

Transfer of auxinic herbicide resistance from *Brassica kaber* to *Brassica juncea* and *Brassica rapa* through embryo rescue

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Abstract Auxinic herbicides are widely used in agriculture to selectively control broadleaf weeds. Prolonged use of auxinic herbicides has resulted in the evolution of resistance to these herbicides in some biotypes of *Brassica kaber* (wild mustard), a common weed in agricultural crops. In this study, auxinic herbicide resistance from *B. kaber* was transferred to *Brassica juncea* and *Brassica rapa*, two commercially important *Brassica* crops, by traditional breeding coupled with *in vitro* embryo rescue. A high frequency of embryo regeneration and hybrid plant establishment was achieved. Transfer of auxinic herbicide resistance from *B. kaber* to the hybrids was assessed by whole-plant screening of hybrids with dicamba, a widely used auxinic herbicide. Furthermore, the hybrids were tested for fertility (both pollen and pistil) and their ability to produce backcross progeny. The auxinic herbicide-resistant trait was introgressed into *B. juncea* by backcross breeding. DNA ploidy of the hybrids as well as of the backcross progeny was estimated by flow cytometry. Creation of auxinic herbicide-resistant *Brassica* crops by non-transgenic approaches should facilitate effective weed control, encourage less tillage, provide herbicide rotation options, minimize occurrence of herbicide resistance, and increase acceptance of these crops.

Keywords Auxinic herbicides · Embryo rescue · Dicamba · Introgression

Introduction

The family Brassicaceae is composed of several agronomically important crop species, including oilseed rape (*Brassica napus*), Indian mustard (*Brassica juncea*), cabbage (*Brassica oleracea*), and turnip mustard (*Brassica rapa*). *Brassica* crops are among the oldest, cultivated plants, and their importance dates back to 1500 BC. This family also includes an excellent reservoir of genes for many economically important traits and is receptive to gene transfer techniques. The wild species of Brassicaceae are sources of many agronomically important traits, such as resistance to pests and diseases and tolerance to abiotic stresses. *Brassica kaber* (syn. *Sinapis arvensis*), commonly known as wild mustard, is a dicot weed that infests both dicot and cereal crops, resulting in an estimated yield loss of 20 to 60% (Buchanan *et al.* 2009). To minimize crop yield loss by *B. kaber* competition, herbicides are commonly used. Specifically, auxinic herbicides, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and dicamba, are widely used in cereal crops to selectively control broadleaf weeds including *B. kaber*. Due to prolonged use of auxinic herbicides in wheat and barley fields in western Canada, some biotypes of *B. kaber* evolved resistance to these herbicides. These *B. kaber* biotypes were highly resistant to dicamba and moderately resistant to 2,4-D (Heap and Morrison 1992). The auxinic herbicide-resistant (R) *B. kaber* biotype has been used as a model species to investigate the physiological, biochemical, and molecular basis of resistance to these herbicides (Penuik *et al.* 1993; Webb and Hall 1995; Hall *et al.* 1996; Zheng and Hall 2001; Mithila and Hall 2007; Mithila *et al.* 2012).

Gene transfer among members of Brassicaceae has been investigated (Prakash and Hinata 1980; Bing *et al.* 1995; Hu

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2002) and the transfer of agronomically important traits has occurred. Resistance to blackleg infection caused by *Leptosphaeria maculans* was transferred from *B. kaber* to *B. napus* (Snowdon *et al.* 2000). Similarly, powdery mildew resistance was transferred from *B. oleracea* to *Brassica carinata* (Tonguc and Griffiths 2004). Nonetheless, trait transfer among *Brassica* members is not always straightforward, due to variations in chromosome number as well as ploidy among species (Mizushima 1950, 1980). Therefore, *in vitro* techniques such as ovule/embryo rescue is commonly employed to successfully produce interspecific hybrids among *Brassica* members (Inomata 1988; Mathias 1991; Bing *et al.* 1995; Momotaz *et al.* 1998; Tonguc and Griffiths 2004).

Classical genetic studies demonstrated that *B. kaber* auxinic herbicide resistance is determined by a single dominant gene (Jasieniuk *et al.* 1995; Jugulam *et al.* 2005). The single gene inheritance facilitates the transfer of genes among related species. Because cultivated *Brassica* crops are sensitive to auxinic herbicides, we investigated the transfer of auxinic herbicide resistance from the weedy diploid *B. kaber* (2n:18) to two *Brassica* crop species, *B. juncea*, an allotetraploid (2n:36), and *B. rapa*, a diploid (2n:20), using conventional breeding combined with ovule/embryo rescue technique.

Materials and Methods

Production of hybrids between B. kaber, B. juncea, and B. rapa. Auxinic herbicide-R *B. kaber* and auxinic herbicide-susceptible (S) *B. juncea* and *B. rapa* were grown from seed. The seeds were sown in 6 in. plastic pots containing Promix (Plant Products, Mississauga, Canada) and placed in a growth chamber with a 16-h photoperiod and 22/15°C day/night temperature. The light intensity and relative humidity were maintained at 350 $\mu\text{mol}/\text{m}^2/\text{s}$ and 65–75%, respectively. Each pot contained one plant, and the plants were irrigated when required. Plants were fertilized weekly with 20:20:20 (NPK). When plants were flowering, crosses were performed between auxinic herbicide-resistant *B. kaber* and *B. juncea* or *B. rapa* following the procedure described by Jugulam *et al.* (2005). To achieve embryo regeneration and plantlet formation *via* embryo rescue, immature siliques (narrow, elongated seed capsules) were harvested 3 to 5 d after pollination. Siliques were disinfected with 70% ethanol for 1 to 2 min, followed by 20% commercial bleach (sodium hypochlorite, 5.25%) containing 3 to 4 drops of Tween 20 per 100 ml for 15 to 20 min, and subsequently rinsed four or five times with sterile deionized water. The siliques were aseptically cultured in a Petri dish (Fig. 1A) containing 15 ml of either of the following two media: A—Murashige and Skoog (1962) salts with Gamborg vitamins (1968), 3% (w/v) sucrose, and 500 mg/l

casein hydrolysate or B—Murashige and Skoog (1962) salts with Gamborg vitamins (1968), 3% (w/v) sucrose, 0.5 mg/l naphthaleneacetic acid (NAA), and 2.5 mg/l kinetin. The pH of each medium was adjusted to 5.8, and 8 g/l of agar (Sigma-Aldrich, Oakville, Canada; micropropagation grade, the same agar was used in all other experiments) was added before autoclaving at 121°C for 20 min. The siliques were allowed to grow on these media for approximately 2 wk. For ovule maturation and regeneration into plantlets, ovules were excised aseptically from the siliques with a forceps and scalpel, and cultured on medium A or B in Petri dishes (two to three ovules per dish; Fig. 1B). All cultures were incubated in a growth room at 24°C in light (16-h photoperiod; 50 $\mu\text{mol}/\text{m}^2/\text{s}$) provided by cool white fluorescent lamps (Philips Canada, Toronto, Canada).

Four weeks after ovule culture, hybrid plant regeneration occurred (Fig. 1C), and these young plantlets were subsequently transferred individually to a Magenta box (300 ml plastic vessels, Magenta Corp., Chicago, IL) containing Murashige and Skoog (1962) salts with Gamborg vitamins (1968) and 1.5% (w/v) sucrose (medium C). The pH of medium C was adjusted to 5.8, and 8 g/l agar was added before autoclaving at 121°C for 20 min. After 4 wk on medium C, the hybrids developed roots and shoots. Hybrid plants were clonally multiplied by aseptically culturing nodal segments in Magenta boxes containing medium C. After 6 wk of culture, nodal segments with well-developed roots and shoots were transferred to soil (Promix) and grown under the same conditions as described before for growing *B. kaber* and *B. juncea* or *B. rapa* parental plants.

Assessment of transfer of dicamba resistance and fertility of hybrids. Whole-plant screening was performed to determine the transfer of auxinic herbicide resistance from *B. kaber* into hybrids. Auxinic herbicide-resistant *B. kaber*, *B. juncea*, and *B. rapa*, as well as hybrids, were grown in a growth chamber (as described earlier). The seedlings were treated with dicamba [200 g acid equivalent per hectare (ae/ha)] at the three- to four-leaf stage of development using a motorized hood sprayer. Dicamba was used because it is a widely used broadleaf weed herbicide, and *B. kaber* biotypes were found to be highly resistant to dicamba (Heap and Morrison 1992). The sprayer was equipped with a flat-fan nozzle (8002 E) and calibrated to deliver 200 l/ha at 276 kPa. At 1 and 2 wk after treatment, the seedlings were visually rated for injury. The hybrid plants were classified as R or S by comparing the injury response with those of *B. kaber* (dicamba-R) or *B. juncea* or *B. rapa* (dicamba-S) seedling response. Susceptibility of plants to dicamba was assessed based on symptoms of epinasty (downward curling of leaf and stem tissue) followed by death;

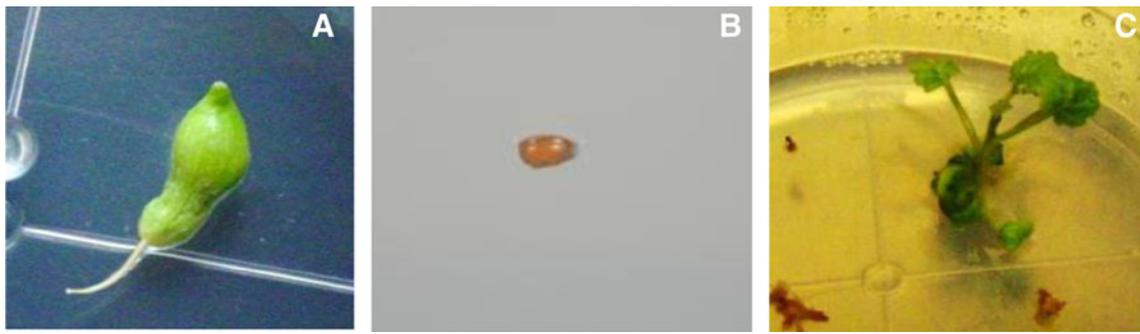


Figure 1. Ovule/embryo rescue procedure to produce hybrids between *B. juncea*, *B. rapa*, and *B. kaber*. *A*, immature cultured silique; *B*, ovule excised from a silique; and *C*, hybrid plant regenerated from the ovule.

R plants were not affected by dicamba application. The fertility of the hybrids was tested by performing reciprocal crosses between hybrids and *B. juncea* or *B. rapa*.

Repeated backcrosses to introgress dicamba resistance from hybrids into B. juncea or B. rapa. The first generation of backcross progeny (BC_1F_1) was produced by performing reciprocal crosses between dicamba-R hybrids and *B. juncea* or *B. rapa*. The hybrids that survived dicamba treatment, as well as *B. juncea* and *B. rapa*, were grown in a growth chamber (as described previously). Cross-pollinations were performed (Jugulam *et al.* 2005) between hybrids and *B. juncea* or *B. rapa* to obtain BC_1F_1 seed. Mature seed (BC_1F_1) was obtained only from the cross between *B. juncea* \times hybrid. BC_1F_1 seed of *B. juncea* \times hybrid was raised in a growth chamber (as described previously). Because there was poor BC_1F_1 production and germination, only three BC_1F_1 seedlings were grown to maturity. These BC_1F_1 seedlings were not screened for dicamba resistance. When BC_2F_1 seedlings were at the three- to four-leaf stage of development, they were treated with dicamba (200 g ae/ha; as described above).

Assessment of DNA ploidy of hybrids and backcross progeny. The DNA ploidy of hybrids, and backcross progeny, was assessed by flow cytometry according to Kron *et al.* (2007). Auxinic herbicide-R *B. kaber*, *B. juncea*, and *B. rapa*

were used as controls. Based on DNA content obtained by flow cytometry, DNA ploidy of hybrids as well as backcross progeny was estimated.

Results and Discussion

Production of hybrids between B. juncea or B. rapa and B. kaber. Cross-pollination between *B. juncea* or *B. rapa* and dicamba-R *B. kaber* produced siliques but failed to develop *in vivo*, as they were unable to grow and eventually aborted. Ovule/embryo rescue from immature siliques facilitated hybrid plant production. More hybrids (32) were produced *in vitro* in crosses between *B. rapa* \times *B. kaber* compared to *B. juncea* \times *B. kaber* (six hybrids; Table 1). A similar number of hybrids were produced when embryos were cultured on either medium A or B. The hybrids exhibited several morphological traits of both the parents (leaf shape, stem, and plant height; Fig. 2). The clones of the hybrids were also successfully established *in vitro* by nodal cuttings.

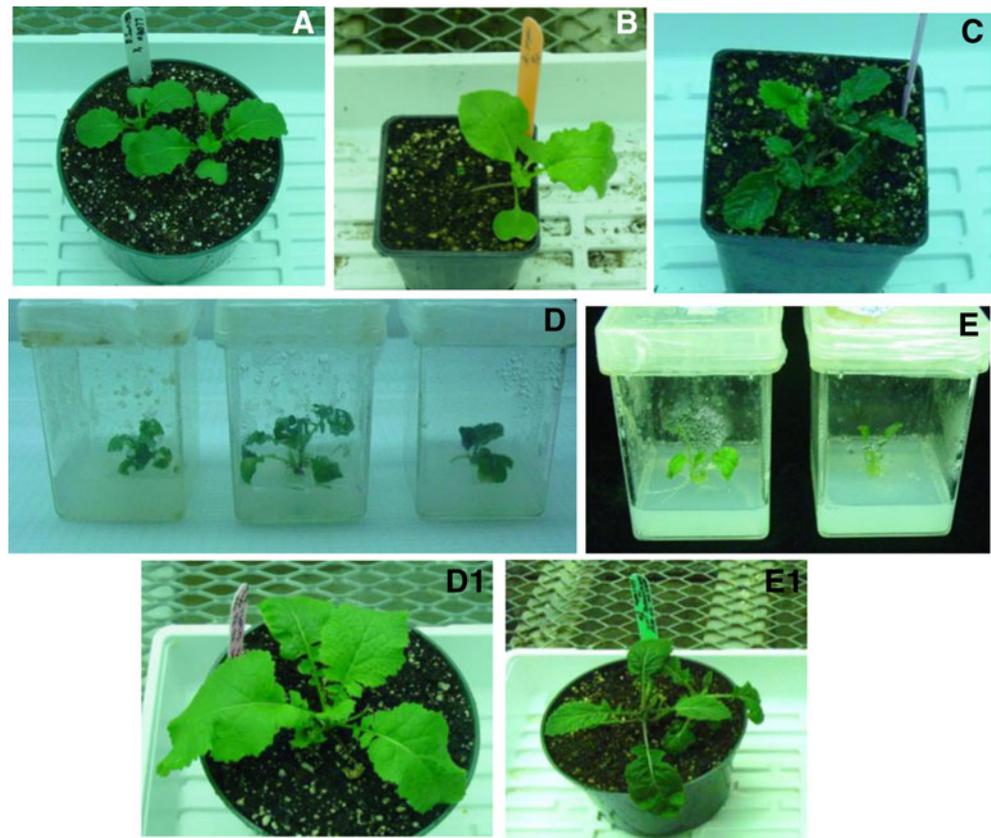
Dicamba resistance in hybrids and backcross progeny. The results of whole-plant screening demonstrated that 1 hybrid of *B. juncea* \times *B. kaber* and 10 hybrids of *B. rapa* \times *B. kaber* were dicamba-R out of 6 and 32 tested, respectively. After treatment with dicamba, the dicamba-R hybrids exhibited little or no epinasty. This response was similar to dicamba-R

Table 1. Hybrid production between *B. juncea*, *B. rapa*, and *B. kaber*: frequency of embryo regeneration and hybrid plant establishment *via in vitro* embryo rescue

Cross combination	# of buds pollinated	# of siliques cultured	# of embryos excised	# of embryos germinated	# of hybrids obtained
<i>B. juncea</i> \times <i>B. kaber</i>	100–150	80 (45 on A and 35 on B)	40 (22 on A and 18 on B)	6 (3 from medium A and 3 from medium B)	6
<i>B. rapa</i> \times <i>B. kaber</i>	80–100	70 (40 on A and 30 on B)	50 (30 on A and 20 on B)	32 (18 from medium A and 14 from medium B)	32

A MS salts + Gamborg vitamins + 30 g/l sucrose + 500 mg/l casein hydrolysate (pH 5.8 and 8 g/l agar), *B* MS salts + Gamborg vitamins + 30 g/l sucrose + 0.5 mg/l NAA + 2.5 mg/l kinetin (pH 5.8 and 8 g/l agar)

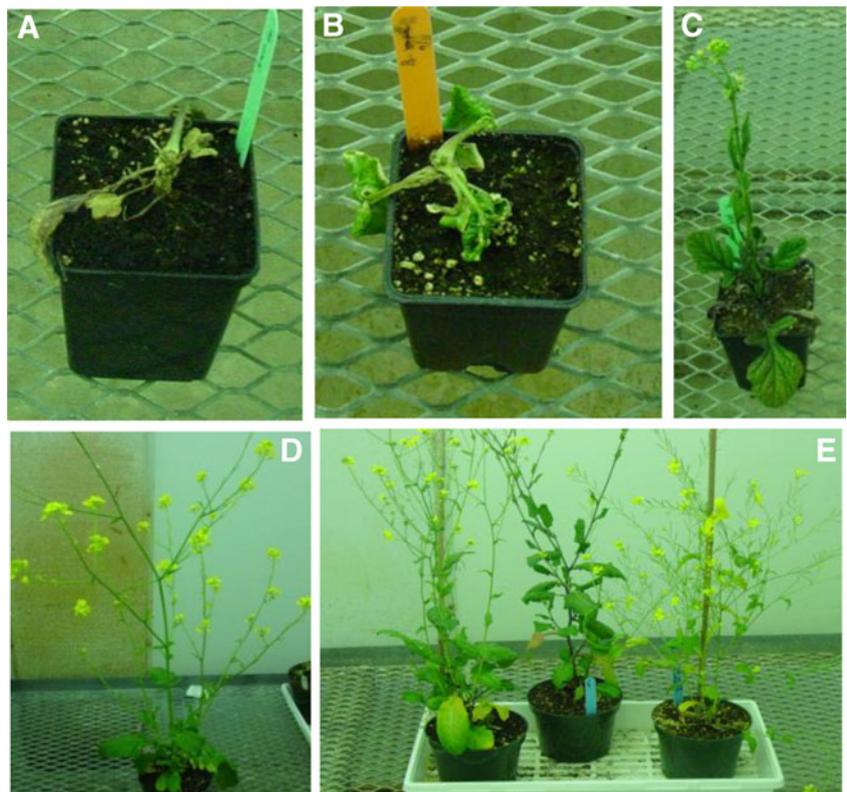
Figure 2. Production of hybrids between *B. juncea*, *B. rapa*, and *B. kaber*. *A–C* represent *B. juncea*, *B. rapa*, and *B. kaber*, respectively. *D*, *D1* and *E*, *E1* illustrate hybrids produced via embryo rescue from crosses between *B. juncea* × *B. kaber* and *B. rapa* × *B. kaber*, respectively. Note the hybrid exhibiting intermediate characteristics of parents.



B. kaber plants. These dicamba-R plants matured and flowered (Fig. 3*D, E*). *B. juncea*, *B. rapa*, as well as S hybrids died

3 wk after dicamba treatment (Fig. 3*A, B*). The dicamba-R hybrids were maintained *in vitro* by nodal cuttings.

Figure 3. Plant response to dicamba (200 g ae/ha) 5 wk after treatment. *A–C* represent *B. juncea*, *B. rapa*, and *B. kaber*, respectively. *D* and *E* indicate hybrids produced by crossing *B. juncea* × *B. kaber* and *B. rapa* × *B. kaber*, respectively.



Reciprocal crosses were performed between parents and dicamba-R hybrids to assess fertility of hybrids as well as generate backcross progeny. For *B. juncea* × *B. kaber*, crosses were successful only when *B. juncea* was used as the female parent. These results suggest that the one dicamba-R hybrid recovered from a cross between *B. juncea* and *B. kaber* was pollen fertile but female sterile. No other crosses were successful. Approximately 15 BC₁F₁ seeds were harvested from crosses between *B. juncea* and the dicamba-R hybrid, and only three seeds germinated. Upon flowering, two of the BC₁F₁ plants were found to be male fertile because the pollen from these plants was able to produce BC₂F₁ seed when used as pollen parent. However, 10 seedlings of BC₂F₁ were established, and when treated with dicamba, the BC₂F₁ progeny showed varying responses. One week after dicamba treatment, six seedlings exhibited severe epinasty and eventually died. The remaining four plants initially showed minor dicamba injury but recovered from the symptoms. Chi-square tests were performed to determine the goodness of fit to a 1:1 (*R/S*) segregation because resistance to dicamba is determined by a single dominant gene in *B. kaber* (Jasieniuk *et al.* 1995; Jugulam *et al.* 2005). The progeny from BC₂F₁ showed 4:6 (*R/S*) segregation with a χ^2 value of 0.4 (which is less than the table value of “3.841” at 0.05 probability for 1 df). The 1:1 segregation of *R/S* in backcross 2 suggests that the gene controlling dicamba resistance may have been transferred to *B. juncea*. However, further efforts are required to produce advanced generations of backcrosses and determine the segregation ratios of dicamba-R or dicamba-S in these progeny to ascertain stable introgression of dicamba resistance trait from *B. kaber* into *B. juncea*.

In the case of *B. rapa* × *B. kaber* hybrids, 5 out of 10 dicamba-R hybrids also produced pollen. However, no seed was obtained when this pollen was used in backcrosses with *B. rapa*; these hybrids were also female sterile. Therefore, introgression of dicamba resistance into *B. rapa* was not successful. Future experiments will determine the possibility of transfer of dicamba resistance into *B. rapa*.

Assessment of DNA ploidy of hybrids and backcross progeny. Flow cytometry was used to quantify the amount of DNA in hybrid as well as parental plants and to estimate ploidy level. Only one hybrid derived from *B. juncea* × *B. kaber* was a DNA triploid (estimated DNA ploidy of 2.96; Table 2), whereas the five other hybrids were tetraploids (Table 2). These results suggest that the five putative hybrids that were DNA tetraploids (Table 2) may have been derived *in vitro* from somatic tissue rather than the fertile embryo. Moreover, all these hybrids were found to be dicamba-S. The flow cytometry results of BC₁F₁ and

BC₂F₁ progeny of *B. juncea* indicated that these plants were either DNA tetraploids (4×, possibly somatic) or aneuploids (plants possessing abnormal number of chromosomes, that is, an extra or missing chromosome, such as 4.2×, 4.3×, 4.4×, 4.6×, or 4.7×; Table 2). Even though DNA was quantified by flow cytometry in *B. rapa* × *B. kaber* hybrids (Table 2), the hybridity of plants was ascertained by plant morphology as well as their resistance response to dicamba treatment (Fig. 3E).

Interspecific crosses for transferring agronomically important traits are routinely performed among agricultural crops. However, interspecific gene transfer is often difficult, specifically among species having different genome or chromosome numbers because of potential irregularities during meiosis. The family Brassicaceae consists of diploids (*B. rapa*, *B. oleraceae*, *Brassica nigra*, and *B. kaber*) as well as allotetraploids (*B. juncea*, *B. napus*, and *B. carinata*). The

Table 2. DNA ploidy of parents, hybrids, and backcross progeny

Plants tested	DNA content (pg) determined by flow cytometry	Estimated DNA ploidy
Parents:		
<i>B. juncea</i>	2.24	3.95
<i>B. rapa</i>	1.08	1.93
<i>B. kaber</i>	1.10	1.98
Hybrids:		
<i>B. juncea</i> × <i>B. kaber</i> #1	1.60	2.96
<i>B. juncea</i> × <i>B. kaber</i> #2	2.21	4.06
<i>B. juncea</i> × <i>B. kaber</i> #3	2.18	3.86
<i>B. juncea</i> × <i>B. kaber</i> #4	2.17	3.84
<i>B. juncea</i> × <i>B. kaber</i> #5	2.17	3.84
<i>B. juncea</i> × <i>B. kaber</i> #6	1.18	3.86
Hybrids:		
<i>B. rapa</i> × <i>B. kaber</i> #1 to # 32	1.04 to 1.12	1.83 to 1.97
Backcrosses with <i>B. juncea</i>		
Backcross 1:		
BC ₁ F ₁ #1	2.61	4.62
BC ₁ F ₁ #2	2.66	4.70
BC ₁ F ₁ #3	2.58	4.56
Backcross 2:		
BC ₂ F ₁ #1	2.45	4.32
BC ₂ F ₁ #2	2.45	4.33
BC ₂ F ₁ #3	2.47	4.37
BC ₂ F ₁ #4	2.41	4.25
BC ₂ F ₁ #5	2.30	4.06
BC ₂ F ₁ #6	2.33	4.11
BC ₂ F ₁ #7	2.44	4.31
BC ₂ F ₁ #8	2.27	4.01
BC ₂ F ₁ #9	2.31	4.08
BC ₂ F ₁ #10	2.42	4.27

allotetraploids of *Brassica* species are naturally evolved and possess two of the three genomes; that is, AA, BB, or CC. Natural crossing among the *Brassica* species was reported to occur with varying difficulty (Myers 2006). However, the majority of hybrid production from crosses between a diploid and tetraploid *Brassica* species has been successful when *in vitro* techniques such as embryo rescue or protoplast fusions were employed (Bing *et al.* 1995; Momotaz *et al.* 1998; Hu *et al.* 2002). In this study, we report for the first time the successful production of hybrids and transfer of auxinic herbicide resistance from *B. kaber* to *B. juncea* × *B. kaber* hybrids. *In vitro* culture of immature siliques 3–5 d after pollination for 2 wk, followed by excision of embryos/ovules yielded higher number of hybrids in crosses between *B. rapa* × *B. kaber* compared to *B. juncea* × *B. kaber* (Table 1). Nonetheless, production of backcross progeny was successful only with *B. juncea*.

B. juncea is an allotetraploid with an AABB genome, whereas *B. rapa* is a diploid with an AA genome alone. Wild mustard is a diploid, possessing SarSar genome (Snowdon *et al.* 2000). The SarSar genome of *B. kaber* is closely related to *Brassica* species that contain B genome (Warwick and Black 1991). Additionally, based on cytogenetic analyses by Mizushima (1980), it appears that three bivalents can be formed between *B. kaber* (SarSar genome) and *B. rapa* chromosomes. Furthermore, *B. kaber* has potential to form up to seven bivalents with chromosomes of BB genome (Mizushima 1980). These cytological studies suggest that there is high possibility of obtaining successful interspecific hybrids between *B. kaber* and *B. juncea*. Nevertheless, in this present study, we obtained higher number of interspecific hybrids between *B. rapa* and *B. kaber* (up to 32 hybrids) than with *B. juncea* (six hybrids; Table 1). This may be due to the increased response of *B. rapa* and *B. kaber* embryos to *in vitro* culture manipulations. Finally, the results from this research offer potential for production of auxinic herbicide-R oil seed *Brassica* crops, specifically, *B. juncea* and *B. rapa*.

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