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Tri-parental protoplast fusion of *Brassica* species to produce somatic hybrids with high genetic and phenotypic variability

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Abstract

Plating efficiencies and the plant regeneration frequencies of bi-parental fusion of *Brassica campestris pekinensis* with Ogura cytoplasmic male sterility (CMS), *B. oleracea* L. var. *italica* with Ogura CMS, and *Brassica juncea* var. *crispifolia* were compared for illustrating the advantages of tri-parental somatic hybridization. The results showed that in the tri-parental fusion combination, 73 plants regenerated from 712 calli and the plant regeneration frequency was 10.3%, three or four times that obtained with the 2 fusion combinations. These hybrids were classified into 12 types based on morphology, and most showed intermediate characteristics between 2 or more of the parental species. The somatic hybiridity were confirmed by flow cytometry, genomic *in situ* hybridization (GISH) and PCR analysis, indicating that these regenerated plants were all true hybrids. Most of the progenies with normal pollen showed varied number of seed set after backcrossing with *B. juncea*. The high variability in the hybrids obtained illustrated that somatic hybridization may be useful in broadening existing *Brassica* gene pools and obtaining material for breeding.

Key words: *Brassica*, tri-parental protoplast fusion, genomic *in situ* hybridization, flow cytometry, morphology

Introduction

Although genetic variability within species has been efficiently utilized by breeders in their efforts to improve crops, the existing variability in a breeding population may not be sufficient for modern plant breeding purposes and therefore, great efforts have been made to broaden the existing gene pools (Xia 2009). Interspecific hybridization with related crucifer species is often used to broaden genetic variability in *Brassica* species (Snowdon et al. 2006). However, incompatibility between species coupled with low fertility in the F_1 hybrids severely limits the introgression of desirable traits (Wang et al. 1983). Somatic hybridization via protoplast fusion makes it possible to bypass sexual-crossing barriers and facilitate transfer of desirable traits that are only present in the cytoplasm such as cytoplasmic male sterility (CMS) (Pelletier et al. 1983; Liu et al. 2005; Prakash et al. 2009), transfer chloroplast (Ovcharenko et al. 2011), and generate new unexpected alleles (Cardi and Earle, 1997). These techniques have been used for scion and rootstock breeding in citrus (Grosser and Gmitt 2011; Wang et al. 2010) as well as for the introgression of genes from wild species into commercial cultivars in cotton (Sun et al. 2011).

Brassicaceae is a model family for somatic hybridization. The possibility of circumventing barriers to sexual reproduction and the effective use of valuable germplasm, which are the benefits of somatic hybridization (Hansen and Earle 1997; Glimelius, 1999), have been demonstrated successfully within the Brassicaceae family, which is very amenable to protoplast fusion. Protoplast fusion has enabled the cultivated *Brassica* allopolyploids to be resynthesized and make easy intergeneric or even intertribal hybrids between *Brassica* crops and relatives in several genera or tribes (Sigareva and Earle 1997; Wang et al. 2006; Du et al. 2009; Lian et al. 2012). Highly asymmetric somatic hybrid calli and plants can be produced via symmetric fusion in a tri-parental fusion system in wheat (Li et al. 2004). In the present study, the plating efficiencies and the plant regeneration frequencies of bi-parental fusion were compared to illustrate the advantages of tri-parental somatic hybridization. The

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morphological features of mature regenerants including leaf shapes and flower size were also investigated to examine the variation in morphology and genetic introgression of fusion-derived plants.

Materials and methods

Plant materials

Inbred lines of *B. oleracea* L. var. *italica* (Broccoli, 2n = 18) with Ogura cytoplasmic male sterility (CMS), *Brassica juncea* var. *crispifolia* (Heading mustard, 2n = 36) and *Brassica campestris pekinensis* (Chinese cabbage, 2n = 20) with Ogura CMS were used as plant materials for protoplast isolation. Seeds provided by the Choong Ang Seed Company of South Korea were surface-sterilised using 70% ethyl alcohol for 30 s followed by 15 min in 30% (v/v) commercial Clorox bleach (1.2% NaOCl) solution to which 0.1% of Tween-20 was added. Seeds were germinated on MS (Murashige and Skoog 1962) medium supplemented with 1% sucrose and solidified with 0.8 g/L^{-1} agar under controlled conditions (25°C, 16 h photoperiod, 84 µmol/(m^2·s), and white fluorescent light).

Protoplast isolation, fusion, and plant regeneration

Protoplasts of Chinese cabbage, broccoli, and leaf mustard were isolated from the cotyledons and hypocotyls of 10-day-old seedlings by using an enzyme solution containing 0.4 mol/L mannitol, 50 mmol/L $CaCl_2 \cdot 2H_2O$, 2% cellulysin (Calbiochem, USA), and 0.5% macerozyme (Calbiochem, USA) at pH 5.8. The protoplasts were isolated by using the method described by Glimelius et al. (1986) with some modifications. The purified protoplasts of the 3 fusion partners were suspended in washing solution W5 (18.4 g/L $CaCl_2 \cdot 2H_2O$, 9.0 g/L NaCl, 1.0 g/L glucose and 0.8 mg/L KCl, pH 5.8) to adjust the final concentration to 1 x 10^5 protoplasts/mL and mixed gently at a 1:1:1 ratio. Protoplast fusion and culture was carried out according to the methods described by Lian et al. (2012).

Morphology analysis

The morphological characteristics of fusion-derived plants including leaf shape, size, and flower color were investigated and compared with those of the parents. First progenies were obtained by crossing in mid-March to late of March with *B. juncea* (mustard). Thirty seeds were harvested after 30-35 days after backcrossing. The seedlings were planted in greenhouse for morphological and cytological analysis.

Ploidy estimation using flow cytometry and chromosome counting

A total of 0.2 g of fresh leaves from the regenerated plants with typical chromosome numbers (Table 1) and fusion parents were determined according to the methods described by Lian (2012). The *B. oleracea* and *B. campestris* and *B. juncea* were used as controls, against which the relative fluorescence intensities from the regenerated plants were compared. To investigate the chromosome number of the putative somatic hybrids, root-tips were pre-treated with 0.002 M 8-hydroxyquinoline at room temperature for 1 h, fixed with 3:1 (v/v) ethyl alcohol:acetic acid, and transferred to absolute alcohol at 4°C for at least 24 h. They were stored in 70% (v/v) ethanol at 4°C ready for chromosomal observation. Chromosome preparations were made by the method as described by Lian et al. (2011a).

PCR analysis

Total DNA was isolated from the leaves of greenhouse-grown parental lines and 12 types regenerated plants, which were classified by their morphological feature, following the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1990). The CMS trait was detected using the following Ogura mitochondrial-specific primers: 5′-GTC GTT ATC GAC CTC GCA AGG-3′ and 5′-GTC AAA GCA ATT GGG TTC AC-3′, synthesised according to the sequences published by Sigareva and Earle (1997). The amplification was

Table 1. Comparisons of plant regeneration frequencies between biparental fusion and tri-parental fusion

Fusion combinations	Cell densities[a] (protoplasts/mL)	Planting efficienty of callus formation (%)	No. of calli	No. of regenerants	Regeneration frequency (%)
Leaf mustard + broccoli	5 x 10^5	0.58	476	12	2.5
Chinese cabbage+broccoli	1 x 10^5	2.81	300	11	3.7
Chinese cabbage + leaf mustard + broccoli	1 x 10^5	17.86	712	73	10.3

A = means cell densities of every fusion parent

performed as follows: 94°C for 3 min, followed by 40 cycles of 30 s at 94°C for denaturation, 60 s at 51°C for annealing, and 120 s at 72°C for extension, with a final extension of 10 min at 72°C before being held at 4°C. Amplification products were analysed by electrophoresis in 1.0% (w/v) agarose gel and detected by staining with ethidium bromide. The gels were photographed under UV light.

Probe labelling and genomic in situ hybridization (GISH) analysis

The DNA fragments derived from *B. juncea* (Genome = ABBB), *B. oleracea* (Genome = CC), and *B. campestris* (Genome = AA) were labelled with Bio-11-dUTP (Roche Germany) by using the nick translation method and were used as probes, the average fragment lengths were 100-200 bp. The slide preparations for GISH mainly followed the method by Zhong et al. (1996) Hybridization of the probe to the chromosome and detection of hybridized DNA was performed by a modified in situ hybridization method (Melody, 1999). Chromosomes were counterstained with 0.2% 4′ -6-diamidino-2-phenylindole (DAPI) solution (Roche, Basel, Switzerland) and propidium iodide (PI), and hybridization signals were detected by using anti-digoxigenin rhodamine (Sigma, USA) and FITC anti-avidin (Sigma, USA), respectively.

Results and discussion

Protoplast isolation, fusion, and plant regeneration

The protoplasts were divided within 2 days of the initial culture. A large number of micro-colonies developed within 7-12 days of culture and >50% of the plated cells had divided at once. In the fusion combination of leaf mustard + broccoli, 12 plants regenerated from 476 calli, yielding a plant regeneration frequency of 2.5%. In the fusion combination of Chinese cabbage + broccoli, 11 plants were regenerated from 300 calli. The hybridities of such regenerated plants were identified by Lian et al. (2012) by using morphological, cytological, and molecular biological methods. The combination of Chinese cabbage + leaf mustard did not form calli. Individual cultures of the 3 fusion parents also did not form calli. High plant regeneration frequency of tri-parental fusion has also been reported in wheat (Li et al. 2004). The study proposed the value of a tri-parental fusion system for the production of asymmetric hybrids. In our study, more than 712 calli were derived from 6250 colonies in the tri-parental fusion combination. These were transferred to MS basal medium containing 5 mg/L ZEA, and 2 mg/L IAA. Subsequently, the first shoot primordia were observed after 2 months of macrocalli development on 73 plants derived from 25 different calli. The plant regeneration frequency was 10.3%, three or four times that obtained with the 2 fusion combinations as described above (Table 1). These results suggest that genetic complementation can be achieved through tri-parental combination. This phenomenon could offer better growth of hybrids and complementation of regeneration capacity in somatic hybridization of *Brassica*. The phenomenon of complementary regeneration of hybrid plants is not uncommon (Xia and Chen, 1996; Xia et al. 2003). Xu et al. (2003) found that protoplasts of both parents were either unable to regenerate or had low regeneration ability, whereas hybrids had good regeneration ability and could form normal plants. The petioles, leaves, and other vegetative features of the regenerated plants showed a considerable amount of variation. Some regenerated plants showed a broccoli-like phenotype, whereas no regenerated plants showed the morphological features of Chinese cabbage or leaf mustard. These results may be related to the plant regeneration abilities of *B. campestris* and *B. juncea*. As previous reports showed, *B. campestris* remains recalcitrant towards protoplast regeneration (Müller and Sonntag, 1998). Plant regeneration capacity was also strongly affected by genotype in *B. juncea* protoplast cultures (Hu et al. 2004). All regenerants were successfully transferred to MS medium supplemented with 0.1 mg/L NAA for rooting.

Morphological characterization of putative somatic hybrids

Leaf and petiole morphologies of putative somatic hybrids

Based on the morphological features, mature plants were classified into 12 types (Table 2). Generally, broccoli has a deep green, dense cluster of flower buds with narrow petioles, arranged in a tree-like fashion on branches sprouting from a thick edible stalk. This mass of flower heads is surrounded by leaves (Fig. 1A). Leaf mustard has broad green leaves with large white petioles, tightly wrapped in a cylindrical formation and usually forming a compact head (Fig. 1B) like Chinese cabbage (Fig. 1C). Morphological features of some regenerated plants were intermediate between broccoli and leaf mustard or broccoli and Chinese cabbage (Figs. 1D, E). Some plants showed a broccoli-like phenotype (Fig. 1F). No plants with intermediate phenotypes between Chinese cabbage and mustard

Fig. 1. Plant morphological types of somatic hybrids of *B. oleracea* + *B. juncea* + *B. campestris* and their fusion parents. A = *B. oleracea* (broccoli), B = *B. juncea* (leaf mustard), C = *B. campestris* (Chinese cabbage), D-I = Somatic hybrids were produced through tri-parental fusion, J = Leaf types of three fusion parents of *B. oleracea*, *B. juncea*, *B. campestris*, K-M = Leaf types of somatic hybrids (broccoli-like leaves, intermediate leaves, curly leaves) and N-Q = Blenching and Flowering types of somatic hybrid

were observed. Most of the regenerated plants showed uniquely specific phenotypes, typical of putative hybrids of triple-fusion (Figs. 1G-I).

With regard to leaf division and marginal incisions of plants, the regenerated plants were classified into crenate/lyrate and undulate/lyrate types (Table 2). The petioles and midvein enlargement were also intermediate and narrow, as seen in Fig. 1J-M. The leaves of the regenerants were thick and deep green and covered with a waxy coating, similar to broccoli. Some of the hybrids had curled-up leaves (Fig. 1M). Under greenhouse conditions, the majority of the plants began to bolt without vernalization after 2 months of cultivation, similar to broccoli, whereas the parental *B. campestris* and *B. juncea* lines did not show bolting. However, the branching pattern of the floral apex in *B. juncea* and *B. campestris* was characterized by single compact heads made up of irregularly packed subheads and by loosely branched, small, terminal heads (Figs. 1N-Q).

Flower morphology of putative somatic hybrids

The shape, size, and colour of the flowers also exhibited morphological diversities (Fig. 2) that were distinguishable from the parental plants (Figs. 2A-C). The regenerated plants had the following flower types: large-sized flowers with round petals (Figs. 2D-H), medium-sized flowers with oval petals (Fig. 2I-N), and flowers with no obvious cross shape with yellow-striped petals (Fig. 2O). The pistil-stamen ratio also showed 3 typesviz., thick long pistils with short stamens (Fig. 2P), medium thick pistils with long stamens, or similar

Fig. 2. Flower morphological types of somatic hybrids of *B. oleracea*+*B. juncea*+ *B. campestris* and their fusion parents. A = *B. oleracea*, B = *B. juncea*, C = *B. campestris*, D-E = Somatic hybrids with large petals, F-G = Somatic hybrids with curly large petals, H-K = Somatic hybrids with small petals and long pistil, L = Stamen changed into carpelred (black arrow) was found in somatic hybrids, M = Somatic hybrids without stamens, N = Somatic hybrids with long stamens and petals, O = Somatic hybrids with striped petals, P = Types of pistil and stamens in somatic hybrids, Q = Types of petals in somatic hybrids, R = Siliques in somatic hybrids and S = Empty siliques

length for pistils and stamens. Five types of petal shape were also observed, namely: long and thin, short and thin, long and round, short and round or short and round, but curled (Fig. 2Q). Most of the putative somatic hybrids had diverse silique (Fig. 2R) morphologies and some showed male sterility, with empty silique (Fig. 2S). In particular, 2 regenerants showed well-developed petals and pistils, but their stamens changed into carpel or disappeared (Figs. 2L, M).Three regenerants flower with normal pollen, and developed seeds through open pollination. In *Brassica* species, Ogura CMS induces numerous floral abnormalities such as petaloid anthers, short and stumpy crooked styles, reduced nectaries, low female fertility, and severe leaf chlorosis (Kirti et al. 1995; Liu et al. 1995). In brief, 3 types of male sterile types were observed in the present study: one type had stamens that were significantly shorter than the stigma length, the second type had flowers with normal stamens and empty pollen, and the third type had normal flowers with stamen carpeloid. In contrast, somatic hybrids derived from 2 parental cell fusions had empty anthers (Lian et al. 2011a) or low fertility (Lian et al. 2011b). These results indicate that tri-parental somatic distant hybridization can not only introduce new characteristics from donors into phylogenetically close plants such as the CMS trait with wider range and greater ease, but can also create new variant types beyond the variation present in the bi-parental fusion. These somatic hybrids may provide a wide range of genetic resources for breeding programs.

The regenerants were backcrossed with *B. juncea*. Leaf blade and petiole of *B. juncea* was thin with prominent pubescence, the edge of leaf and petiole was red-brown. Progenies also, seem like parental plant, but their petioles were wider than petioles of parental. Their leaves had serrate incrustation. Most seeds can be obtained from hybrids with chromosome numbers ranging 52-56 after backcrossing with *B. juncea*. However, the seed set within the group of hybrids significantly varies. Regenerated plants chromosome numbers ranging 58-64, had very poor fertility, rarely obtained seeds after backcrossing.

Ploidy estimation using flow cytometry and chromosome counting

Flow cytometry analysis revealed variations in the ploidy level of the somatic hybrids and the typical position of the histograms of the fluorescence was obtained. *B. campestris* showed one peak with a diploid at around channel 75 (Fig. 3A), according to the Partec User Manual. The peak of the *B. oleracea* diploid was located at around channel 50 (Fig. 3B), *B. juncea* was located at around channel 75 (Fig. 3C). (Table 1, No. 2, 2n = 38; No. 12, 2n = 54; and No 5, 2n = 64, respectively). The peaks of seven plants grouped from No. 2 appeared at the channel near 150, indicating that the tetraploid somatic hybrid was derived from a combination of two parents (Fig. 3D). The peaks of another thirteen plants from No. 12 appeared around at near the channel 250, indicating that the hexaploid somatic hybrid regenerated from cell fusion (Fig. 3E). The peaks of the other five plants from No.5 peaks appeared around at channel 350, suggesting that the polyploid plant was obtained through protoplast fusion and cell (Fig. 3F).

Based on the morphological characteristics shown in Table 1, 12 plants were selected for determination of chromosome numbers. Chromosome counting of the hybrids revealed that the number of chromosomes in the hybrid cells was not simply the sum of parental sets but that it mostly varied between 18 and 64 (Table 2, Figs. 5A-C). No allopolyploid (genome: AAAABBCC, 2n = 74) somatic hybrids were produced, and no parental plants were obtained except in broccoli. Nearly 50% of putative hybrids, having about 52-56 number of chromosomes, and had higher regeneration ability than hybrid clones with more or fewer chromosomes (Table 1). This suggested that the regeneration capacity of hybrids relates not only to the use of “three parents” but also to the genetic balance in the hybrids (Zhou et al. 2001).

Chromosome elimination is more common in hybrids between distantly related species than in hybrids between closely related ones (Akagi et al. 1995). Chromosome elimination in symmetric fusion was attributed to differences in the cell cycle of the remote parents, and mutation events were induced during fused cell growth and regeneration after protoplast fusion, as well as by the interaction between extranuclear and nuclear genomes in somatic cell lines (Li et al. 2004). Furthermore, Harms (1983) proposed that gross genomic imbalances would result if the fused cells differ in their ploidy levels or if the fusion event involves more than 2 cells. Therefore, the products of multiple fusions may have to face developmental disadvantages, which would preclude their manifestation as somatic hybrid plants. This concept could explain the present results showing the absence of allopolyploids despite the production of somatic hybrids by interspecific triple fusion.

Table 2. Characterization of somatic hybrids plants derived from tri-parental fusion among *B. campestris*, *B. juncea*, and *B. oleracea* based on leaf margin, petiole, leaf color, flower color, and floral apex branching pattern

Shape of leaf margin	Petiole	Leaf colour	Floral apex branching patternmn	Total no. of plants	Chromosome numbers (2n)
Crenate/lyrate	Narrow	Deep green	Single compacted he4ad of irregularly packed subheaded	13	18
Crenate/lyrate	Intermediate	Green	Loosely branched small terminal heads	7	38
Crenate/lyrate	Intermediate	Deep green	Single compacted head of irregularly packed subheaded	4	54
Undulate/lyrate	Narrow	Deep green	Single compacted head of irregularly packed subheaded	3	54
Crenate/lyrate	Narrow	Deep green	Single compacted head of irregularly packed subheaded	5	64
Crenate/lyrate	Narrow	Deep green	Single compacted head of irregularly packed subheaded	5	58
Crenate/lyrate	Intermediate	Green	Single compacted head of irregularly packed subheaded	6	56
Crenate/lyrate	Narrow	Green	Single compacted head of irregularly packed subheaded	7	52
Crenate/lyrate	Intermediate	Deep green	Loosely branched small terminal heads	7	52
Crenate/lyrate	Narrow	Deep green	Loosely branched small terminal head	2	48
Crenate/lyrate	Narrow	Green	Loosely branched small terminal heads	1	44
Crenate/lyrate	Intermediate	Green	Loosely branched small terminal heads	13	54

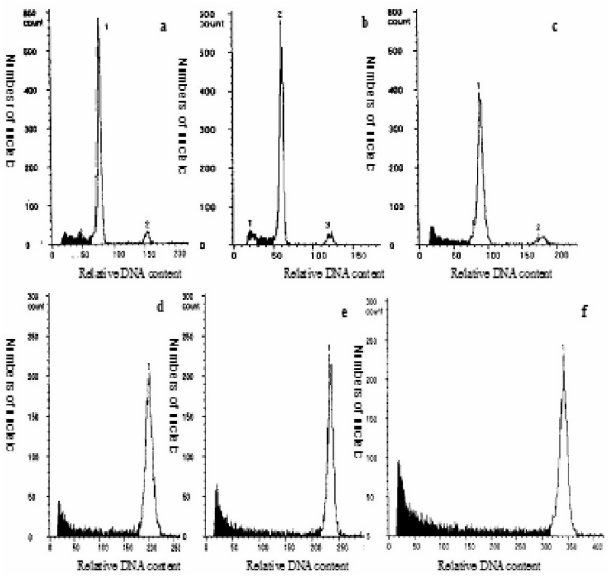

Fig. 3. Histogram of the fluorescence intensities for isolated cells from chopping leaves of fusion partners and somatic hybrids. A = *B. campestris* (Chinese cabbage), B = *B. oleracea* (cabbage), C = *B. juncea* (leaf mustard), D = Tetraploid somatic hybrids, E = Hexaploid somatic hybrids and F = polyploid somatic hybrids

PCR analysis

PCR performed with primers specific for the Ogura CMS region of mtDNA enabled the screening of a large numbers of regenerated plants and the identification of CMS hybrids. A total 70 regenerated plants, a 0.5-Kb band was detected (Fig. 4), only three plants showed missing bands, this is consistent with the results of flower morphological investigation. This approach was used by Akagi et al. (1995) to select CMS plants at an early stage of plant regeneration from rice protoplasts. Sigareva and Earle (1997) also used this technique to select CMS plants obtained from somatic hybridization between Ogura CMS broccoli and fertile cabbage. In the present study, >90% of plants produced by tri-parental protoplast fusions were scored as sterile, by PCR using the Ogura CMS-specific primers (Fig. 4). Somatic hybrids have high rate male sterility, probably due to transferring of CMS trait through cell fusion. In addition, introduction of too much exotic genetic material accompanying the expected gene(s) and genetic imbalance leading to somatic incompatibility. These limitations could cause either abnormal growth and development of the somatic hybrids or regeneration of hybrids with low fertility (Liu et al. 2005).

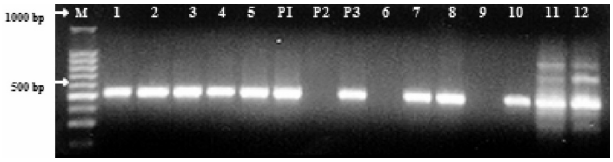

Fig. 4. PCR analysis using CMS-specific primers to confirm the presence of the CMS trait. M: Marker, P1 = *B. campestris* with CMS, P2 = *B. juncea*, P3 = *B. oleracea* with CMS, Lanes 1-5, 7-8, 10-12 = Somatic hybrids with the CMS-specific band; lane 6 and 9 = fertile somatic hybrids

GISH of somatic hybrids and their progenies

GISH not only enables the distinction of the parental chromosomes in a large number of interspecific and intergeneric hybrids, but also the detection of genomic constitution and chromosome rearrangements (Ji et al. 2004; Zhang et al. 2006; Ge et al. 2007). In the present study, introgression of genome A was confirmed by the hybridization of the *B. campestris* probe with somatic hybrids. Figures 5D-F showed that in the majority of chromosomes, the A genome was dominant, suggested that in tri-parental fusion experiment, *B. juncea* cell and *B. campestris* cell with more compatibilities which was dominant in the hybrid cells so that the alien chromosomes could be rejected. Chromosome number and morphological traits have also supported these results. Whereas, *B. campestris* signals (A genome) were distributed in certain parts of the chromosomes, indicating that the introgression of the 3 parental genomes occurred through protoplast fusion, despite the absence of distinct *B. campestris* morphological features in the regenerated plants.

B. juncea probes (AB genome) (labelled in pink) distributed throughout the chromosomes of putative somatic hybrids (Fig. 5G). As shown in Figs. 2H-I, signals derived from *B. oleracea* (C genome) were detected in the centromere of chromosomes. No intact chromosomes from *B. campestris*, or *B. oleracea* were observed in these regenerants. GISH in *Brassica* is normally characterized by strong signals at centromeric heterochromatin and weak hybridization on chromosome arms, for the low copy numbers of dispersed repeated sequences in *Brassica* and related genera (Snowdon et al. 1997; Yao et al. 2010).

Hybrid plant regeneration always needs chromosomes elimination in some degree to reduce the imbalance of hereditary substances between both parents (Song et al. 1999), but the elimination has a

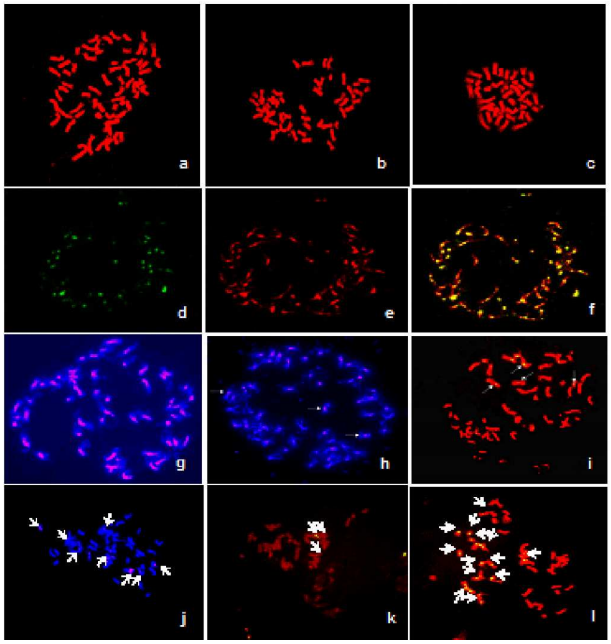

Fig. 5. Cytology of somatic hybrids within *B. oleracea, B. campestris* and *B. juncea*. A-C = Mitotic cells of somatic hybrids (2n=64, 2n=54, 2n=46), counterstained by propidium iodide (PI), D-F = GISH analysis of somatic hybrids in *Brassica* species using A-genome probe (yellow signals), D = FITC signals, E = PI counter staining, F = Synthesis of D and E, G = GISH analysis of somatic hybrids in *Brassica* species using *B. juncea* (AB genome, leaf mustard) probe (pink), H-I = GISH analysis of somatic hybrids in *Brassica* species using C-genome probe (pink and yellow signals), J-L = GISH analysis of first progenies obtained by backcrossing with *B. juncea*, J = Chromosome sets of first progeny; pink signals (arrows) are *B. juncea* probe, K-L = Chromosome sets of first progeny; yellow signals (arrows) are *B. juncea* probe

limitation. Although there are many factors influencing the chromosome elimination in somatic hybrids, but we suggest that the most important reason for chromosome elimination in tri-parental hybrids was hereditary imbalance.

To identify crossing-over and introduction of *B. juncea* genome in BC1 progenies, three plants were selected for GISH analysis. The regenerants, whose chromosome numbers ranged from 46 to 50, were selected for GISH analysis. As expected, BC_1 progenies showed irregular chromosome numbers between of 46, 48, and 50 (Figs. 5J-L). GISH analysis

also revealed red or yellow staining of chromosomes in the BC_1 population of *B. juncea* origin and similar chromosome numbers among BC_1 plants, despite the fact that the *B. juncea* signal distributed on 12, 3, and 22 sites of the BC_1 progrnies, respectively (Figs. 5J-L). These results show that the possibility of utilizing triple somatic hybridization to improve the gene pool of *Brassica* crops is affected by the degree of genetic diversity within the species.

In conclusion, tri-parental somatic hybrids between *B. campestris*, *B. oleracea*, and *B. juncea* were successfully produced, enabling exploitation of valuable trait diversity in *Brassica* species to broaden the genetic pool for breeding of *Brassica* species. The most significant advantage of this method is a higher probability of recombination between nuclear and cytoplasmic organelles than in somatic hybridization between 2 fusion partners, which may provide more genetic diversity for future breeding programs. This technique can facilitate the production of new crops with new genetic compositions, as well as the transfer of traits of interest such as CMS. In addition, this method can increase the frequency of the transfer of traits of interest to more than twice that achieved using fusion between 2 parents. Somatic hybridization would, therefore, facilitate the generation of new genetic resources with extensive variations by backcrossing with parental plants, thereby making hybrids accessible to advanced utilization during plant breeding.

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