Production and confirmation of hybrids through interspecific crossing between tetraploid *B. juncea* and diploid *B. oleracea* towards a hexaploid *Brassica* population

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ABSTRACT

The production of a trigenomic hexaploid population using a cross between *B. carinata* and *B. rapa* followed by chromosome doubling at Huazhong Agricultural University stimulated the interest in synthesizing hexaploid *Brassica*. An attempt has been made to synthesize trisomic hexaploid *Brassica* by crossing *B. juncea* and *B. oleracea* followed by chromosome doubling. Five diverse genotypes of *B. juncea* (var. Zun Yi and Wu Ding – Chinese varieties, R 4355, R 4321 and Sel 21 – Russian varieties) and five diverse genotypes of *B. oleracea* (Chinese Broccoli, Broccoli (var. Shogun), Cauliflower (var. Snowball and var. Phenomenon Early) and Cabbage (var. Sweet Eureka) were selected for the investigation. Hand pollination was attempted by emasculating the buds of one species and pollination using another species where flowering was synchronous. Crossing was made in both directions. The crosses of *B. juncea* (♀) x *B. oleracea* (♂) resulted in 307 mature pod set. The success of pod formation was 25%. The success of seed set was 26%. In total 1193 putative hybrid seeds were harvested. Reciprocal crosses were not successful. Evaluation of 70 putative hybrids by microsatellite markers and plant morphological characterization confirmed four true hybrids, one Wu Ding x Chinese Broccoli, two Zun Yi x Chinese Broccoli and one R 4355 x Broccoli (var. Shogun). Although the success rate of pod and seed formation of the crosses between *B. juncea* and *B. oleracea* was low, the production of true interspecific hybrids was successful. Evaluation and characterization of the remaining putative hybrids are currently being in progress.

Key words: interspecific hybridization – polyploidy - trigenomic hexaploids

INTRODUCTION

The *Brassica* genus contains species very important to human food consumption worldwide as edible oils and vegetables. *B. juncea,* commonly known as Indian Mustard, is one of the major oilseed crops in the Indian sub-continent and in China. *Brassica oleracea* is a species highly consumed as a leafy vegetable in many countries. It is rich in different forms of Vitamins B and C, calcium, iron, magnesium, phosphorus, potassium and zinc. *B. oleracea* also contains very high levels of anti-oxidant and anti-cancer compounds. Vegetables of *B. oleracea* are grown in an intensive farming system, very different environments from *B. juncea* as oilseed crop.

The cytology and relationships between six *Brassica* species are well understood. The six species possess three types of diploid genomes designated as A, B, and C either singly or in pairs. *B. juncea* is a tetraploid containing AABB genomes and *B. oleracea* contains only CC genome which is a diploid (U, 1935). These different genomes have different characteristics. Interspecific hybridition between tetraploid species is used in the *Brassica* genus to broaden the genetic diversity and to transfer valuable traits from one species to another. Success in crosses between *B. napus* and *B. juncea* was reported in 1970s (Roy 1978). Meng et al. (1988) succeeded in crossing *B. napus* x *B. carinata* using traditional breeding and the hybrids were fertile. The UWA canola group used double haploid technology to further advance this hybridization and rapidly developed homozygous populations for cultivar development (Nelson et al., 2006 and Nelson et al., 2009).

Hybridization between a tetraploid and a diploid species is difficult and failures occur at many stages starting from pollination incompatibility to pre/post-germination barriers. Most interspecific crosses do not produce mature seeds due to failure of endosperm development (Nishiyama ***et al.***, 1991). Ovule culture was used to overcome postzygotic interspecific incompatibility in reciprocal crosses between *B. rapa* and *B. oleracea.* (Diederichsen and Sacristan, 1994). Similarly, the cross between *B. napus* and *B. oleracea* is normally unsuccessful, but the use of embryo culture techniques can produce hybrids (Gowers and Christey, 1999).

Brassica species provide an opportunity to study rapid genome changes related with polyploidy. Amphidiploid species, *B. napus, B. juncea* and *B. carinata* have been resynthesized by hybridizing diploid species followed by doubling the chromosomes (Song et al., 1993) resulting in completely homozygous polyploid lines. So far relatively little work has been done on the synthesis of hexaploid *Brassica* species except in interspecific crosses between *B. rapa* (AA) and *B. carinata* (BBCC) (Meng et al., 1988; Li et al., 2005). According to Li et al. (2005) the crossability between *B. carinata* and *B. rapa* was varied with the cultivar of *B. rapa*. An extensive work on molecular mechanisms regarding the cytology in intergeneric hybrids between synthetic *Brassica* allohexaploids (2n = 54, AABBCC) and another crucifer *Orychophragmus violaceus* has also been carried out by Ge and Li, (2006) and Li and Gi, (2007) as a further step towards understanding the genome-specific chromosome behavior in wide hybrids. Hexaploid *Brassica* plants of the genomic constitution AABBCC were also synthesized by crosses between *B. rapa* (AA), and *B. alboglabra*/*B. oleracea* (CC) and *B. carinata* (BBCC) (Rahman, 2002).

It has been observed in nature that an increase in genome size of plant species is associated with an increase in cell size and dry matter production. Among crop species, common wheat (*Triticum aestivum* L.) is a fine example of hexaploid plants having wider adaptation, better quality and higher yielding capacity than tetraploid durum wheat (*Triticum* durum Desf.). Durum wheat accounts for 5 % of total wheat production in the world (Anon, 1992). The hexaploid wheat is by far the most important wheat in terms of utilization and geographical distribution (Gooding and Davies, 1997, Leitch and Leitch, 2008).

Development of hexaploid *Brassica* plants is a process of learning from nature and applying innovation to advance *Brassica* production for edible oils, bio-diesel and vegetables in a wide range of environments with higher yield. The synthesis of hexaploid plants with all the A, B and C genomes together will have very good potential to create new crops for domestication. The synthesized new hexaploids may provide traits for resistance to a number of biotic and abiotic stresses including drought, salt, pests and diseases, increased yield in terms of seed production, higher oil content, higher nutrition value, enhanced anti-carcinogenic, anti-oxidant and other medicinal properties as well as phytoremediative properties.

In this study we examined the feasibility of producing a trisomic hexaploid *Brassica* population through interspecific hybridization between *B. juncea* and *B. oleracea*. Further, we attempted to examine success rate of crossing between tetraploid *B. juncea* and diploid *B. oleracea* and to obtain interspecific F_1 hybrid seedlings of triploid nature (ABC).

MATERIALS AND METHODS

Five diverse genotypes of *B. juncea*: Zun Yi and Wu Ding – Chinese varieties and R 4355, R 4321 and Sel 21 – Russian varieties. Five diverse genotypes of *B. oleracea:* Chinese Broccoli, Broccoli (var. Shogun), Cauliflower (var. Snowball), Cauliflower (var. Phenomenon Early) and Cabbage (var. Sweet Eureka)

Plants were grown in a glasshouse from October, 2007 to November 2008. Crossing was made in both directions. Flowers were bagged with perforated plastic bags. The number of flowers crossed, mature pods formed and the seed set for each cross was recorded. About 10% of the hybrids seeds were germinated and 70 hybrid seedlings were obtained. They were transplanted in pots and morphological traits such as flowering time, stem height at flowering, no. of leaf nodes at flowering, leaf/petiole/stem colour and hairiness, flower colour and seed colour were recorded for each plant.

For molecular confirmation of 70 putative hybrid plants, four SSR (Simple Sequence Repeats) markers, sN11722, Ol10-D03, Ol10-F09 and sN12353 were selected and Polymerase chain reaction (PCR) amplification was carried out with genomic DNA of parents and F_1 hybrids and five parents of *B. juncea* and five parents of *B. oleracea* and the products were separated by agarose/polyacrylamide gels. Based on the results, the SSR marker sN12353 was selected for confirming the true hybridity of 70 F_1 hybrid plants.

RESULTS

Hybridization was possible between *B. juncea* and *B. oleracea*. All the five varieties of *B. juncea* were able to hybridize with *B. oleracea* varieties with a varied proportion of pod set out of total pollinated flowers. The cross (*B. juncea* (♀) x *B. oleracea* (♂)) of 1245 flowers resulted in 307 mature pod set. The success of pod formation was 25%. The success of seed set was 26% (1193 seeds). Only 1-5 seeds were present per pod and pods were very small compared to that of parents. The reciprocal cross (*B. oleracea* (♀) x *B. juncea* (♂)) of 1160 flowers resulted in 108 mature pods and the success of pod formation was 9%. However, no seeds were developed in mature pods. About 10% of the F_1 hybrid seeds were germinated to obtain 70 F_1 seedlings. The % of seed germination was very low. Although usually *B. juncea* and *B. oleracea* seeds take 2-3 days to germinate, the germinated F_1 seeds took 5-20 days to germinate.

Morphological evaluation:

The 70 resulted F_1 hybrid plants were evaluated based on their morphological characters. A range of variation in morphological traits were observed in the F_1 hybrids of each cross combination. In order to give a clear picture of morphological variation among F_1 hybrids in the same cross, a comparison between some selected F_1 hybrids with their parents were given in Table 1. Data in Table 1 clearly demonstrate that there is a wide range of morphological differences among the F_1 individuals resulted from crosses of *B. juncea* (♀) x *B. oleracea* (♂) genotypes. Figure1 also shows morphological differences between the leaves of *B. juncea* (var. R 4355) and *B. oleracea* (var. Chinese Broccoli) parents and the F_1 hybrids between them. True hybrid plants are more vigorous than none true hybrids (Figure 2).

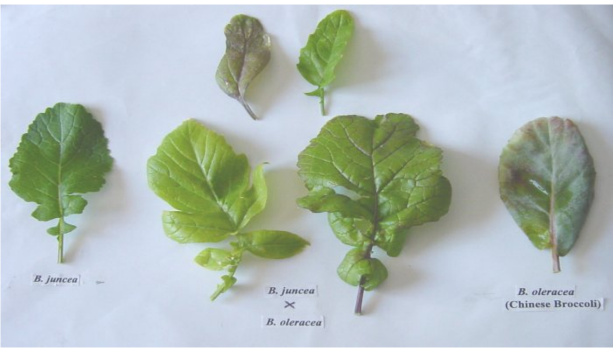

Figure 1. Morphological differences shown in *B. juncea* (var. R 4355) (♀) and *B. oleracea* (genotype Chinese Broccoli) (♂) and their F_1 hybrids.

a. **b*.***

Fig. 2. True hybrid plants a) Cross of B juncea (var Wu Ding) x *B. oleracea* (Chinese Broccoli). b) Cross of *B juncea* (var Zun Yi) x *B. oleracea* (Chinese Broccoli).

Molecular confirmation:

Out of 70 putative hybrids, four F_1 hybrids, ie. one Wu Ding x Chinese Broccoli, two Zun Yi x Chinese Broccoli and one R 4355 x Broccoli (var. Shogun) were confirmed to be true hybrids with the SSR marker sN12353.

Table 1: A comparison of morphological characters between selected F_1 hybrids and their parents

Morphological Trait	**Parents and Hybrids**					
	***B. juncea* (var. Wu Ding) (♀)**	***B. oleracea* (var. Chinese Broccoli) (♂)**	**$F_{1\,(3)}$**	**$F_{1\,(4)}$**	**$F_{1\,(10)}$**	**$F_{1(12)}$**
Fl. Time (days)	79	90	115	108	98	90
Stem Height at flowering (cm)	90	36	113	70	90	100
No. of leaf nodes at flowering	21	13	35	36	36	31
Leaf colour / Hairyness	Leaf green Petiole purple Hairy	Leaf green Petiole green Non-hairy	Leaf green Petiole purple Non-hairy	Leaf green Petiole purple Non-hairy	Leaf green Petiole green Hairy	Leaf green Petiole green Hairy
Stem colour	Green	Green	Green	Green	Green	Green
Flower colour	Yellow	White	Yellow	Yellow	Yellow	Yellow
Seed colour	Light Brown	Black	Yellow	Yellow	Yellow	Yellow
	***B. juncea* (var. Zun Yi) (♀)**	***B. oleracea* (var. Broccoli - Shogun) (♂)**	**$F_{1\,(2)}$**	**$F_{1\,(3)}$**	**$F_{1\,(4}$**	**$F_{1\,(5)}$**
Fl. Time (days)	75	121	45	49	46	46
Stem Height at flowering (cm	76	15	65	80	69	63
No. of leaf nodes at flowering	23	20	18	22	18	16
Leaf colour / Hairyness	Leaf green Petiole purple Hairy	Leaf green Petiole green Non-hairy	Leaf green Petiole green Non-hairy	Leaf green Petiole purple Non-hairy	Leaf green Petiole purple Non-hairy	Leaf green Peti. L. purple Non-hairy
Stem colour	Light Purple	Green	Green	Green	Green	Green
Flower colour	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
Seed colour	Yellow	Black	Yellow	Yellow	Yellow	Yellow

DISCUSSION

Hybridization was possible between tetraploid *B. juncea* (AABB) and diploid *B. oleracea* (CC). Crosses were only successful when *B. juncea* was used as the female parent. Although there was a 9% success in pod set in reciprocal crosses (*B. oleracea* (♀) x *B. juncea* (♂)), seeds were not developed. Out of the total flowers crossed, 25% of crosses yielded hybrid seeds from crosses of *B. juncea* (♀) x *B. oleracea* (♂). These results were consistent with the work carried out by Schelfhout et al. (2006) that the success rate was better in interspecific crosses of *Brassica* only when tetraploids were used as female parents.

Interspecific hybridization between a tetraploid species and a diploid species is difficult and failures occur at many stages starting from pollination incompatibility to pre/post germination barriers. Hybridization between allotetraploid species, *B. napus*, *B. juncea*, *B. carinata* and the diploids, *B. nigra*, *B. oleracea* and *B. rapa* are naturally highly infertile (Diederichson and Sacristan, 1994). Fertilization may take place, but abortion occurs early in the development of the embryo. In the present study too the crosses in both directions a very high rate of seed abortion occurred early in the development.

Natural crossing between *B. juncea* and *B. oleracea* under field condition is not possible (Bing et al., 1996) and also a successful artificial pollinalion has not been recorded so far. This demonstrates the high natural barrier in crosses between *B. juncea* and *B. oleracea*. The barriers might overcome by selecting suitable cross combinations involving different genotypes. Therefore, in our study, a wide range of genotypes were selected to maximize the success rate of crossing.

To confirm the true hybrid nature of putative F_1 hybrid plants, morphological traits and molecular markers (SSR) were used. The morphological characterization of putative F_1 hybrids clearly demonstrated that some F_1 hybrids are very different from both parents. The molecular confirmation with the SSR marker sN12353 confirmed four true hybrids. *B. juncea* (♀) var. Wu Ding, Zun Yi and R 4355 were successfully hybridized with *B. oleracea* (♂) genotypes Chinese Broccoli and Broccoli (var. Shogun) to produce true hybrids. These true hybrids are more vigorous than the none true hybrid plants.

Evaluation and characterization of the remaining putative hybrid seeds will be carried out and the triploid true hybrid seedlings will be treated with chromosome doubling technique to synthesize hexaploid (6x, AABBCC) population.

Polyploidy is accepted as a key mechanism in plant evolution and adaptation (Leitch and Leitch, 2008). Many successful natural hexaploid plants are existing such as wheat, oat and kiwifruit as well as man-made hexaploids such as hybrid (Triticale) of *Triticum* and *Secale*. However, naturally occurring hexaloid *Brassica* are not available. We report here a potential approach to be used to produce hexaploid *Brassica* cultivars ie. The use of one tetraploid (4x) and one diploid (2x) as parents.

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