-tolerance of *B. scorzonerifolium* maybe related to the secondary metabolites in it. There are more than 70 chemical components existed in the *B. scorzonerifolium*. Most of them are secondary metabolites and metabolic intermediate [8]. Ebel and Hahlbrock [23] found that accumulation of UV-absorbing flavonoids induced by UV-B radiation enhanced the UV-resistance in plants. UV-irradiation was also used to improve the productivity of second metabolites in plants [24,25]. It, therefore, seems probably that UV radiation enhance the synthesis of secondary metabolite productions in *B. scorzonerifolium*, which makes its DNA tolerant to UV-irradiation. This is a new valuable trait in *B. scorzonerifolium*, which is important for the further study of both this herb's application and the mechanism of UV-resistance in plant.

4.3. Elimination of receptor chromosomes

It is well known that irradiation leads to a preferential loss of donor DNA in hybrids [10,26,27]. Moreover, the elimination of the recipient chromosomes was also detected in some fusion combinations. For example, somatic hybrid plants between carrot (donor) and rice (recipient) contained many carrot chromosomes, while only several chromosomes of rice were present [28]. In our experiment, the hybrids contained a complete set of chromosomes of donor (*B. scorzonerifolium*) and a few additional intact chromosomes or only chromosomal fragments of recipient (*A. thaliana*). Some factors are deduced to influence the chromosome exclusion of somatic hybrids [10], e.g., phylogenetic distance of the parents, radiation dose, cytokinetics, premature chromosome condensation (PCC), etc. [26,29,30]. In this experiment, we deduced that both phylogenetic relationship and UV impact together on the elimination and fragmentation of receptor chromosomes. The latter is possiblely responsible for the chromosome fragmentation and introgression. Ionization and excitation induced by primary UV-radiation led to the production of new chemical species in donor and hybrid cell. As a result of radiolysis of the water existing in a cellular complex, new chemical such species as hydroxyl radicals, hydrogen radicals, and solvated electrons produced have a finite probability of interacting with DNA sites. Hydrogen radicals, as well as hydroxyl radicals, can react either with sugar moieties or with bases [31]. These reactions between water radicals and various DNA sites can lead to such damages as base alterations, base deletions, and strand breaks of *A. thaliana*. Serious DNA breaking of the receptor could induce by a high UV-dosage (380 μW/cm^2/UV 120 s) and the fragments integrated into donor genome via an instant breakage of the DNA chain of *B. scorzonerifolium* in hybrid cells. The similar phenomenon also occurred in the somatic hybridization of *Triticum aestivum* L. (+) *B. scorzonerifolium* (UV-irradiation). The introgression chromosome and DNA fragments of *T. aestivum* increased with the UV dose in the hybrids and no intact wheat chromosome existed in the same dose of 380 μW/cm^2/UV 120 s (Zhou et al., personal communication). Both results imply that DNA breakage could be directly and indirectly induced by UV-irradiation, which may be the basis of random introgression of chromatin in the asymmetric somatic hybridization [27,32]. In the introgression of donor chromosome fragments resulted from UV-irradiation into receptor [9–11,32], it was suggested that the breaks for a while in receptor DNA induced indirectly by UV was also involved.

4.4. Genetic composition in relation to plant regeneration of hybrids

Both *B. scorzonerifolium* and *A. thaliana* calli have lost the regeneration ability in the tissue culture, but their hybrids could regenerate plantlets or shoots. This complementary effect was reported in the hybridization between wheat and its intergeneric and intertribal grass or cereal [9–11,32,33].

Six regenerable calli (15.1% of total hybrid calli) were produced from Combinantion VI. It is mentioned above that the total numbers of chromosomes in these hybrids were the same as *B. scorzonerifolium*, $2n = 12$ (Fig. 4F; Table 2). Whereas, the number of chromosomes ranged from 12–17 in Combinations III–V (Fig. 4C–E; Table 2), in which only abnormal green leaves and shoots were regenerated. The results also revealed that the regeneration frequency of hybrid calli (Table 1) in Combination VI was much higher than others (Combinations III–V). Compared to *B. scorzonerifolium,* the chromosomes of *A. thaliana* were much smaller. But it is difficult to detect intact chromosomes of *A. thaliana* from chromosomal segments of *B. scorzonerifolium*. Biparental chromosomes and chromosomal segments could be verified through GISH. It was shown that the cell lines with regeneration ability of complete plantlets contained 12 chromosomes of *B. scorzonerifolium* and some small chromosome fragments integrated (Fig. 5D–F). But other cell lines regenerated abnormal green leaves and shoots have 12 chromosomes of *B. scorzonerifolium* and 1–5 intact chromosome(s) of *A. thaliana*, as well as one to several chromosome fragments (Fig. 5C). Since chromosomal fragmentation but not chromosomal loss was the first event induced by UV-irradiation [10], a small portion of segments of DNA from one partner integrated into the genome of another has some advantages over adding a

complete set of chromosomes or some intact chromosomes. The former may not result in metabolic disorders or unstable genomic constitution in hybrids [34]. So, hybrid plantlets could only be obtained in the introgression lines. The introgression mechanism of alien genetic material from *A.thaliana* to *B. scorzonerifolium* resulted by indirect effect of UV is worth to further study.

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