

## Intergeneric somatic hybridization and its application to crop genetic improvement

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### Abstract

Related or distant species of cultivated crops are a large pool of many desirable genes. Gene transfer from these species through conventional breeding is difficult owing to post- and pre-zygotic sexual incompatibilities. Somatic hybridization via protoplast fusion is a possible alternative for gene transfer from these species to cultivated crops. Since the early days of somatic hybridization many intergeneric somatic hybrids have been developed through symmetric fusion, asymmetric fusion and microfusion. Somatic hybrids are mainly selected by using markers such as specific media or fusion parents with special features, biochemical mutants, antibiotic resistance and complementation strategy. The hybridity of the regenerants is determined based on morphological, cytological and molecular analysis. The inheritance patterns of nuclear and cytoplasmic genomes in the somatic hybrids are diverse. Nuclear DNA from both fusion parents co-exists congruently in some hybrids with translocation and rearrangement of chromosomes, but spontaneous elimination of chromosomes from either or both fusion parents has been observed very often. In asymmetric fusion, chromosome elimination is an important issue that is a complicated process influenced by many factors, such as irradiation dose, phylogenetic relatedness, ploidy level of fusion parent and regenerants. As for chloroplast genome, uniparental segregation is mainly detected, though co-existence is also reported in some cases. The mitochondrial genome, in contrast to chloroplast, undergoes recombination and very frequent rearrangements. Somatic cell fusion has potential applications for crop genetic improvement by overcoming sexual incompatibility or reproductive barriers, and by realizing novel combinations of nuclear and/or cytoplasmic genomes.

### Introduction

Crop production is affected by biotic and abiotic stresses, such as bacterial, fungal and viral diseases and adverse environment. Genetic improvement of cultivated species to withstand these stresses is key to successful crop production. Related or distant genera of cultivated crops contain a large reservoir of genes covering a variety of desirable traits. Tapping and utilization of this germplasm has great potential for crop improvement. Nevertheless, it is

hard to transfer the desirable traits present in this germplasm to the cultivated species via conventional breeding ways owing to some unexpected impediments, such as sexual incompatibility. Other barriers like polyembryony, female and/or male sterility in some crops further restrain the chances for recombination and segregation of desirable traits. As a result, gene flow from the related or distant genera to the cultivated species is minimized. It is of great significance to explore other breeding alternatives to complement the traditional

way, so as to fully use the related or distant genera for crop genetic improvement. Somatic hybridization, involving mainly somatic cells, could circumvent the aforementioned barriers and is a possible choice for gene(s) transfer between intergeneric, sexually compatible or incompatible, combinations for effective use of valuable germplasm. Since the first tobacco somatic hybrid was generated much progress has been made in this area (reviewed by Grosser et al., 2000; Johnson and Veilleux, 2001; Orczyk et al., 2003). Intergeneric somatic hybridization has been done via symmetric fusion, asymmetric fusion, and microfusion, which could give rise to symmetric hybrids, asymmetric hybrids and cybrids in terms of nuclear constitution (Figure 1). Many plant species have been used in intergeneric somatic hybridization, which are listed in Table 1. The present review specifically looks at intergeneric somatic hybridization with emphasis on reviewing treatments of donor and recipients in asymmetric fusion, methods of selection and identification of somatic hybrids, nuclear and cytoplasmic inheritance patterns in hybrids and realized and future applications of somatic hybridization in crop improvement.

#### Symmetric versus asymmetric fusion and treatments in asymmetric fusion

Since the first tobacco interspecific somatic hybrids were produced through symmetric fusion (Carlson et al., 1972), a large number of somatic hybrids have been produced. In most cases, fusion of two divergent parents leads to hybrids that combine nuclear genomes from both fusion parents, resulting in regeneration of symmetric hybrids. Incorporation of total genomes of the two parents, especially nuclear ones, in a hybrid has two obvious disadvantages, 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 (Wang et al., 1989; Sherraf et al., 1994; Spangenberg et al., 1994; Begum et al., 1995; Kisaka et al., 1998; Hu et al., 2002b; Wang et al., 2003). For example, the somatic hybrids between *Arabidopsis thaliana* and *Brassica napus*, *Lycium barbarum*

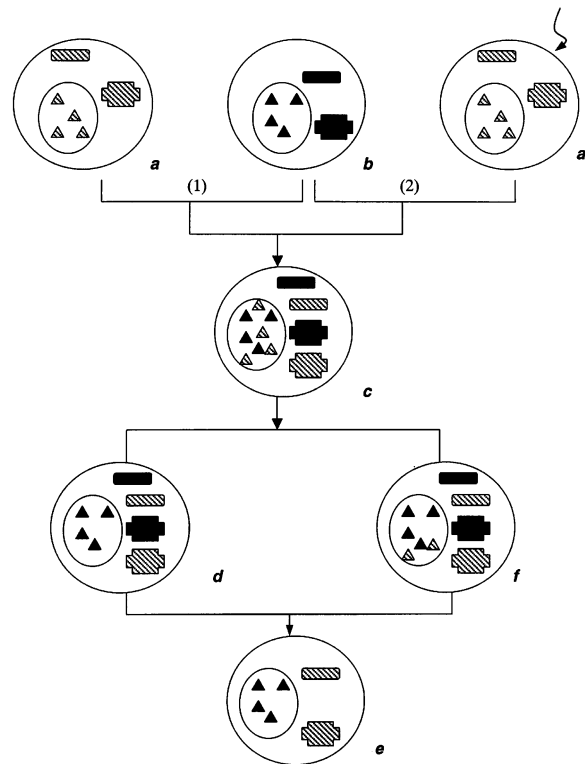


Figure 1. Schematic diagram of symmetric, asymmetric hybrids and cybrids derived from protoplast fusion in terms of nuclear compositions. Triangles indicate the nuclear genomes, rectangles indicate the mitochondrial genomes and the cruciform frames indicate the chloroplast genomes. a and b are the fusion parents. c is the symmetric hybrids derived from fusion between a and b. d and e are cybrid (alloplasmic hybrid) between a and b. f is the asymmetric hybrid between a and b. (1) and (2) are symmetric and asymmetric fusions, respectively. Zigzag arrow denotes the irradiation treatment of parent a. Note: this figure is only simple illustration of the three kinds of hybrids and the nuclear and cytoplasmic genomes are not representatives of any crop species.

and tobacco (*Nicotiana tabacum*) did not develop roots (Bauer-Weston et al., 1993; Liu et al., 1995b). In addition, for some combinations no plants can be produced by symmetric fusion (Gupta et al., 1984). Therefore, efforts should be made to reduce the input of nuclear genome of the wild relatives into the hybrids. Asymmetric fusion allows transfer of partial genomes from one species to another. In some asymmetric fusions both the donors and recipients are subjected to treatment to limit the input of nuclear genome into the hybrid. But in most of the cases treatment is only given to the donor.

Table 1. Tabulation of intergeneric symmetric and asymmetric fusions

Parents		Fusions	
1	2	Symmetric fusions	Asymmetric fusions or microfusions
<i>Apium</i>	<i>Daucus</i>	Wang et al. (1989)	
<i>Atropa</i>	<i>Datura</i>	Krumbiegel and Schieder (1979)	
	<i>Hyoscyamus</i>	Ahuja et al. (1993)	
	<i>Nicotiana</i>	Gleba et al. (1982), Babiychuk et al. (1992), Kushnir et al. (1987, 1991) and Yemets et al. (2000)	Gleba et al. (1988) and Yemets et al. (2000)
<i>Brassica</i>	<i>Arabidopsis</i>	Gleba and Hoffmann (1978, 1980) and Hoffmann and Adachi (1981)	Bauer-Weston et al. (1993), Forsberg et al. (1998a, b), Siemens and Sacristan (1995) and Yamagishi et al. (2002)
	<i>Camelina</i>	Narasimhulu et al. (1994)	
	<i>Crambe</i>		Wang et al. (2003, 2004)
	<i>Moricandia</i>	Kirti et al. (1992a, 1998)	O'Neill et al. (1996)
	<i>Sinapis</i>	Hansen and Earle (1997), Primard et al. (1988), Gaikwad et al. (1996), Hu et al. (2002a), Lelivelt et al. (1993) and Toriyama et al. (1987a)	
	<i>Raphanus</i>	Pelletier et al. (1983), Arumugam et al. (2002), Sakai and Imamura (1990), Lelivelt and Krens (1992), Kameya et al. (1989), Hagimori et al. (1992) and Yamanaka et al. (1992)	Sakai and Imamura (1992) and Sakai et al. (1996)
	<i>Trachystoma</i>	Kirti et al. (1992b)	
	<i>Barbarea</i>	Fahleson et al. (1994a)	
	<i>Diploxys</i>	Begum et al. (1995), Kirti et al. (1995) and Klimaszewska and Keller (1988)	Chatterjee et al. (1988)
	<i>Orychophragmus</i>	Hu et al. (2002b) and Vasilenko et al. (2003)	Hu et al. (2002b)
	<i>Thlaspi</i>	Fahleson et al. (1994b) and Brewer et al. (1999)	
	<i>Armoracia</i>	Navrátilová et al. (1997)	
	<i>Camelina</i>	Hansen (1998) and Sigareva and Earle (1999a)	
	<i>Capsella</i>	Sigareva and Earle (1999b)	
	<i>Eruca</i>	Fahleson et al. (1988)	Sikdar et al. (1990)
	<i>Lesquerella</i>		Skarzhinskaya et al. (1996)
<i>Bupleurum</i>	<i>Vitis</i>		Song et al. (1999)
<i>Cichorium</i>	<i>Helianthus</i>	Rambaud et al. (1993)	
<i>Citrus</i>	<i>Feroniella</i>	Takayanagi et al. (1992)	
	<i>Fortunella</i>	Deng et al. (1992), Grosser et al. (1996), Ollitrault et al. (1996), Liu and Deng (2000b), Costa et al. (2003) and Takami et al. (2004)	
	<i>Poncirus</i>	Ohgawara et al. (1985), Grosser et al. (1988b), Ohgawara et al. (1991), Grosser et al. (1996), Ollitrault et al. (1996) and Guo et al. (2002)	Vardi et al. (1987) and Liu and Deng (2000a)
	<i>Citropsis</i>	Grosser et al. (1990), Ling and Iwamasa (1994) and Grosser et al. (1996)	
	<i>Swinglea</i>	Takayanagi et al. (1992) and Motomura et al. (1995, 1997)	
	<i>Aegle</i>	Motomura et al. (1995, 1997)	
	<i>Glycosmis</i>		
	<i>Merrillia</i>		

Table 1. (Continued)

Parents		Fusions	
1	2	Symmetric fusions	Asymmetric fusions or microfusions
	<i>Murraya</i>	Shinozaki et al. (1992) and Guo and Deng (1998)	
	<i>Atlantia</i>	Louzada et al. (1993) and Grosser et al. (1996)	
	<i>Clausena</i>	Guo and Deng (1999) and Fu et al. (2003)	
	<i>Feronia</i>	Grosser et al. (1996)	
	<i>Microcitrus</i>	Grosser et al. (1996), Motomura et al. (1995, 1997), Liu et al. (1999, 2000, 2002 a, b) and Xu et al. (2004)	Vardi et al. (1989) and Liu and Deng (1999)
	<i>Severinia</i>	Grosser et al. (1988a, 1996, 2000) and Motomura et al. (1995, 1997)	
<i>Cucumis</i>	<i>Cucurbita</i>	Yamaguchi and Shiga (1993) and Zhang and Liu (1998)	
<i>Daucus</i>	<i>Hordeum</i>	Kisaka et al. (1997)	
<i>Dendranthema</i>	<i>Artemisia</i>	Furuta et al. (2004)	
<i>Dianthus</i>	<i>Gypsophila</i>	Nakano and Mii, (1993) and Nakano et al. (1996)	
<i>Duboisia</i>	<i>Nicotiana</i>	Endo et al. (1988)	
<i>Festuca</i>	<i>Lolium</i>	Takamizo et al. (1991)	
<i>Fortunella</i>	<i>Poncirus</i>	Miranda et al. (1997)	
<i>Glycine</i>	<i>Nicotiana</i>	Kao (1977) and Chien et al. (1982)	
	<i>Lotus</i>	Kihara et al. (1992)	
	<i>Oryza</i>	Niizeki et al. (1985)	
<i>Helianthus</i>	<i>Cichorium intybus</i>		Varotto et al. (2001)
<i>Hibiscus</i>	<i>Lavatera</i>	Vazquez-Thello et al. (1996)	
<i>Hyoecyanmus</i>	<i>Nicotiana</i>	Potrykus et al. (1984), Kishinami and Widhlo (1987) and Zubko et al. (1996)	Imamura et al. (1987) and Zubko et al. (2002)
	<i>Scopolia</i>	Zubko et al. (1996)	
<i>Lathyrus</i>	<i>Pisum</i>	Durieu and Ochatt, (2000)	
<i>Lolium</i>	<i>Triticum</i>	Chen et al. (1992)	Ge et al. (1997) and Cheng and Xia (2004)
	<i>Festuca</i>		Spangenberg et al. (1994, 1995)
<i>Lotus</i>	<i>Oryza</i>	Nakajo et al. (1994)	
<i>Lycium</i>	<i>Nicotiana</i>	Liu et al. (1995a, b) and Xie et al. (1996)	
<i>Lycopersicon</i>	<i>Nicotiana</i>		Wolters et al. (1993a, b), Ramulu et al. (1995) and Vlahova et al. (1997)
	<i>Solanum</i>	Handley et al. (1986), O'Connell and Hanson (1986), Sakomoto and Taguchi (1991), Gavrilenko et al. (1992), Guri et al. (1988, 1991), Hossain et al. (1994), Sherraf et al. (1994), Schoenmakers et al. (1993, 1994a, b) and Kobayashi et al. (1996),	Melchers et al. (1992), McCabe et al. (1993), Liu et al. (1995) and Samoylov and Sink (1996)
<i>Medicago</i>	<i>Lotus</i>		Kaimori et al. (1998)
	<i>Onobrychis</i>		Li et al. (1993)
<i>Nicotiana</i>	<i>Solanum</i>	Vries et al. (1987), Wan et al. (1988), Toki et al. (1990) and Gilissen et al. (1992)	Perl et al. (1991), Thanh and Medgyesy (1989), Ramulu et al. (1995, 1996a, b), Tempelaar et al. (1991), Wolters et al. (1991) and Schoenmakers et al. (1994a,b)
	<i>Hordeum</i>		Somers et al. (1986)
	<i>Datura</i>	Ye et al. (1987)	
	<i>Salpiglosis</i>		Thanh et al. (1988)

Table 1. (Continued)

Parents		Fusions	
1	2	Symmetric fusions	Asymmetric fusions or microfusions
	<i>Daucus</i>	Kisaka and Kameya (1994)	Kisaka and Kameya (1994) and Dudits et al. (1987)
	<i>Petroselinum</i>		Dudits et al. (1980)
	<i>Petunia</i>	Li et al. (1982), Pental et al. (1986) and Dragoeva et al. (1999)	Glimelius and Bonnett (1986) and Hinnisdaels et al. (1991)
<i>Oryza</i>	<i>Echinochloa</i>	Terada et al. (1987)	
	<i>Hordeum</i>	Kisaka et al. (1998)	
	<i>Panicum</i>		Xin et al. (1997)
	<i>Porteresia</i>	Jelodar et al. (1999)	Finch et al. (1990)
	<i>Zizinia</i>		Liu et al. (1999)
<i>Physalis</i>	<i>Datura</i>		Gupta et al. (1984)
<i>Pyrus</i>	<i>Prunus</i>	Ochatt et al. (1989)	
<i>Rauwolfia</i>	<i>Vinca/Rhazya/Catharanthus</i>	Kostenyuk et al. (1991)	
<i>Saccharum</i>	<i>Pennisetum</i>	Tabaeizadeh et al. (1986)	
<i>Triticum</i>	<i>Avena</i>		Liu and Liu, (1995) and Xiang et al. (2003a, b)
	<i>Aeluropus</i>		Yue et al. (2001)
	<i>Agropyron</i>		Xia et al. (1996, 2003) and Cheng et al. (2004)
	<i>Bromus</i>	Xing et al. (2001)	Xiang et al. (1999)
	<i>Leymus</i>	Huang et al. (1999)	Xia and Chen (1996) and Huang et al. (1999)
	<i>Psathyros-tachys</i>	Xing et al. (2001)	Xia et al. (1996)
	<i>Pennisetum</i>	Vasil et al. (1988)	
	<i>Setaria</i>		Li et al. (2001) and Xiang et al. (2004)
	<i>Haynaldia</i>	Zhou et al. (2001b)	Xia et al. (1998) and Zhou et al. (2001a, b, 2002a, b)
	<i>Zea</i>	Wang et al. (1993) and Szarka et al. (2002)	Xu et al. (2003)
<i>Vicia</i>	<i>Helianthus</i>	Schnabl et al. (1999)	
<i>Vinca</i>	<i>Catharanthus</i>	Kostenyuk et al. (1991)	

### Treatment of the donor protoplasts

The above discussion indicates that it is necessary to minimize the introduction of genomes from the fusion parents into the hybrids, which is mainly done by breaking or fragmentating chromosomes using irradiation with X or gamma rays (Dudits et al., 1980; Liu and Deng, 1999; Zubko et al., 2002). Dudits et al. (1980) produced the first intergeneric asymmetric hybrid between X-ray irradiated parsley (*Petroselinum hortense*) protoplasts and tobacco protoplasts. UV is being used more

and more due to its easy access and convenience in use for causing chromosomal breakages (Jain et al., 1988; Xia and Chen, 1996; Xia et al., 1996, 1998, 1999, 2003; Zhou et al., 1996, 2001a, b, 2002a, b; Vlahova et al., 1997; Yue et al., 2001; Xu et al., 2003; Xiang et al., 2003a, b, 2004; Cheng and Xia, 2004). Hall et al. (1992a, b) found that more chromosome breakage was detected in the UV-treated cells than gamma-treated cells at the same biological dosage. In addition to the irradiation, restriction endonuclease, spindle toxin or chromosome condensation agents have also been

used for chromosomal fragmentation (Ramulu et al., 1994; Forsberg et al., 1998b). Treatment of the donor protoplasts with bromodeoxyuridine prior to UV and gamma irradiation could promote formation of highly asymmetric hybrids (Trick and Bates, 1996). In most of the asymmetric fusions, protoplasts that have been purified or are being isolated were irradiated before fusion (Somers et al., 1986; Vlahova et al., 1997; Kaimori et al., 1998; Liu and Deng, 1999; Zubko et al., 2002). But callus, leaf, cell suspension cultures or *in vitro* plantlets have also been used for irradiation prior to protoplast isolation (Tempelaar et al., 1991; Wolters et al., 1991; Hansen and Earle, 1997). Response to the irradiation varied in different tissues. Protoplasts were more sensitive to the irradiation than the cell suspension cultures (Liu and Liu, 1995). It is reasonable since the protoplasts, without the protection by the cell wall, are more fragile than intact cells. Thus, use of protoplasts is, in principle, favorable for causing chromosome fragmentation.

#### *Treatment of the recipient protoplasts*

In order to facilitate hybrid selection the recipient protoplasts are always treated with some metabolic inhibitors, such as IA (Iodoacetic acid) and IOA (Iodoacetamide), which affect the metabolic process of the cells and make them physiologically impaired for cell division when they are cultured independently. The protoplasts treated with the metabolic inhibitors become malformed and finally burst (Glimelius and Bonnett, 1986; Kushnir et al., 1987; Liu and Deng, 1999). But when the protoplasts were fused with the donor protoplasts the heterokaryons can grow due to metabolic complementation.

#### *Effects of the treatments on the production of asymmetric hybrids*

The key issue in irradiation treatment is whether it can really cause chromosome elimination in the resulting hybrids and transfer of only limited amount of genome of the donor parent. A number of studies have shown that irradiation could induce chromosome elimination, which leads to a limited introduction of donor chromosomes in the hybrids. The somatic hybrids between *Atropa belladonna*, used as a donor, and *Nicotiana ta-*

*bacum* contained 11–90% of chromosomes from the former parent (Gleba et al., 1988). The somatic hybrids between Italian ryegrass (*Lolium multiflorum*, donor) and tall fescue (*Festuca arundinaceae*) contained only 20% of the donor chromosomes (Spangenberg et al., 1994). Limited transfer of partial genomes from donor to the recipient has also been reported in other fusions (Xia et al., 1998; Liu and Deng, 1999; Wang et al., 2004). In some cases highly asymmetric hybrids containing only chromosome fragments, or a few chromosomes of the donor parent were obtained (Hinnisdaels et al., 1991; Liu et al., 1999). Somatic hybrids between gamma-ray-irradiated *Zizania latifolia* and rice (*Oryza sativa*) had chromosome number equal to rice, but Southern analysis using both total genomic DNA and moderate-copy *Z. latifolia*-abundant DNA sequences as probes detected signal from the donor, indicating that possibly only chromosome segments from the donor have been integrated into the hybrids (Liu et al., 1999). In addition, creation of cybrids in many asymmetric fusions provides convincing evidence that exposure of the donors to irradiation prior to fusion could cause complete loss of donor chromosomes, in which only cytoplasm from the donor is transferred to the somatic hybrids (Glimelius and Bonnett, 1986; Kushir et al., 1987; Vardi et al., 1987, 1989; Hinnisdaels et al., 1991; Perl et al., 1991; Varotto et al., 2001; Zubko et al., 2002). Compared to symmetric fusion, asymmetric fusion strategies with irradiation of donor protoplasts lead to regeneration of normal plants, as demonstrated in fusions between *Arabidopsis* and *Brassica* (Hoffmann and Adachi, 1981), *Lycopersicon* hybrid and *Solanum melongena* (Guri et al., 1991; Liu et al., 1995a, b). Moreover, irradiation causing reduced input of one of the two parents increased the possibility of producing fertile hybrids (Fahleson et al., 1994b; Forsberg et al., 1998a). For example, all of the symmetric hybrids between *B. napus* and *Lesquerella fendleri* were self-sterile, whereas 38% of the asymmetric hybrids of the same combination were self-fertile (Skarzhinskaya et al., 1996). Likewise, somatic hybrids derived from symmetric fusion between *Orychophragmus violaceus* and *B. napus* were sterile, but fertile hybrids were recovered from asymmetric fusion of the same combination, which could set seeds after selfing or backcrossing (Hu et al., 2002b).

However, pretreatment of the donor protoplasts by irradiation and/or at times the recipient protoplasts by metabolic inhibitors had negative effects on protoplast division and plant regeneration, indicated by retarded cell division, reduced plating efficiency, delayed or reduced plant regeneration, low shoot regeneration frequency and difficulty in rooting, which is possibly due to severe physiological lesions (McCabe et al., 1993; Schoenmakers et al., 1994a; Hansen and Earle, 1997; Forsberg et al., 1998a; Liu and Deng, 1999, 2000b; Wang et al., 2003).

### **Selection and characterization of somatic hybrids**

#### *Selection of somatic hybrids*

Several sorts of cell types can be found in the fusion products, heterokaryonic hybrid, homofusants and unfused parental protoplasts. The frequency of properly fused hybrid cells is always much lower than the parental protoplasts. Without a strategy for identification and selection of hybrids cells, one will have to go through a very time-consuming and tedious process of regenerating plants from a large number of protoplast-derived cell colonies and subsequently identifying hybrids from the population. As a consequence, some strategies that select hybrid cells or enrich these have been very important. A number of selection strategies have been used for the selection of hybrids, of which utilization of biochemical mutants and antibiotics or herbicide resistance are used frequently. As far as biochemical mutants are concerned, mainly three kinds have been used, cytoplasmic chlorophyll deficiency mutant (Toki et al., 1990; Kisaka and Kameya, 1994; Dragoeva et al., 1999), nitrate-reductase deficiency mutant (Kushnir et al., 1991) and albino mutant (Schoenmakers et al., 1993; Zubko et al., 2002). Difference in resistance to specific antibiotic(s), amino acid analogues or herbicides between fusion parents could also expedite hybrid selection when the fusion products are cultured in a medium supplemented with the above-mentioned chemicals (Kisaka et al., 1994; Vlahova et al., 1997; Kulawiec et al., 2003). If each of fusion parents is resistant to a different antibiotic it is more efficient to select their hybrids using a medium containing the two antibiotics (Kushnir et al., 1991; Schoenmakers et al., 1994a, b; Vazquez-Thello

et al., 1996). Selection of double or triple mutants, which are regarded as universal hybridizers, has been proven to be very effective for selecting somatic hybrids (Pental et al., 1984, 1986; Kushnir et al., 1987; Toriyama et al., 1987a; Ye et al., 1987). In addition, genetic and/or metabolic complementation is frequently employed in asymmetric fusions. Protoplasts of the fusion parents have been treated with ionizing radiation or metabolic inhibitors that prevent the division of the unfused or homofused protoplasts (Liu and Deng, 1999; Yamagishi et al., 2002). The heterokaryons could grow smoothly and finally develop into hybrid callus or plants, due to genetic or physiological complementation (Kostenyuk et al., 1991; Wang et al., 1993). In some cases low or no regeneration capacity of parental protoplasts could be used for hybrid selection because the morphogenic potential of the fusants could be restored due to complementation (O'Neill et al., 1996; Xia and Chen, 1996; Xia et al., 1996, 2003; Hansen, 1998; Zhou et al., 2001a, b; Hu et al., 2002a; Xu et al., 2003).

#### *Characterization of the somatic hybrids*

Since the parental protoplasts and hybrid cells can all undergo morphogenesis and in metabolic complementation escapes can also regenerate, it is imperative to identify the hybrid nature of the regenerants, which is mainly done by morphological, cytological, biochemical and genetic markers.

#### *Morphological markers*

Distinct morphology in leaf, flower and other organs or difference in callus color can serve as markers for hybrid identification (Xiang et al., 1999; Zhou et al., 2001b). The somatic hybrids have mainly two kinds of morphology, intermediate between the fusion parents, identical or similar to one fusion parent. The former is primarily seen in symmetric fusions and the latter in asymmetric fusions (Yan et al., 1999; Sigareva and Earle, 1999b; Varotto et al., 2001; Zubko et al., 2002; Hu et al., 2002a; Xia et al., 2003). However, plants with intermediate morphology have also been derived from asymmetric fusion, and regeneration of somatic plants with morphology identical to one of the fusion parents has been reported in many symmetric fusions (Kushnir et al., 1987; Hansen and Earle, 1997; Kisaka et al., 1997; Liu

et al., 1999, 2000; Szarka et al., 2002). It is noteworthy that morphology of plants regenerated from the same fusion event may be different from each other and variation in plant morphology has also been reported (Brewer et al., 1999).

#### *Cytological markers*

Chromosome counting and flow cytometry (FCM) are widely used for determining total chromosome number or ploidy level of the regenerants (Hu et al., 2002a). Some of the reports on somatic hybrids have shown that the chromosome numbers of the hybrids were less than the sum of the chromosome numbers of the fusion parents (Song et al., 1999; Yue et al., 2001; Xiang et al., 2003a, b). Therefore, chromosome number or ploidy level is unsuitable to identify somatic hybrids alone in a reliable manner. But major differences in chromosome morphology and size between the fusion parents could facilitate hybrid verification based on karyotype analysis, which can also reveal parental chromosome contribution to the hybrids. Verification of hybrids via chromosome morphology difference has been used in characterizing a number of somatic hybrids (Kao, 1977; Binding and Nehls, 1978; Chien et al., 1982; Gleba et al., 1982, 1988; Gupta et al., 1984; Kushnir et al., 1987; Endo et al., 1988; Hinnisdaels et al., 1991; Kostenyuk et al., 1991; Babychuk et al., 1992; Ahuja et al., 1993; Wang et al., 1993; Skarzhinskaya et al., 1996; Kisaka et al., 1998; Xing et al., 2001; Szarka et al., 2002).

#### *In situ hybridization*

*In situ* hybridization (ISH), including genomic *in situ* hybridization (GISH) and fluorescence *in situ* hybridization (FISH), a powerful tool for somatic cytogenetics, has been employed frequently to investigate the chromosomal composition of the somatic hybrids (Chevre et al., 1994; Ramulu et al., 1996a, b; Escalante et al., 1998; Skarzhinskaya et al., 1998; Jelodar et al., 1999; Szarka et al., 2002; Xiang et al., 2003b, 2004). ISH can clearly confirm parental origin of the chromosomes present in the hybrids, demonstrating nuclear genomic contribution of each parent (Rutgers et al., 1997; Jelodar et al., 1999; Horsman et al., 2001). Escalante et al. (1998) analyzed tetraploid and hexaploid hybrids between *L. esculentum* and wild nightshade

(*S. lycopersicoides*) and detected an equal number of chromosomes from both fusion parents in the tetraploid hybrids, whereas the hexaploid hybrids contained four sets of tomato chromosomes and two sets of chromosomes of wild nightshade. By ISH it is very effective to know if limited gene transfer occurs in asymmetric fusion and identify the alien chromosomes that are present in the somatic hybrids (Jacobsen et al., 1995; Cheng and Xia, 2004; Wang et al., 2004; Xiang et al., 2004). For example, GISH analysis of the asymmetric hybrids between *Triticum aestivum* and *Setaria italica* (donor) revealed that only chromosome fragments or few chromosomes from the donor were present in the hybrids (Xiang et al., 2004). In addition, ISH can give an insight into intra or intergenomic translocation and chromosome rearrangements which has been detected in somatic hybrids of *Z. latifolia* and *O. sativa* (Liu et al., 1999), *L. esculentum* and *S. tuberosum* (Wolters et al., 1994), *T. aestivum* and *Haynaldia villosa* or *Agropyron elongatum* (Xia et al., 1998; Zhou et al., 2001a), *T. aestivum* and *Zea mays* or *Avena sativa* (Szarka et al., 2002; Xiang et al., 2003b), *B. napus* and *Lesquerella fendleri* (Skarzhinskaya et al., 1998), etc. Besides, ISH can detect preferential chromosome elimination, screen monosomic addition line and investigate cytogenetic behavior of the somatic hybrids that are used in the sexual crosses (Wolters et al., 1994; Jacobsen et al., 1995; Garriga-Caldere et al., 1997; Ali et al., 2000, 2001; Gavrilenko et al., 2001; Wang et al., 2004).

#### *Isoenzyme and Fraction-I protein*

Isoenzymes are the most widely used biochemical way for identifying somatic hybrids (Wetter and Kao, 1980; Dragoeva et al., 1999). Isoenzymes of esterase (Bauer-Weston et al., 1993), peroxidase (Begum et al., 1995; Xia et al., 1998; Xu et al., 2003), phosphoglucose isomerase (Fahleson et al., 1994b), phosphoglucomutase (Ochatt et al., 1989), glutamate aspartic aminotransferase (Jain et al., 1988), phosphatase (Yamaguchi and Shiga, 1993), menadione reductase (Klimaszewska and Keller, 1988) and leucine aminopeptidase (Hansen and Earle, 1997) have been used for confirming hybrid nature of regenerants from intergeneric fusions. Subunits of Fraction-I protein, RUBPCase, have been used to characterize somatic hybrids based on isoelectric focusing (Shepard et al., 1983). Small



subunits of RUBPCase identify the nuclear composition of the somatic hybrids, whereas large subunits of RUBPCase can detect parental chloroplasts (Chen et al., 1977; Melchers et al., 1978).

#### *Molecular markers*

Use of molecular markers is ideal for hybridity confirmation. To date, several molecular markers have been used, such as randomly amplified polymorphism DNA (RAPD), restriction fragment length polymorphism (RFLP), simple sequence repeat (SSR), amplified fragment length polymorphism (AFLP), cleaved amplification polymorphic sequences (CAPS), intersimple sequence repeat (ISSR) and 5S rDNA spacer sequence (Sakomoto and Taguchi, 1991; Bauer-Weston et al., 1993; Hansen and Earle, 1997; Zubko et al., 2002; Xia et al., 2003). Species-specific satellite DNA sequence was also used for hybrid identification (Schweizer et al., 1988). Dot blot hybridization with species specific repetitive DNA probes and use of DNA clone as probe can identify quantitative contribution of parental DNA in the somatic hybrids (Moore and Sink, 1988; Wolters et al., 1993a; Samoylov and Sink, 1996).

#### **Inheritance of nuclear and cytoplasmic genomes in somatic hybrids**

Nuclear and cytoplasmic genomes of phylogenetically distant species are brought together in a common cytoplasmic milieu in somatic hybrids, leading to nuclear–nuclear, nuclear–cytoplasmic and/or cytoplasmic–cytoplasmic interactions, from which diverse nuclear and cytoplasmic genomes could arise.

#### *Inheritance of nuclear genomes*

##### *Inheritance of nuclear genomes in symmetric fusion*

In some hybrids the nuclear genomes from both fusion parents are compatible and could remain together in somatic hybrids (Sakomoto and Taguchi, 1991; Kirti et al., 1992a, b; Lelivelt and Krens, 1992; Gaikward et al., 1996; Nakano et al., 1996; Kisaka et al., 1997; Kisaka et al., 1998;

Jelodar et al., 1999). However, in a number of somatic hybrids derived from symmetric fusions, full nuclear complements are not present. Firstly, rearrangements and recombinations have been detected in many hybrids (Hoffmann and Adachi, 1981; Kostenyuk et al., 1991; De Jong et al., 1993; Begum et al., 1995; Vlahova et al., 1997; Escalante et al., 1998; Liu et al., 1999; Szarka et al., 2002). Secondly, spontaneous chromosome elimination of either or both parents has been extensively reported, leading to regeneration of asymmetric hybrids or cybrids (Gleba et al., 1988; Babiychuk et al., 1992; Gilissen et al., 1992; Ahuja et al., 1993; Nakano et al., 1996; Kisaka et al., 1997; Navrátilová et al., 1997; Liu et al., 1999; Hu et al., 2002a). For instance, somatic hybrids between *A. belladonna* and *Datura innoxia* contained few chromosomes of *A. belladonna* and full complement of *D. innoxia* (Krumbiegel and Schieder, 1979). Protoplast symmetric fusion between nightshade (*A. belladonna*) and tobacco (*N. tabacum*) gave rise to highly asymmetric and fertile somatic hybrids containing only one small chromosome of nightshade in addition to the whole tobacco genome (Babiychuk et al., 1992). It is proposed that spontaneous asymmetry was possibly necessary for normal division and morphogenesis of the hybrid cells derived from remote combinations (Chen et al., 1992; Liu et al., 1995b; Song et al., 1999).

##### *Inheritance of nuclear genomes in asymmetric fusion*

Translocations and recombinations have also been detected in the hybrids derived from asymmetric fusions, as an example between *N. tabacum* and *Petunia hybrida* (Hinnisdaels et al., 1991), *T. aestivum* and *H. villosa* (Xia et al., 1998). Compared with symmetric fusion chromosome loss of the donors is possibly the most prominent event for the nuclear genomes in asymmetric fusion. No fixed rules on chromosome elimination are conclusive since contradictory results are reported on different species or by different scientists. Some reported that many donor chromosomes were present in the asymmetric hybrids (Imamura et al., 1987; Wolters et al., 1991), whereas in others extensive chromosome elimination took place (Dudits et al., 1980, 1987; Gupta et al., 1984; Gleba et al., 1988; Hinnisdaels et al., 1991). In addition,

elimination of recipient chromosomes was detected in some fusion combinations, as shown in the somatic hybrid between carrot (donor) and rice (Kisaka et al., 1994). Even the regenerants from the same fusion event may vary from each other in terms of extent of chromosome elimination (Hoffmann and Adachi, 1981; O'Connell and Hanson, 1986, Endo et al., 1988; Spangenberg et al., 1994; Chen et al., 2000; Xia et al., 2003). Therefore, chromosome elimination is quite complex and the underlying mechanism for chromosome elimination is not clear and in all probability is affected by several factors, such as irradiation dosage, phylogenetic relationship, ploidy level of the parents and the fusion products, etc.

#### *Irradiation dosage*

In some reports chromosome elimination was exacerbated with increase in irradiation dose (Spangenberg et al., 1994; Trick et al., 1994; Schoenmakers et al., 1994a, b; Forsberg et al., 1998a; Wang et al., 2003). In a case study, when 10 Gy irradiance was employed no chromosome elimination happened in the somatic hybrids of X-ray irradiated *L. multiflorum* and *F. arundinaceae* protoplasts, while 85–100% of the donor chromosomes were lost when the irradiation dose was increased to 500 Gy (Spangenberg et al., 1994). Similar results have been obtained in asymmetric hybrids between UV-irradiated *Avena sativa* and wheat protoplasts (Xiang et al., 2003a, b). However, positive correlation between irradiation dosage and chromosome elimination was not observed in many reports, in which limited elimination of chromosomes in the hybrids occurred when high irradiation dose was exerted to the donor protoplasts (Imamura et al., 1987; Gleba et al., 1988; Wolters et al., 1991; Bauer-Weston et al., 1993; McCabe et al., 1993). The conflicting results may be attributed to the fact that irradiation-caused chromosome elimination is not exclusive but possibly affected by other elements like genotype, irradiation type, phylogenetic relationship between the fusion parents and physiological status of the irradiated materials (Samoylov and Sink, 1996; Xia and Chen, 1996). Additionally, dose-effect can be modified by the repairing facility present in the irradiated cells (Tempelaar et al., 1991).

#### *DNA content of the fusion parents and the regenerants*

It is reported that the ratio between the donor and the recipient DNA contents can affect chromosome elimination. The larger the ratio, the less serious was chromosome elimination, as revealed in the asymmetric fusions of *L. pennellii* with eggplant (*S. melongena*), *L. esculentum* with *S. tuberosum* and carrot (*Dacus carota*) with *N. tabacum*. As for the combination *L. esculentum* with *S. tuberosum* (donor) with a ratio of 1.8:1 most of the potato chromosomes were present in the hybrids (Wolters et al., 1991). Extensive chromosome elimination was detected in the hybrid callus derived from fusion between *L. pennellii* (donor) and eggplant, the ratio of which was 1:1.22 (Samoylov et al., 1996). In the combination between carrot (donor) and *N. tabacum*, the ratio being 1:9.4, highly asymmetric hybrids containing only one chromosome from carrot were produced (Dudits et al., 1987). Ploidy level of the regenerants also affects the presence of species-specific chromosomes. The somatic hybrid plants derived from fusion between *Physalis minima* and *Datura innoxia* contained three and one donor chromosomes in presence of tetraploid and octoploid recipient background (Gupta et al., 1984).

#### *Phylogenetic relatedness*

Since irradiation primarily causes loss of chromosome fragments instead of whole chromosome, highly asymmetric somatic hybrids can be produced only in few cases (Gleba et al., 1988; Forsberg et al., 1998a). Melzer and O'Connell (1992) proposed that the degree of asymmetry in the somatic hybrids is decided by phylogenetic relatedness rather than the irradiation dose or other factors. Phylogenetically distant species possibly differ from each other in chromosome behavior and severe incompatibility exists between their whole chromosome sets. Sorting out of chromosome of either or both fusion parents could alleviate the incompatibility so that their hybrids can be established after fusion (Takamizo et al., 1991; Shinozaki et al., 1992). So highly asymmetric somatic hybrids could be obtained in remote fusion combinations (Dudits et al., 1987; Trick et al., 1994). When wheat protoplasts were fused with three intergeneric grasses *Psathyrostachys juncea*, *Bromus inermis* and *B. willdenowii*, the

highest chromosome elimination rate was observed in *B. inermis*, which is phylogenetically more distant to wheat than the other two (Xing et al., 2001). Genetic incompatibility derived from phylogenetic relatedness also affects chromosome elimination of somatic hybrids during asexual or sexual propagation, as revealed by the somatic hybrid between *N. plumbaginifolia* and *Atropa belladonna* (Gleba et al., 1988).

#### *Inheritance of cytoplasmic genome*

Mitochondrial and chloroplastic genomes undergo maternal inheritance in sexual hybridization. There are of course some exceptions where biparental inheritance mode has also been observed. Much more complicated inheritance modes are encountered in somatic hybrids leading to diversity in organelle combinations, which cannot be achieved in sexual hybridization.

#### *Inheritance of chloroplast genome*

A large number of reports have shown that in the somatic hybrids or cybrids the chloroplast genomes could be from either of the two parents used for somatic hybridization (Schiller et al., 1982; Li and Sink, 1992; Bauer-Weston et al., 1993; Kaimori et al., 1998; Zhou et al., 2001a, b; Zubko et al., 2002; Ishikawa et al., 2003). The transmission mode could be random or in some case biased towards one of the parents (Wolters et al., 1993a; Escalante et al., 1998; Mohapatra et al., 1998). Non-random or biased segregation of cpDNA detected in some of the somatic hybrids was possibly caused by difference in rate of chloroplast division between the fusion parents or plastome-genome incompatibility (Fahleson et al., 1988; Bonnett and Glimelius, 1990; Earle et al., 1992; Kirti et al., 1998). In comparison to symmetric fusions, in asymmetric fusions, cpDNA of the recipient is predominant in the hybrids, as has been shown in hybrids of *Brassica* and *Arabidopsis* (Bauer-Weston et al., 1993), *N. tabacum* and *D. carota* (Smith et al., 1989), *T. aestivum* and *Z. mays* or *L. multiflorum* (Xu et al., 2003; Cheng and Xia, 2004).

Co-existence of chloroplasts from both fusion parents has been found in a few somatic hybrids (Primard et al., 1988; Motomura et al., 1996; Mohapatra et al., 1998; Moreira et al., 2000; Cheng

and Xia, 2004). Kumar and Cocking (1987) ascribed such phenomenon to parental chloroplast genomic similarity or absence of any selection advantage to either of the two plastid genomes in the heterokaryotic cells and their regeneration into plants. However, chloroplast co-existence is in all probability a temporary status derived either from incomplete or ongoing sorting out of cpDNA in the hybrids. Some work tracing the change in the chloroplast segregation during regeneration process provides evidence for this. Vardi et al. (1989) analyzed cpDNA in the differentially aged regenerants from asymmetric fusion and showed that cpDNA in the hybrid callus was similar to both fusion parents, whereas only cpDNA from one parent could be detected in the hybrid plants. It is more or less clear now that only plastid of one of the parents will finally be present in each somatic hybrid in a random or biased manner.

Chloroplast DNA recombination has also been reported in some intergeneric somatic hybrids. Thanh and Medgyesy (1989) first reported chloroplast recombination in somatic hybrids between tobacco and potato. Later, such kind of phenomenon was detected in many other somatic hybrids (Wolters et al., 1995; Kanno et al., 1997; Kisaka et al., 1997, 1998; Baldev et al., 1998; Escalante et al., 1998; Zhou et al., 2001a, b, 2002b). In some reports the recombinant plastomes were derived from stringent selection. For instance, chloroplast recombination occurred in somatic hybrid between light-sensitive *N. tabacum* mutant and *S. tuberosum* that was treated with lethal irradiation dose (Thanh and Medgyesy, 1989). However, in most cases recombination also took place in the fusion events without selection pressure, as shown by the cases in the somatic hybrids *Trachytoma ballii* and *B. juncea* (Baldev et al., 1998) and *Hordeum vulgare* + *D. carota* or *O. sativa* (Kisaka et al., 1997, 1998). The somatic hybrids were proven to have recombinant chloroplast genomes based on occurrence of novel bands that were not present in the fusion parents via RFLP analysis. The underlying mechanism for chloroplast genome recombination has not been determined, and its potential roles in somatic hybridization can be questioned. One possibility of the survival of recombinants is that the recombinant chloroplast genomes may overcome or abate nuclear-plastome incompatibility (Thanh et al., 1988; Thanh

and Medgyesy, 1989). In addition, the process of chloroplast recombination though rare could be used to generate novel germplasm combining two different traits controlled by chloroplast genomes, such as herbicide resistance. If somatic hybridization is carried out between two fusion parents each carrying a different genetic marker coded by chloroplast genome (e.g. herbicide tolerance) it is possible to get somatic hybrids with recombined chloroplast that are resistant to two different herbicides. However, this is only hypothetical and work on this aspect has not been carried out.

#### *Inheritance of mitochondrial genome*

Compared with chloroplast genome, mitochondrial genomes of most of the somatic hybrids mainly maintain biparent co-existence, show extensive recombination or rearrangements (Gaikwad et al., 1996; Kanno et al., 1997; Kishaka et al., 1997, 1998; Shikanai et al., 1998; Zhou et al., 2001a, b, 2002a, b; Yamagishi et al., 2002; Zubko et al., 2002, 2003; Leino et al., 2003; Xu et al., 2003). But presence of mitochondrion from one fusion parent has been reported in some intergeneric somatic hybrids (Kirti et al., 1992a; Nakajo et al., 1994; Wolters et al., 1995; Liu et al., 2002b; Vasilenko et al., 2003; Xu et al., 2004; Takami et al., 2004). It is not clear why mitochondrial genomes undergo frequent recombination or rearrangement in the somatic hybrids. One explanation is that some regions or sequences in the mitochondrial genome are prone to recombination and rearrangements, which take place during the *in vitro* culture process or in the heteroplasmic status after protoplast fusion (Lelivelt and Krens, 1992; Mohapatra et al., 1998). Difference in inheritance patterns of mt and cp genomes in the somatic hybrids could be due to differences in their membrane structure and due to differences in their genomic organization (Kumar and Cocking, 1987).

MtDNA patterns of the somatic hybrids had effects on the growth habit and yield parameters of the somatic hybrids (Bonnet and Glimelius, 1990; Rambaud et al., 1993; Cheng et al., 2003). Cybrids between tobacco and *Petunia hybrida* could grow and develop normally only when they contained mtDNA from tobacco, whereas those

having mtDNA different from tobacco grew and developed poorly as indicated by low fertility and production of few pollen grains (Bonnet and Glimelius, 1990). Cheng et al. (2003) reported that the mtDNA pattern was correlated with the phenotypic abnormality of the somatic hybrid between *Citrus sinensis* and *Fortunella crassifolia*. Leino et al. (2003) reported that different mitochondrial rearrangements led to hybrids with aberrant growth and flower development in the backcross progenies of the somatic hybrids between *A. thaliana* and *B. napus*.

#### *Factors affecting cytoplasmic inheritance*

Cytoplasmic inheritance in somatic hybrids is affected by many factors like genotype, irradiation dose, protoplast source, ploidy level of fusion parents, nuclear background and phylogenetic relatedness (Perl et al., 1991; Bauer-Weston et al., 1993; Wolters et al., 1993b; Spangenberg et al., 1994; Skarzhinskaya et al., 1996; Kirti et al., 1998). As indicated elsewhere, extensive elimination of donor chromosome often leads to preferential transmission of the recipient's cytoplasm to the hybrids, whilst donor cytoplasm is possibly present if limited elimination of donor chromosome occurs (Takamizo et al., 1991; Wolters et al., 1991; Spangenberg et al., 1995; Kirti et al., 1998). For the combination between *L. multiflorum* (donor, treated by X-ray) and *F. arundinaceae*, when radiation dose was below 50 Gy, mtDNA from both fusion parents was detected, whereas mtDNA of the latter parent was predominant when irradiation dose was above 500 Gy, the dosage for production of highly asymmetric hybrid plants (Spangenberg et al., 1995). As mentioned above, cpDNA normally undergoes uniparental random or biased transmission. But random segregation of cpDNA tends to take place in fusions of closely related species, whilst biased segregation was detected in phylogenetically more distant fusion combination (Sundberg and Glimelius, 1991). For example *Brassica* chloroplast DNA was preferentially transmitted to the somatic hybrids derived from fusion between *Brassica* and *Raphanus* (Earle et al., 1992). Similarly, cpDNA of *Moricandia arvensis* was primarily maintained in the hybrids derived from fusion between green mustard and a mustard cytoplas-

mic male sterility (CMS) line containing *M. arvensis* cytoplasm (Kirti et al., 1998). Nuclear background can also influence chloroplast or mitochondrion segregation (Sundberg and Glimelius, 1991). In the fusions between *N. plumbaginifolia* and *L. esculentum* or *S. tuberosum*, presence of cp DNA or mt DNA in the somatic hybrids was correlated with the nuclear compositions (Wolters et al., 1993a, b). But in a later work on fusion between *L. esculentum* and *S. tuberosum* Wolters et al. (1995) reported that nuclear DNA composition of the somatic hybrids had no impact on chloroplast and mitochondria type. Therefore, effect of nuclear background on cytoplasmic segregation depends on species or combinations.

#### *Interactions between nuclear and cytoplasmic genomes*

Protoplast fusion leads to completely *de novo* combinations of nuclear and cytoplasmic genomes, which offers new materials for studying nuclear and/or cytoplasmic interactions. Compatible interaction between nuclear and cytoplasmic genomes of different origins could lead to production of fertile and functional hybrids (Glimelius and Bonnett, 1986; Thanh et al., 1988; Kameya et al., 1989; Thanh and Medgyesy, 1989). On the contrary, nucleo-cytoplasmic incompatibility results in either failure in regeneration of hybrids or inferior development after regeneration. For example, cybrid plants of tobacco and *Atropa belladonna* were green when they contained tobacco nuclear genome and cp genome of *A. belladonna*, whereas they became chlorophyll-deficient if they contained *A. belladonna* nuclear genome and tobacco cp genome, showing that *A. belladonna* nucleus is not compatible with tobacco chloroplast (Kushnir et al., 1987, 1991). Similarly, the cybrids between tobacco and *Hyoscyamus niger* showed late germination of seeds, dramatic decrease in chlorophyll *in vivo* and pigment deficiency in cotyledons *in vitro* owing to nucleo-cytoplasmic incompatibilities (Zubko et al., 2001). In addition, alloplasmic incompatibility between nucleus and mitochondria was reported to be responsible for CMS in somatic hybrids between *A. thaliana* and *B. napus* and their progenies (Leino et al., 2004).

#### **Application of somatic hybridization to crop genetic improvement**

##### *Creation of novel germplasm by circumventing reproductive barriers*

As is outlined above, gene introgression between cultivated varieties and their related or distant species of agronomic interest via conventional way is difficult due to sexual incompatibility. In contrast, many intergeneric, intertribal or interfamilial somatic hybrids have been obtained via protoplast fusion and some of them were fertile and therefore can be used as bridging materials for breeding (Gleba and Hoffmann, 1978, 1980; Hinnisdaels et al., 1991; Ohgawara et al., 1991; Babiychuk et al., 1992; Gavrilenko et al., 1992; Fahleson et al., 1994b; Forsberg et al., 1994, 1998a, b; Skarzhinskaya et al., 1996; Nothnagel et al., 1997; Vlahova et al., 1997; Grosser et al., 1998; Bohman et al., 1999, 2002; Liu et al., 1999; Hu et al., 2002a; Xia et al., 2003). In addition, many hybrids were successfully established in some species with special reproductive features. For example, sexual hybrids between *F. arundinaceae* and *L. multiflorum* could be produced only when the latter was used as pistillate parent, which does not allow the use of cytoplasm from the former parent. Takamizo et al. (1991) obtained somatic hybrid plants containing cytoplasm of *F. arundinaceae*, thus enriching the gene pool of the combination. Citrus, an important fruit tree, is special in its reproduction features. It shows polyembryony and male and/or female sterility. Production of sexual hybrids between citrus and its relatives is restricted if citrus species are used as pistillate parent. But a large number of somatic hybrids have been recovered between citrus and its relatives by protoplast fusion, which can be used either as rootstocks or used as parents for interploidy crosses to produce seedless triploids (Grosser et al., 2000).

As far as cytoplasmic genomes are concerned somatic hybridization provides novelty. In sexual crosses chloroplast and mitochondrial genomes undergo uniparental maternal inheritance and it is not possible to create novel plastid, mitochondrial combinations through this process. Protoplast fusion has proved to be an effective method for generating novel organelle combinations as demonstrated by the production of many cybrids containing chloroplast and mitochondrial genomes

from a different origin. An interesting example is combining atrazine resistance coded by chloroplast genome and CMS coded by mitochondrial genome in *Cruciferae*. Pelletier et al. (1983) incorporated mtDNA-related CMS and cpDNA-related atrazine resistance from two different parents into the hybrid in addition to the nuclear background of one parent. This methodology is useful for production of hybrid seeds that are tolerant to a herbicide.

Asymmetric fusion or microfusion could also allow transfer of some partial genomes from a donor species to produce monosomic additional line (MAL) without intensive or tedious backcrosses. Ramulu et al. (1996b) obtained MAL containing whole genome of tomato and one potato chromosome. Garriga-Caldere et al. (1998) produced 27 MALs containing seven independent potato chromosomes by crossing the backcross progeny of somatic hybrids between tomato and potato with tetraploid potato. These MALs can be used for introgression of genes from donor genome to the recipient genome and also facilitate physical and genetic mapping of individual chromosomes. In intergeneric combinations MAL may alleviate somatic incompatibility in that addition of a single chromosome to the whole genome of another species could be much better tolerated than convergence of two whole genomes from divergent species.

#### *Transfer of resistance to biotic and abiotic stresses*

Many related or distant species of cultivated crops possess elite attributes like resistance to biotic or abiotic stresses. Owing to the presence of reproductive incompatibilities these cannot be efficiently employed in crop genetic improvement programmes. Somatic hybridization could play a role in transfer of resistance from divergent relatives into crop plants. For example, resistance to diseases caused by bacterium, fungus or nematode has been transferred from donor species to the cultivated crops by protoplast fusion (Lelivelt and Krens, 1992; Lelivelt et al., 1993; Forsberg et al., 1994; Hansen and Earle, 1997; Sigareva and Earle, 1999b; Bohman et al., 2002; Hu et al., 2002a; Furuta et al., 2004). Somatic hybrids between Japanese radish and cauliflower showed resistance to clubroot, a serious disease in cauliflower. The selfing progenies of the somatic hybrid showed

stable and perfect resistance to clubroot over three generations. In addition, the backcross progenies also showed resistance to the disease (Hagimori et al., 1992; Hagimori, 1995). Somatic hybrids between *Capsella bursa-pastoris* and *Camelina sativa* or *Sinapsis alba* and *B. oleracea* showed higher resistance to black spot disease, which is caused by *Alternaria brassicola*, in comparison to *B. oleracea* (Hansen and Earle, 1997; Sigareva and Earle, 1999b). Somatic hybrids between chrysanthemum (*Dendranthema × grandiflorum*) and wormwood (*Artemisia sieversiana*) were more resistant to rust caused by *Puccinia horiana* than chrysanthemum and showed much smaller disease spots compared with those observed on chrysanthemum (Furuta et al., 2004).

Resistance has been checked in the progenies of somatic hybrid. For example, the backcross progenies of the somatic hybrids between rapeseed and *Sinapsis arvensis* or *A. thaliana* showed significantly higher resistance to blackleg or stem canker than rapeseed, indicating stable inheritance of disease resistance from the somatic hybrid to the progenies (Bohman et al., 2002; Hu et al., 2002a). In addition, tolerance to herbicide, salt, drought, heat and cold has also been transferred from the desirable donors to the cultivated species via somatic hybridization (Louzada et al., 1993; Hossain et al., 1994; Sherraf et al., 1994; Begum et al., 1995; Vazques-Thello et al., 1996; Yemets et al., 2000; Arumugam et al., 2002; Xia et al., 2003). Atrazine resistance was transferred from the resistant *R. sativus* to *B. campestris* (Pelletier et al., 1983). The somatic hybrids between *N. plumbaginifolia* (donor) and *A. belladonna* showed higher resistance to the phosphorothioamidate herbicide, amiprofosmethyl (APM), through asymmetric fusion (Yemets et al., 2000). Somatic hybrids derived from intertribal asymmetric combinations between wheat and *Aeluropus littoralis* had higher salt tolerance than wheat as revealed by relative growth, accumulation of free proline and Na<sup>+</sup> and K<sup>+</sup> (Yue et al., 2001). Similar results were reported for the somatic hybrids between wheat and *Agropyron elongatum* (Chen et al., 2000; Xia et al., 2001). *Thlaspi caerulescens* is zinc (Zn) and cadmium (Cd)-tolerant, which can be used for phytoextraction or phytoremediation. In order to transfer Zn and Cd tolerance from to *B. napus* for phytoextraction and metal-contamination tolerance Brewer et al. (1999) fused protoplasts of

*T. caerulescens* and *B. napus*. The resulting somatic hybrids showed increased accumulation of Zn and Cd compared with the sensitive parent, *B. napus*.

#### *Transfer of CMS or production of novel CMS type*

CMS is a maternal inheritance trait present in many plants. Because plants with CMS cannot produce functional pollen, CMS trait is useful for the production of hybrid seeds. Molecular characterization of CMS-related genes showed that they had chimeric open reading frames (orfs) derived from recombination (Schnable and Wise, 1998; Budar et al., 2003), leading to formation of chimeric mitochondrial genomes, which disturbed the mitochondrial function during tapetum development, leading to male sterility (Dragoeva et al., 1999). Extensive work has been carried out on identifying the mechanism of CMS in many crops, such as petunia CMS and ogura-CMS. In CMS *Petunia* male sterility was associated with a fused gene (*pcf*), which was composed of partial fragments of *atp9* gene, *coxII* gene and an unidentified open reading frame *urfS* (Young and Hanson, 1987; Nivison and Hanson, 1989). Work on *Brassica* cybrids carrying ogura CMS trait showed that the ogura-specific mitochondrial fragments contained two orfs encoding 138 (orf138) and 158 (orf158) amino acids, respectively. Transcription analysis showed that orf158 was present in fertile plants, whereas orf138 could not be detected in the fertile plants, indicating correlation between male sterility and orf138 (Bonhomme et al., 1992). Subsequent work showed that dissociation of *orf138* gene led to fertility restoration in the rapeseed cybrids, further supporting the role of orf138 in male sterility (Bellaoui et al., 1998). Transfer of CMS via conventional crosses requires several backcrosses in order to restore the nuclear background. Besides, CMS present in some species with reproductive defects cannot be transferred by traditional way. To date successful CMS transfer via protoplast fusion has been accomplished in several combinations, such as sunflower and chicory (Rambaud et al., 1993; Varotto et al., 2001), *A. thaliana* and *B. napus* (Forsberg et al., 1998a; Leino et al., 2003, 2004), *R. sativus* and *B. oleracea* (Kameya et al., 1989; Kanno et al., 1997), tobacco and *Petunia hybrida* (Dragoeva et al., 1999), *B. juncea* + *Moricandia arvensis* (Kirti et al., 1998). In addition

intergeneric transfer of CMS has been accomplished between *R. sativus* and *B. napus* via cytoplasm–protoplast fusion (Sakai and Imamura, 1990).

Though CMS was transferred from CMS donor to the recipient the nuclear background may be inappropriate. One way to solve this problem is to use the somatic hybrids for sexual hybridization to develop more desirable CMS line containing optimum nuclear and cytoplasmic combinations (Baldev et al., 1998; Prakash et al., 1998; Leino et al., 2003; Zubko et al., 2003). For instance, a *B. napus* CMS line was developed by backcrossing male sterile somatic hybrids between *B. napus* and *A. thaliana* with *B. napus*, which contained nuclear and chloroplast genomes from *B. napus* and rearranged mitochondrial DNA, (Leino et al., 2003). In addition to undesirable nuclear background, co-transmission of chloroplast and mitochondria from CMS donor could lead to unexpected performance (e.g. developmental or floral abnormalities) in the somatic hybrids or their progenies, as is shown by chlorosis present in backcross progenies of the CMS cybrids between *B. juncea* and *M. arvensis* (Kirti et al., 1992a, 1998). In order to substitute *M. arvensis* chloroplast Kirti et al. (1998) carried out protoplast fusion between iodoacetate-treated green fertile line of mustard and the CMS cybrid. Some of the resulting somatic hybrids were green and male sterile, indicating that the chloroplast of *M. arvensis* in the original cybrid has been replaced by the one from green fertile mustard, which facilitated production of hybrid mustard (Kirti et al., 1998). Similar work was done by Morgan and Maliga (1987) and Earle et al. (1992) on somatic hybrids between *Brassica* and *Raphanus*.

Aside from transfer of CMS that is already present in a donor species, CMS could be synthesized between two fertile species via protoplast fusion. Asymmetric fusion between fertile red cabbage and fertile radish resulted in regeneration of CMS cabbage (Kameya et al., 1989). Molecular characterization revealed that the CMS cabbage was similar to ogura type CMS, which was different from the fusion parents (Motegi et al., 2003). Similarly, Zubko et al. (1996) created novel homeotic CMS plants between tobacco and *Hyoscyamus niger* or *Scopolia carniolica* via protoplast fusion.

### *Transfer of traits pertinent to quality improvement*

Some distant species possess desirable traits related to quality that are absent in common cultivars. Transfer of gene(s) related to these traits via protoplast fusion has been tried in some species, leading to production of germplasm of better quality. *Crambe abyssinica* is an annual herb with high content of erucic acid. In order to improve *B. napus*, UV-irradiated *C. abyssinica* was fused with *B. napus* and the erucic acid content in some of the resulting asymmetric hybrids and their progenies was significantly higher than in *B. napus* (Wang et al., 2003, 2004). Similarly, nervonic acid of the intertribal somatic hybrids between *B. napus* and *Thlaspi perfoliatum* was higher than the former fusion parent (Fahleson et al., 1994b). Accumulation of raucassic acid, raw material for antihypertensive and antineoplastic, was 10 folds higher in the hybrids between *Rauwolfia serpentina* and *Vinca minor* in comparison to *R. serpentina* (Kostenyuk et al., 1991).

Transfer of C3–C4 or C4 traits to cultivated species could lead to higher survival rate and better yield potential in crops. In this regard, somatic hybridization has been carried out between C3–C4 or C4 and C3 species (Kirti et al., 1992a; O'Neill et al., 1996; Yan et al., 1999; Schnabl et al., 1999; Ishikawa et al., 2003). Somatic hybrids between *B. napus* and *M. arvensis* expressed the *M. arvensis* C3–C4 intermediate character at the physiological level, indicating transfer of C3–C4 gene from *M. arvensis* to *B. napus* (O'Neill et al., 1996). Somatic hybrid plants between *B. oleracea* and *M. nitens* or *M. arvensis* had CO<sub>2</sub> complementation point intermediate between the fusion parents (Yan et al., 1999; Ishikawa et al., 2003).

### *Creation of novel and desirable rootstocks*

Most of the fruit trees are composed of two parts, rootstock and scion. Rootstock affects the adaptability, yield, canopy and resistance of the grafted trees. In many cases the desirable traits are present in the phylogenetically distant species, which are sexual and/or graft-incompatible with the scion cultivars. Protoplast fusion paves the way for rootstock improvement by circumventing some barriers encountered with traditional breeding, such as high heterozygosity, long juvenile period and large tree size. Few fruit crops have been

subjected to such work, but the most promising work is done in citrus (Ochatt et al., 1989; Grosser et al., 2000; Grosser and Chandler, 2003). For citrus mainly two strategies have been tried for rootstock improvement. The first one is complementary combination of diploid rootstock so that novel tetraploid rootstock possessing elite traits from both fusion parents could be produced. Nearly 50 such somatic hybrids have been produced, among which trifoliolate orange (*Poncirus trifoliata*) is frequently used as one of the fusion parents (Grosser et al., 1988b, 1998; Grosser and Chandler, 2000, 2003; Guo et al., 2002). Trifoliolate orange has many superior traits like CTV-resistance and cold tolerance, but it is sensitive to CEV (citrus exocortis virus). *Citrus reticulata* cv. Red tangerine, also one of the rootstocks, confers tolerance to CEV. But it is not tolerant to CTV, and the fruits from trees grafted on red tangerine are not comparable to those from trees grafted on trifoliolate orange. Protoplast fusion between trifoliolate orange and red tangerine is expected to produce novel rootstocks that are tolerant to both CTV and CEV (Guo et al., 2002). The second strategy for citrus rootstock improvement is to fuse citrus with its sexually incompatible genera, such as *Citropsis*, *Atlantia*, *Clausena* and *Murraya* (Guo and Deng, 1998, 1999; Grosser et al., 1988a, 1990, 1996, 2000; Grosser and Chandler, 2003). Of the distant hybrids the ones between *Nova* and *Citropsis gillettiana*, *Sucarri* and *Atlantia ceylanica* showed potential (Grosser and Chandler, 2003). Most of the citrus somatic hybrids are tetraploids, which were effective in reducing tree size. Average tree diameters of the trees grafted on tetraploid somatic hybrid rootstocks are smaller than the diploid rootstocks (Grosser and Chandler, 2003).

### **Concluding remarks**

Somatic hybridization and genetic transformation are the two most promising alternatives and supplements of sexual hybridization for gene transfer in higher plants. They have their own attributes and we cannot overestimate the function of one technique and underestimate the other. Genetic transformation, gene manipulation at molecular level, is precise and aim-oriented with current sophisticated technology. However, lack of target gene, difficulty in transferring polygenes impeded



its wide use for crop genetic improvement despite the fact that some transgenic crops have been commercialized, such as soybean, corn and cotton. In addition, presence of selection or reporter markers in the transgenic products posed negative impacts on public acceptance of the transgenic products. Somatic hybridization can play a role in transferring polygenic traits, such as resistance to biotic or abiotic stresses. Moreover, it has unique advantages for creating new combinations of nuclear and/or cytoplasmic organelles, leading to more variations and enrichment of current gene pool. In terms of bio-safety somatic hybridization has advantages over genetic transformation because some of the genes for the latter method are not cloned from plants directly.

It has been well documented that protoplast fusion can be used to create useful bridging materials for breeding programmes. However, we should be aware that certain problems do exist for this kind of technology. Somatic incompatibility at different levels is present in the somatic hybrids derived from fusion combinations involving phylogenetically distant species, which leads to unexpected hybrid performance (Fahleson et al., 1994a). For example, somatic hybrids between *B. oleracea* and *M. arvensis* had the CO<sub>2</sub> compensation point as high as *B. oleracea* rather than intermediate one (Toriyama et al., 1988). Some remote hybrids cannot grow normally during *in vitro* or field stage (Grosser et al., 2000). Though asymmetric fusion could mitigate somatic incompatibility to some degree, unpredictable elimination of chromosome happens in most of the fusion events. Since one cannot control the chromosome loss as it is expected some desirable chromosomes will be lost, or undesirable chromosomes are transferred to the recipient than expected in addition to the required chromosomes. Furthermore, aneuploidy in somatic hybrids derived from symmetric or asymmetric fusion has inhibitory effects on the application of the hybrids owing to their low fertility, retarded growth and development, loss of growth vigor, aberrant development and abnormal morphology (Leino et al., 2003). However, despite these negative points, some of the intergeneric somatic hybrids have shown favorable performance and will possibly have potential for cultivar improvement (Xia et al., 2001; Grosser and Chandler, 2003).

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