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Full Length Research Paper

Synthesis and characterization of interspecific trigenomic hybrids and allohexaploids between three cultivated *Brassica* allotetraploids and wild species *Brassica fruticulosa*

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Interspecific reciprocal crosses between three cultivated *Brassica* allotetraploids and one wild species *Brassica fruticulosa* (FF, 2n =16) were made and the trigenomic hybrids were produced only with embryo rescue. From the crosses with *Brassica juncea* (AABB, 2n = 36) and *Brassica napus* (AACC, 2n = 38), hybrids (F.AB, 2n = 26; F.AC, 2n = 27) were obtained only with *B. fruticulosa* as female, but the reciprocal crosses with *Brassica carinata* (BBCC, 2n = 34) gave rise to hybrids (F.BC/BC.F, 2n = 25). These hybrids showed an intermediate morphology and were sterile for male and female except those with *B. carinata*. All the triploids showed high frequency of bivalents during diakinesis and metaphase I (MI) of meiosis. The allohexaploids (FF.AABB, 2*n* = 52; FF.AACC, 2*n* = 54; BBCC.FF, 2*n* = 50) were synthesized by colchicine treatments of respective hybrids *in vitro*, which showed growth vigor and had larger stature than the hybrids, but were male sterile except one (FF.AABB). They produced progenies with the expected chromosome complements (F.AABB, 2*n* = 44; F.AACC, 2*n* = 46; BBCC.F, 2*n* = 42) after pollination by respective *Brassica* allotetraploids. In these allohexaploids, chromosomes were mainly paired as bivalents at diakinesis and segregated equally at anaphase I (AI) during meiosis of pollen mother cells (PMCs). These allohexaploids and progenies were valuable for the breeding of *Brassica* crops.

Key words: *Brassica* allotetraploids, *Brassica fruticulosa*, interspecific hybrids, crossability, morphology, genomic affinity.

INTRODUCTION

In times of molecular genetic engineering, potentially useful agronomic traits from wild species can be introgressed into the genome of cultivated crops by transformation. With transformation alien DNA can be integrated directly into the target nuclear DNA, thereby excluding the problems of genetic incompatibility. However, the number of isolated genes of agronomic importance are still quite limited because identifying and cloning the genes of interest is difficult, especially those controlled by polygenes. Many *Brassica* species and allies are wild and weedy with useful genes which could be incorporated into breeding programs, including cytoplasmic and nuclear male sterility, resistance to diseases, insect and nematode pests; and tolerance of cold, salt and drought conditions. So, interspecific hybridization is still a useful approach to enrich genetic pool of crops and to study the affinity of parental genomes (Garg et al., 2007; Mei et al., 2010). During the last 30 years, *in vitro* techniques such as ovary and

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Abbreviations: PMCs, Pollen mother cells; **MI,** metaphase I; **AI**/**TI,** anaphase I/telophase I; **GISH,** genomic *in situ* hybridization; DAPI, 4'-6-diamidino-2-phenylindole.

embryo culture and protoplast fusion have successfully been employed to obtain a large number of sexual and somatic interspecific hybrids (Prakash et al., 2009).

Crop brassicas, the major source of edible oils and vegetables, are experiencing severe biotic and abiotic stresses (Chopra et al., 1996) due to change in environmental conditions, which impacts the qualitative and quantitative crop and oil production to a greater extent. So, it is necessary to find wild species with valuable traits / genes which can be transferred to crops (Jensen et al., 2002; Pink et al., 2003) or to develop new *Brassica* species with higher ploidy levels and consequently, higher vigour and stress tolerance. *Brassica fruticulosa* Cirillo (twiggy turnip) is a wild species endemic to the Mediterranean coast. It can be a potential genetic source for crop improvement because it possesses resistance to cabbage aphid (*Brevicoryne brassicae*) (Ellis et al., 2000; Pink et al., 2003), and cabbage root fly (*Delia radicum*) (Jenson et al., 2002; Felkl et al., 2005).

Interspecific hybrids between *B. fruticulosa* and *Brassica rapa* L. (2n= 20, AA) (Nanda Kumar et al., 1991; Chandra et al., 2004) and *Brassica nigra* (L.) Koch (2n= 16, BB) (Truco and Quiros, 1991) have been produced and their chromosomal homology has also been investigated. The present study is devoted to the development of the interspecific hybrids and allohexaploids between *B. fruticulosa* and the three cultivated *Brassica* allotetraploids and to evaluate their breeding potential for the crop brassicas as a trigenomic bridge.

MATERIALS AND METHODS

Plant materials and crosses

Reciprocal crosses of *B. fruticulosa* Cirillo (2n=16, FF) were attempted with *Brassica* allotetraploids *B. napus* L. cvs. 'Zhongyou 821', 'Oro' (2n= 38, AACC); *Brassica juncea* (L.) Czern and Coss accession GJ19, *B. juncea* var. *crispifolia* (2n= 36, AABB) and *B. carinata* A. Braun accession Go-7 (2n= 34, BBCC). Their seeds were sown in the experimental field at Huazhong Agricultural University in the first week of October. *B. fruticulosa* was planted in a greenhouse at due time to ensure the match of its flowering coincides with those of *Brassica* allotetraploids. Sexual crosses were carried out by hand emasculation and pollination.

About 15 to 20 days after pollination, some swollen siliques with the immature embryos resulting from the controlled pollinations were excised for embryo rescue and others were left on plants to harvest seeds. Swollen siliques were rinsed with tap water, surface-sterilized in 70% ethanol for three minutes and then treated with 0.1% mercuric chloride for 15 min, followed by three rinses in sterile distilled water. The immature embryos were aseptically dissected and transferred to Murashige and Skoog (MS) agar medium (Murashinge and Skoog, 1962). The cultures were held at 25°C in a 12 h light/12 h dark cycle. The plantlets obtained through embryo culture were multiplied *in vitro* by successively subculturing the top and axillary buds on MS agar medium supplemented with 1.5 mgl^{-1} 6-benzylaminopurine (6-BA) and 0.25 mgl^{-1} α-naphthalenacetic acid (NAA). The cloned buds were rooted on MS agar medium and then transferred to the field. To induce allohexaploidy in the hybrids, young plantlets were cultured for 10 days on the MS agar medium containing 1.5 mgl^{-1} 6-benzylaminopurine (6-BA), 0.25 mgl^{-1} α-naphthalenacetic acid (NAA) and 100 mgl^{-1} colchicine; and then transferred to the same MS agar medium without colchicine, until the plantlets were regenerated from callus. Finally, rooted plantlets were transferred to the field.

Pollen viability and cytology analysis

Pollen fertility was determined as the percentage of pollen grains stained with 1% acetocarmine. Normal pollen grains were fully round, densely stained and were easily distinguishable from small, shrunken and lightly stained sterile pollen grains.

For mitotic chromosome counting, the immature ovaries were pre-treated with 2 mM 8-hydroxyquinoline for 3 h at room temperature and fixed in a mixture of 1:3 (v/v) acetic acid/ ethanol for about 24 h and then, transferred to 70% alcohol and stored at -20°C. The young flower buds for meiotic studies were fixed in a mixture of 1:3 (v/v) acetic acid/ ethanol for 24 h and stored at -20°C. Mitotic and meiotic observations were carried out as described by Li et al. (1995).

RESULTS

Crossability

In the reciprocal crosses of *B. fruticulosa* with *B. napus* and *B. juncea*, the hybrids (F.AC, F.AB) were only obtained via embryo rescue when *B. fruticulosa* was used as the female parent and no true hybrids were identified in reciprocal crosses (Table 1). In the reciprocal crosses of *B. fruticulosa* with *B. carinata*, the hybrids (F.BC/BC.F) were produced in both directions. The percentages of the hybrid production (hybrids / pollinations) were similar, 2.2 to 4.6% for the crosses with *B. fruticulosa* as the female parent (Table 1), but in the reciprocal crosses the crossability was much lower with *B. carinata,* while no hybrids were obtained with *B. juncea* and *B. napus*. The mature seeds harvested from the flowers pollinated gave only the maternal plants, showing that the embryo rescue should be exploited for the production of hybrids in these crosses.

Morphology and cytology of hybrids

B. fruticulosa × *B. napus*

The F.AC hybrids from the crosses with two *B. napus* cultivars (Zhongyou 821, Oro) grew vigorously in the field and the young plants were morphologically inclined to *B. napus* parents (Figure 1, a1 to a3) and the flowering plants were intermediate. They showed the basal clustering stems from *B. fruticulosa* and produced flowers of intermediate size, which were smaller than those from *B. napus,* but larger than those from *B. fruticulosa* (Table 2) and had petals more similar to those of *B. fruticulosa*. The hybrids were totally sterile and produced no seeds either under open-pollination or backcrossing to *B.*

Table 1. Crossability for the reciprocal crosses between *B. fruticulosa* and three cultivated *Brassica* allotetraploids.

Combinations	Number of flowers pollinated (A)	Number of embryos cultured	Number of plants formed	Hybrids (B)	B/A×100
FF × AACC[1]	157	27	9	7	4.5
AACC[1] × FF	536	16	13	0	0
FF × AACC[2]	195	32	6	6	3.1
AACC[2] × FF	752	32	11	0	0
FF × AABB[3]	107	42	11	5	4.6
AABB[3] × FF	959	37	19	0	0
FF × AABB[4]	186	12	7	4	2.2
AABB[4] × FF	475	28	17	0	0
FF × BBCC	237	28	15	11	4.6
BBCC × FF	760	17	6	3	0.4

[1], Zhongyou 821; [2], Oro; [3], GJ-19; [4], *B. juncea* var. *Crispifolia.*

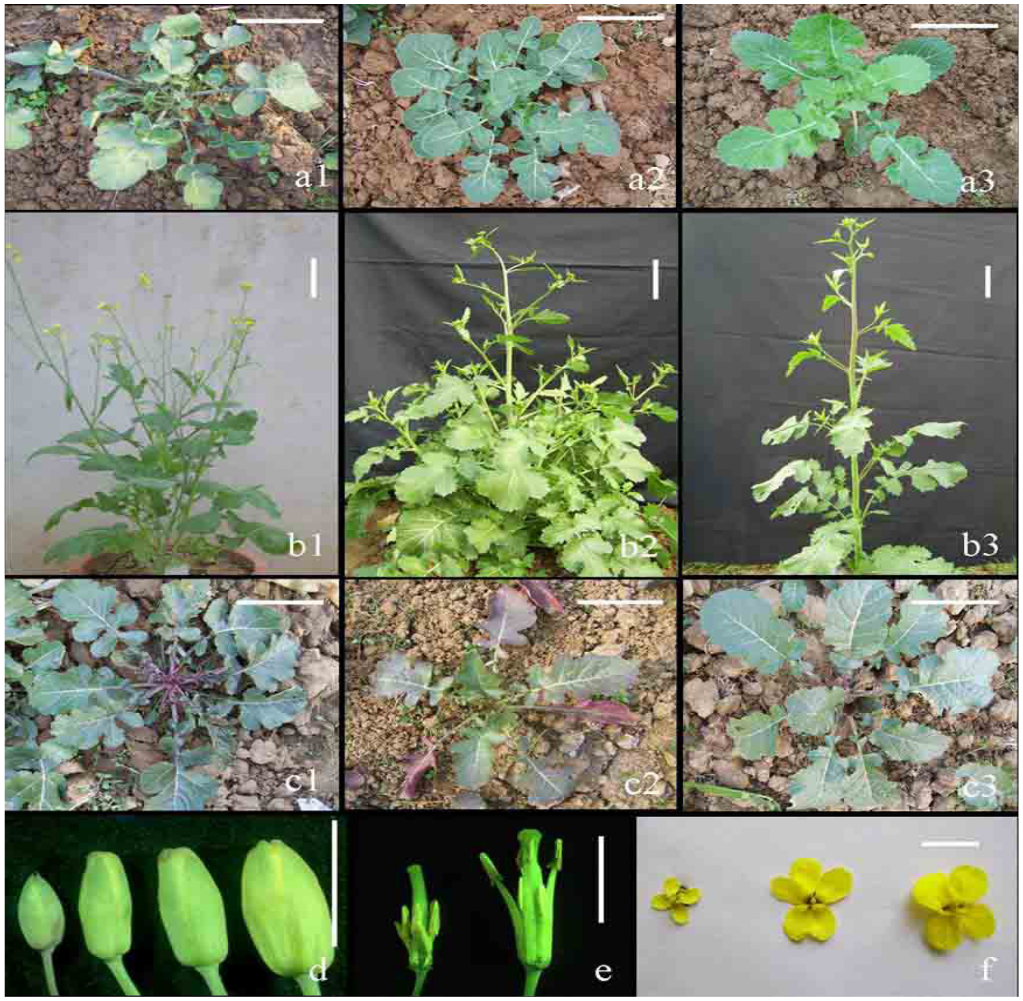

Figure 1. Phenotypes of the hybrids and allohexaploids between *B. fruticulosa* and three cultivated allotetraploids. (A1- a3) Young plants of *B. fruticulosa* (a1), hybrid F.AC (a2) and *B. napus* (Oro) (a3); (B1- b3) flowering plants of *B. fruticulosa* (b1), hybrid F.AB (b2) and *B. juncea* (GJ19) (b3); (C1- c3) young plants of hybrid BC.F (c1), F.BC (c2) and *B. carinata* (c3). Scale bars = 10 cm. (D) Buds of *B. fruticulosa*, hybrid F.AC, allohexaploid FF.AACC and *B. nupus* (Oro) (from left to right); (E) stamina and pistils of hybrid BC.F (left) and allohexaploid BBCC.FF (right). (F) flowers of *B. fruticulosa*, allohexaploid FF.AACC with five petals and *B. napus* (Oro) (from left to right). Scale bar = 1 cm.

napus.

The hybrids had the expected chromosomes number (2n= 27) in their somatic and meiotic cells. In PMCs at diakinesis and MI, various pairing configurations were observed (Figure 2a) and the average chromosome association was 0.3 III + 4.3 II + 17.5 I. The maximum of eight bivalents or 27 univalents were observed in 10.5 and 7.9% PMCs, respectively. The majority of PMCs (70%) at AI/II had laggards and chromosomal bridges. Micronuclei appeared frequently at telophase II (TII) and polyads besides tetrads were also produced.

B. fruticulosa × *B. juncea*

The hybrids F.AB showed vegetative vigor and were intermediate between the two parents for most of the phenotypic traits (Table 2, Figure 1, b1 to b3). They expressed the serrated leaves as *B. juncea*, but basal clustering stems resembled those of *B. fruticulosa* while the plant stature and height were similar to those of *B. fruticulosa*. The flowers were smaller than those of *B. juncea* but larger than those of *B. fruticulosa* and the shape and disposition of petals resembled those of *B. juncea*. The hybrids produced no seeds under open pollination or backcross with *B. juncea*.

All hybrids had the expected somatic chromosome number (2*n*= 26). Their PMCs at diakinesis / MI showed many kinds of pairings with the mean pairing association of 0.1 III + 4.9 II + 15.9 I (Figure 2b). A maximum of 9 bivalents was observed in 7.7% PMCs, while 26 univalents in 5.1% cells. Numerous abnormalities including chromosome bridges and laggards were frequently observed in about 70% PMCs at AI/II. Three to eight nuclei of different size were usually produced by one PMC, which gave rise to several microspores instead of tetrads.

B. fruticulosa × *B. carinata*

The hybrids (F.BC/BC.F) from the reciprocal crosses with one genotype of *B. carinata* (GO-11) were similar to *B. carinata* for most of their morphological attributes (Table 2, Figures 1 a1,c1 to c3), but showed the basic clustering stems, deep serrated leaves and profuse branching similar to *B. fruticulosa*. The shape and size of flowers were intermediate. In comparison with the hybrid (BC.F), F.BC showed poorer growth and smaller stature. In weakly developed stamens, no pollen grains were found. Less than 1% of pods with individual seeds were developed due to open pollination or backcrossing with *B. carinata*.

Both F.BC/BC.F had the expected chromosome number (2n= 25) in their somatic and meiotic cells. In PMCs at diakinesis and MI, many kinds of pairings appeared with the average pairing of 0.1 III + 4.0 II + 16.7 I for F.BC and 0.1 III + 3.9 II + 16.9 I for BC.F (Figure 2c). Meiotic irregularities of the bridges, laggards occurred in 58% AI/II PMCs and frequently, several daughter groups were formed in AII/TII PMCs, which resulted in the formation of sterile pollen grains.

Morphology and cytology of allohexaploids

The allohexaploids (FF.AABB, 2n= 52; FF.AACC, 2n= 54; BBCC.FF, 2n= 50) were produced with high frequency after *in vitro* chromosome doubling of respective hybrids with the colchicine treatment. All allohexaploids showed normal growth and usually had a larger size of leaves, buds (Figure 1d), flowers and stamens (Figure 1e) than respective hybrids. Some of the flowers of FF.AACC had 5 to 7 petals (Figure 1f). The chromosomes were predominantly paired as bivalents and infrequently univalents (1-4), trivalents (1-2) and quadrivalents (1-3) were observed in PMCs (Figure 2 d, e, f). About 70% of PMCs showed equal segregations without laggards at A I/II and others showed unequal segregations (Figure 2g, h, i). The FF.AABB produced about 17% stainable pollen grains and also produced some seeds by selfing but more seeds were obtained when pollinated with *B. juncea*. The majority of the selfed or backcrossed seeds germinated readily and developed normal plants, which had either 2n= 52, the same number as the allohexaploid or 2n= 44 (AABBF). The plants of AABBF produced a lot of seeds after pollination by *B. juncea*, from which the additions with individual chromosomes of *B. fruticulosa* should be available. The other two allohexaploids (FF.AACC, BBCC.FF) produced anthers with no stainable pollen grains and no seeds after selfing, but showed good seed-set when pollinated by *B. napus* and *B. carinata*, respectively. The majority of backcrossing seeds also germinated well and gave rise to normal plants.

DISCUSSION

In the present study, the interspecific hybrids have been obtained when the wild species *B. fruticulosa* was used as the female parent and three cultivated *Brassica* tetraploids as male parents, whereas the reciprocal crosses were unsuccessful except for the cross with *B. carinata* (Table 1). This result indicated incompatibility in the reciprocal crosses. Such unidirectional incompatibility was also frequently encountered in other interspecific and intergeneric crosses (Bhat and Sarla, 2004; Garg et al., 2007). In the intergeneric hybridizations between the six cultivated *Brassica* species and another crucifer *Orychophragmus violaceus* (L.) O. E. Schulz (2n=24), hybrids were only obtained when the *Brassica* species were used as the female parents (Li et al., 1995; Li and Ge, 2007). Herein, *B. fruticulosa* is a diploid species with the low chromosome number and *Brassica* parents are

Table 2. Phenotypes of hybrids and allohexaploids from the reciprocal crosses between *B. fruticulosa* and *Brassica* allotetraploid*.

Combinations	Plant height (cm)	Branching position (cm)	Number of first branches	Number of pods per plant	Flower diameter (cm)	Seeds per pod
FF	116.5	8.3	17.8	195	1.1	17.5
AACC[1]	156.7	24.8	9.2	249	1.8	19.9
F.AC	148.5	0	17.5	46	1.7	0
FF.AACC	151.2	0	18.0	203	1.7	7
AACC[2]	151.0	25.2	8.9	261	1.7	20.1
F.AC	145.5	0	19.6	0	1.5	0
FF.AACC	147.8	0	19.1	84	1.6	3
AABB[3]	176.0	17.5	11.7	277	1.6	17.5
F.AB	126.2	10.5	20.4	0	1.3	0
FF.AABB	133.5	12.7	19.0	176	1.4	11.3
AABB[4]	169.5	12.5	9.5	255	1.6	15.9
F.AB	133.8	0	17.3	0	1.4	0
BBCC	152.5	23.0	11.6	141	1.7	17.0
F.BC	117.5	12.3	15.6	1	1.3	0.5
BC.F	119.2	15.8	18.2	0.8	1.5	0.8
BBCC.FF	120.5	12.7	17.8	61	1.6	10.5

[1], Zhongyou 821; [2], Oro; [3], GJ-19; [4], *B. juncea* var. *Crispifolia;* *, Ten plants of parents and at least 3 plants of hybrids and allohexaploids were observed.

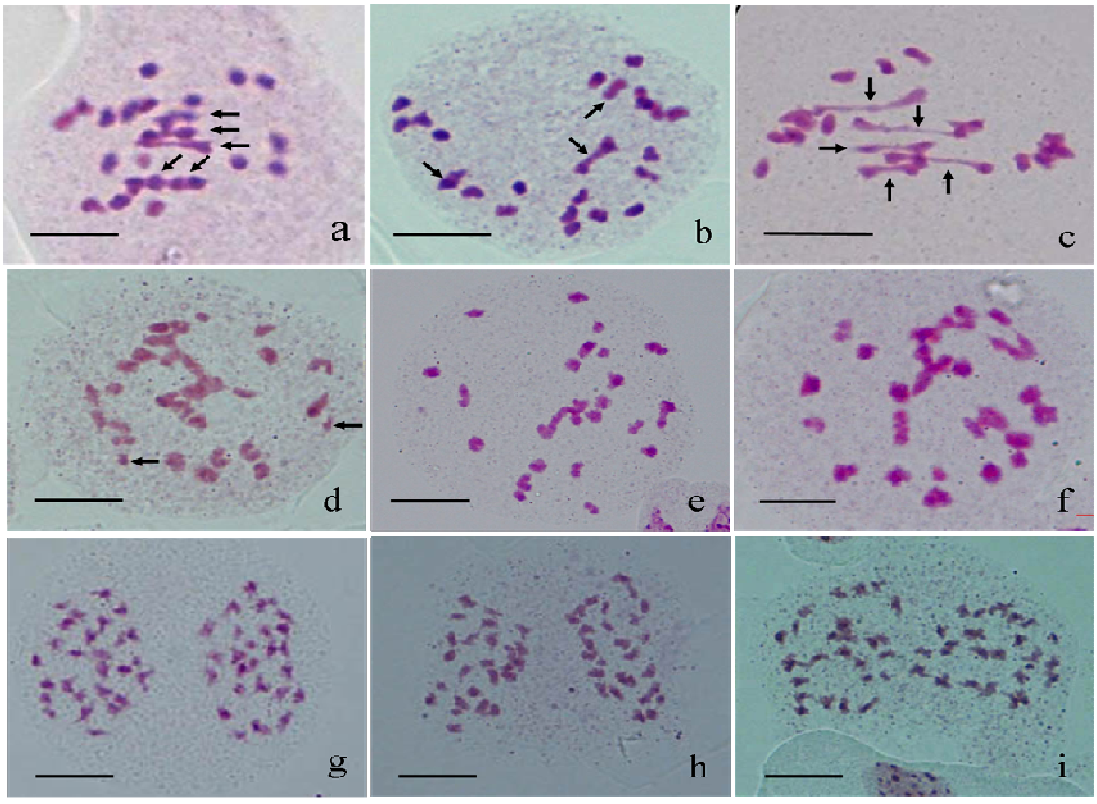

Figure 2. Cytology of the hybrids and allohexaploids between *B. fruticulosa* and *Brassica* allotetraploids. (a) One MI PMC of hybrid F.AC with five bivalents (arrows); (b) one diakinesis PMC of hybrid F.AB with three bivalents (arrows); (c) one MI PMC of hybrid F.BC with five bivalents (arrows); (d-f) PMCs of FF.AACC with two univalents (arrows) (d), of FF.AABB with about 26 bivalents (e) and BBCC.FF (f) with about 25 bivalents; (g-i) AI PMCs of allohexaploids FF.AACC (g), FF.AABB (h) and BBCC.FF (i) with 27:27, 27:25 and 25:25 segregations, respectively. Scale bars = 10 µm.

allotetraploids with much higher chromosome numbers. Inversely, Nishiyama et al. (1991) proposed that, crosses of diploid × tetraploid usually failed, while the reciprocal crosses of tetraploid × diploid species were partly successful. Besides, chromosome numbers of parents some other factors also contributed to crossability.

Embryo culture technique has been widely used to overcome the post-fertilization barriers and was effective in producing interspeciefic and intergeneric hybrids to transfer genes from allied species to crop brassicas (Inomata, 2002; Shaw et al., 2009), when the lack of a functional endosperm or its early degeneration appear to be the major reasons for abortion of hybrid embryos. All hybrids from our crosses were only obtained through embryo rescue and the harvested seeds gave the maternal plants, suggesting that the hybrid embryos failed to develop viable seeds and the embryo culture was necessary to produce the hybrid plants.

Wide crosses and synthetic amphiploids have opened up the possibility of genetic enrichment of cultivated *Brassica* species through gene introgression from *Brassica* allies (Nanda Kumar et al., 1988; Mohanty et al., 2009). Male sterility of F_1 hybrid plants is common, but amphiploids induced from F_1 hybrids often recovered male fertility in wide hybrids (Banga et al., 2003; Chandra, 2004). But the three types of allohexaploids produced here only showed low pollen fertility (FF.AABB) or male sterility (FF.AACC, BBCC.FF), but a much higher fertility for female. The poor male fertility may be caused by the unbalanced chromosome complements in the gametes from the unequal segregations (Figure 2h) and laggards or by the genetic incompatibility between two parents. The successful production of the backcrossing progenies after pollinating these allohexaploids with the cultivated allotetraploids makes it feasible to develop the crop brassicas, *B. fruticulosa* additional lines and to introgress some useful genes / traits into crops.

The allohexaploid (2n= 52, FF.AABB) is possibly stabilized after successive generations, for it is able to produce selfed progeny with 2n= 52. The other two allohexaploid (2n= 54, FF.AACC; 2n= 50, BBCC.FF) with male sterility can be used as a bridge to develop additional lines and transfer some target traits to crops.

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