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Long, Ming Hua
Laboratory of Horticultural Science, Faculty of Agriculture, Kyushu University

Xing, Guo Ming
Laboratory of Horticultural Science, Faculty of Agriculture, Kyushu University

Okubo, Hiroshi
Laboratory of Horticultural Science, Faculty of Agriculture, Kyushu University

Fujieda, Kunimitsu
Laboratory of Horticultural Science, Faculty of Agriculture, Kyushu University

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Cross Compatibility between *Bmssicoraphanus (Brassica oleracea X Raphanus satiuus)* and Cruciferous Crops, and Rescuing the Hybrid Embryos through Ovary and Embryo Culture

Ming Hua Long*, Guo Ming Xing, Hiroshi Okubo and Kunimitsu F'ujieda**

Laboratory of Horticultural Science, Faculty of Agriculture, Kyushu University 46-01, Fukuoka 812, Japan

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Cross compatibility between clubroot resistant *Brassicoraphanus* and susceptible or unsusceptible cruciferous crops was investigated. Pollen germination rate was high in all the crosses. Numbers of ovules survived were almost the same in all the outcrossings as those in self-pollinated *Brassicoraphanus* seven days after pollination (DAP), whereas they decreased rapidly by 15 DAP. The hybrid embryos were observed 25 DAP and degeneration of the endosperm and embryo was initially observed 27 DAP. The crossability was different when different pollen parents were used. Hybrids of *Brassicoraphanus* with ***Raphanus sativus*** were obtained easily, whereas those with ***Brassica*** were not. It is suggested that the genome incompatibility may be one of the causes of the cross incompatibility between *Brassicoraphanus* and cruciferous crops. Hybrids difficult to obtain ***in vivo*** were effectively obtained through embryo culture.

INTRODUCTION

Clubroot caused by *Plasmodiophora brassicae* is one of the serious diseases in Cruciferae. High resistance to clubroot has been reported to be present in some *Raphanus* species (Ashizawa ***et al.,*** 1980; Crute *et* ***al.,*** 1980). Genotypes of ***Brassica oleracea*** have been less identified to be resistant to clubroot than those of ***B.*** *rapa* and ***B.*** *napus* (Crute ***et al.,*** 1983; Crisp ***et al.,*** 1989). For breeding cruciferous plants resistant to clubroot, attempts of transferring clubroot resistant genes through interspecific hybridization have been reported (Lammerink, 1970; Johnston, 1974; Gowers, 1982; Chiang and Crete, 1983) and clubroot resistant dominant gene has been successfully transferred from ***B.*** *napus* to ***B. oleracea*** (Chiang and Crete, 1983).

Brassicoraphanus (an amphidiploid progeny of *B.* ***oleracea*** var. *capitata* × ***R.*** *sativus* var. *longipinnatus*, 2n=36, ccrr genome; Fukushima, 1945) possesses stable and strong resistance to clubroot and the resistance is dominant (Xing et al., 1989). The final goal of our study is to breed clubroot resistant cruciferous crops using *Brassicoraphanus* as a bridge plant. The present study was carried out to investigate the causes of cross incompatibility between *Brassicoraphanus* and cruciferous crops and to obtain a large

* Present address : Guang Xi Agricultural University, Nan Ning, Guang Xi, China.
* * Present address : Department of Horticulture, Shan Xi Agricultural University, Ta Gou, Shan Xi, China

number of their hybrid plants using ovary and embryo culture techniques.

MATERIALS AND METHODS

Plant materials and crosses

All the opened flowers and young floral buds from the selected inflorescences of *Brassicoraphanus* 'K-11' and 'K-13' were removed one or two days before anthesis, and the remained floral buds were emasculated and pollinated with various cruciferous crops listed in Table 1. They were then protected by paraffin paper bags for more than seven days. All the crosses were made in a greenhouse from early April to early May in 1987 to 1989.

Observation of pollen tube and embryo growth

Observation of pollen tubes followed the method of Kho and Baer (1968) with some modification. Styles were collected **48** h after pollination and fixed in acetic alcohol (1:

Table 1. Species, genome constitution and cultivars in *Brassica* and *Raphanus* used in this study.

Species or hybrid	Genome constitution	Cultivar or strain
Brassicoraphanus	ccrr(2n= 36)	K-11"
		K-13"
	aarr(2n= 38)	JS[y]
Brassica x napus	ccaa(2n = 38)	Shoren[x]
B. campestris	aa(2n= 20)	
pekinensis group		Hiroshimana
		Santo
		Kyoto No.3
		Nozaki No.2
rapifera group		Nagasakiaka
		Hakatasuwari
B. oleracea	cc(2n = 18)	
var. *capitata*		Miikewase
		Miikechusei
		Kurobachusei
		Y oshin
var. *alboglabra*		Hakushin
var. *acephala*		kale (W)[w]
		kale (Y)[v]
R. sativus	rr(2n=18)	
daikon group		Shijunichi
		Minowase
		Miyashige
		Sakurajima
		Arutari
radicula group		Comet

[z] *B. oleracea x R. sativus.*
[y] *B. japonica x R. sativus.*
[x] *B. oleracea x B. campestris.*
"kale with white flowers.
"kale with yellow flowers.

3 v/v) for 24 h. They were then rinsed with water and hydrolyzed with 1N NaOH at 60°C for 30 min. After rinsing with water for 3 min, they were stained with one drop of a solution of 0.1 % aniline blue dissolved in 0.1 N K_3PO_4 for several minutes on a glass slide at room temperature. Observation was made with a Nikon Microphoto-FX fluorescence microscope equipped with a mercury super-pressure vapor lamp (Osram, HBO-100W/2), a 365 nm excitation and a 420 nm suppression filters.

Degree of pollen germination and pollen tube growth was classified into five degrees, and the pollen germination index (P.G.I.)(Matsuzawa, 1983) was calculated (see Table 2).

Ovaries were excised and bisected longitudinally 7, 15, 20, 25, 27 and 31 days after pollination (DAP) to examine the growth and development of ovules and embryos, and then the number of survived ovules with normal embryo and endosperm was counted. Length of embryos was measured with a micrometer under a dissecting microscope.

Silique set and seed set per silique were also examined.

Ovary and embryo cultures

A basal medium of White (White, 1963) supplemented with various growth regulators and a full, 1/2 and 1/4 strength of Murashige and Skoog basal media (MS) (Murashige and Skoog, 1962) were used. The media contained 30 gl^{-1} and 50gl^{-1} sucrose for embryo and ovary cultures, respectively. They were adjusted to pH 5.8 before the addition of 8 gl^{-1} agar, and autoclaved at 120°C for 20 min.

Ovaries which were excised 4, 8, 10 and 12 DAP were sterilized with 2 % sodium hypochlorite for 15 min following the surface sterilization with 70 % ethanol. After rinsing with sterile distilled water for five times, they were cultured in test tubes (20 × 150 mm) containing 10 ml MS medium. Those developed into mature siliques-were taken out 30 to 36 days after culture, and the seed set in the siliques was counted. Ovaries from 'K-13' *x* 'Miikewase', 4 DAP, were cultured for 36 days and young embryos protruded out of the undeveloped seed coat were subcultured on White's medium.

For embryo culture, immature siliques from various crosses were collected 27 to 30 DAP, and sterilized with the same procedure as that of ovary culture. Embryos were aseptically excised from ovules under a dissecting microscope and cultured in 100 ml flasks containing 20 ml White's medium. Those developed into plantlets with well developed leaves and roots were taken out from the flasks and transplanted in plastic pots after the removal of the agar. The others without well developed roots were subcultured on a 1/2 strength MS medium containing 20 gl^{-1} sucrose and 8 gl^{-1} agar to enhance rooting.

All the cultures were maintained at 25°C under 16 h daylength with approximately 20 $\mu Em^{-2}sec^{-1}$.

RESULTS

Crossability

Mean P.G.I. and mean percentage of silique set were high in all the crosses between 'K-11' and various cruciferous species, although the individual percentage of silique set varied depending on the pollen parents (Table 2). Mean number of seeds

obtained per silique was highest in 'K-11' × *R. sativus,* followed by 'K-11' × *B. campestris*, 'K-11' × *B.oleracea* and 'K-11' × amphidiploids (*B.x napus* 'Shoren' and *Brassicoraphanus* ' JS').

Degeneration of endosperm and embryos was observed in all the outcrosses. Numbers of surviving ovules were almost the same in all the crosses by 7 DAP, but

Table 2. Seed fertility in *Brassicoraphanus* 'K-11' pollinated with various cruciferous crops.

Pollen parent	P.G.I."	No. of flowers pollinated	No. of siliques set (A)	% of silique set	No. of seeds obtained (B)	A/B
B. campestris						
Hiroshimana	4.0	210	42	20	11	0.26
Santo	3.8	387	271	70	38	0.14
Kyoto No.3	4.0	261	183	70	71	0.39
Nagasakiaka	3.8	203	154	76	21	0.14
Hakatasuwari	3.8	198	143	72	8	0.06
Total or mean	3.9	1259	793	63	149	0.19
B. oleracea						
Miikewase	3.5	111	62	56	2	0.03
Miikechusei	3.8	322	148	46	14	0.09
Kurobachusei	3.7	103	96	93	8	0.08
Y oshin	3.6	97	22	23	1	0.05
Hakushin	4.0	658	592	90	40	0.07
kale (W)	4.0	638	587	92	141	0.24
kale (Y)	4.0	597	585	98	112	0.19
Total or mean	3.8	2526	2092	83	318	0.15
R. sativus						
Shijunichi	3.7	55	11	20	14	1.27
Minowase	3.8	269	207	77	192	0.93
Miyashige	3.5	64	18	28	35	1.94
Sakurajima	3.6	90	19	21	7	0.37
Arutari	3.5	276	213	77	240	1.13
Comet	4.0	102	31	30	42	1.35
Total or mean	3.7	856	499	58	530	1.06
Amphidiploid						
B. napus						
Shoren	3.9	633	557	88	19	0.03
Brassicoraphanus JS	***4.0***	***530***	***450***	***85***	***5***	0.01
Total or mean	4.0	1163	1007	87	24	0.02
K-11	4.0	164	154	94	1348	8.75

"pollen germination index $= (b+2c+3d+4e)/(a+b+c+d+e)$ (OsP.G.I.14).
No. of pistils without pollen grains on the stigma (a), with pollen grains not germinated (b), with pollen grains germinated (c), with pollen tubes reaching to the style (d), and with pollen tubes penetrating into the ovule(e).

rapidly decreased 15 DAP in outcrossings as compared with those of self-pollinated 'K-11' (Table 3). Those in 'K-11'×*B.* oleracea remarkably decreased less than 1.0 by 20 DAP. Percentages of ovules with embyos were lower in all the outcrossings than in self-pollinated 'K-11', whereas the hybrid embyos were observed 25 to 27 DAP in all the crosses (Table 4). By 27 DAP, most of the hybrid embyos developed into a torpedo or nearly mature stage (NM) (Table 5). Mean embyo length was less in 'K-11' X *B. oleracea* than in other hybrids. In 'K-11' ×*R. sativus,* the hybrid embyos developed quickly and about 75% of them reached NM, while in 'K-11'×*B. campestris* and 'K-11' X *B.* oleracea only 19 % and 16% of them, respectively reached NM.

Ovary and embyo cultures

Ovaries obtained from various crosses 4 to 12 DAP produced 0 to 1.43 seeds per explant, and those collected 8 DAP showed the highest degree of successful culture in all the crosses except for 'K-11'× 'Hakatasuwari' (Table 6). Among all the crosses, 'K-11' X 'Minowase' showed the best result of successful culture. Two media consisting of a half or full strength MS salts gave the best effect on the hybrid seed development in two crosses, 'K-11' *x* 'Kyoto No.3' and 'K-11' X 'Hakushin' (Table 7). White's basal medium and that supplemented with 300 mgl^{-1} casein hydrolysate showed the best promoting effect on the development of hybrid embryos in ovary culture of 'K-13' × 'Miikewase' (Table 8). Addition of gibberellin or auxin to the medium showed negative effects on their development. About 50 % of the small hybrid embryos germinated and grew into plantlets in subculture on White's medium.

A high degree of success in plantlet formation was achieved from the immature embryos from all the crosses except for those from 'K-11' X 'Nagasakiaka and 'K-11' *x* 'Hakatasuwari' on White's medium supplemented with 300mgl^{-1} casamino acid (Table 9).

DISCUSSION

Crossability

In many cases of interspecific hybridizations in *Brassiceae,* high crossability does not parallel the high rate of pollen tube penetration into the stigma (Hinata ***et al.,*** 1974). This may be due to post-fertilization failure of ovule development, from which the sterility is rather a common phenomenon in interspecific crosses (Cooper and Brink, 1940). Hakansson (1956) reported that in the interspecific cross between ***B. oleracea*** and ***B. rapa***, a numerical unbalance of chromosome number between endosperm and embyo resulted in the breakdown of the embyo. Raghavan (1976) reviewed such kind of inviable hybrids and concluded that in a large proportion of cases, failure of endosperm development was responsible for embyo death. Our results indicate that normal fertilization occurred in all the crosses and that cross incompatibility resulted from the abortion of the endosperm and hybrid embryos at an early stage of the embyo development. It is also supposed that the difference of genomes between *Brassicoraphanus* and cruciferous crops lead to endosperm abortion and subsequent death of potentially viable hybrid embryos since the degeneration of endosperm and embryos was observed in all the outcrosses at about 27 DAP.

Karpechenko (1927) and Fukushima (1937) reported that *Raphanobrassica,* an

Table 3. Degeneration of ovules in *Brassicoraphanus* 'K-11' pollinated with various cruciferous crops.

Pollen parent	Number of viable ovules per silique in indicated days after pollination					
	7	15	20	25	27	31
B. campestris						
Kyoto ***No.3***	***14.90***	***3.80***	***4.47***	***3.90***	***3.08***	***0.35***
Nozaki No.2	14.30	5.40	3.40	2.10	2.48	0.08
Nagasakiaka	14.40	8.40	7.33	5.75	3.00	0.32
Hakatasuwari	14.30	5.20	4.80	4.90	3.60	0.60
Mean	14.48	5.70	5.00	4.16	3.04	0.34
B. oleracea						
Miikewase	15.30	3.30	0.32	0.30	0.26	0.21
Hakushin	14.30	4.00	0.39	0.34	0.29	0.14
kale (W)	13.90	4.90	0.87	0.81	0.74	0.53
kale (Y)	13.70	3.40	0.82	0.76	0.63	0.31
Mean	14.30	3.90	0.60	0.55	0.48	0.30
R. sativus						
Shijunichi	13.50	10.40	7.32	5.71	4.23	3.04
Minowase	14.30	5.80	5.40	4.82	3.16	2.11
Mean	13.90	8.10	6.36	5.27	3.70	2.58
K-11	14.60	10.20	8.60	9.90	10.60	10.20

Table 4. Embryo development in *Brassicoraphanus* 'K-11' pollinated with various cruciferous crops.

Pollen parent	Percentage of ovules with embryo in indecated days after pollination(N[z])		
	25	*27*	*31*
B. campestris			
Kyoto ***No.3***	1(78)	8(72)	13(15)
Nozaki No.2	0(42)	6(62)	13(8)
Nagasakiaka	0(115)	1(75)	63(8)
Hakatasuwari	14(98)	14(90)	13(15)
Total or mean	5(333)	8(299)	22(46)
B. oleracea			
Miikechusei	50(6)	67(3)	75(4)
Hakushin	43(7)	75(4)	80(5)
kale (W)	80(5)	80(5)	75(4)
kale (Y)	75(8)	86(7)	89(9)
Total or mean	62(26)	79(19)	82(22)
R. sativus			
Shijunichi	0(4)	50(6)	100(3)
Minowase	100(8)	89(9)	79(19)
Total or mean	67(12)	73(15)	82(22)
K-11	90(99)	89(53)	96(51)

[z]Number of ovules examined.

Table 5. *In vivo* development of embryos in *Brassicoraphanus* 'K-11' collected 27 days after pollination with various cruciferous crops.

Pollen parent	No. of embryos examined	Stage of embryo development[z]				Embryo length (mm)
		G	H	T	NM	
B. campestris						
Kyoto No.3	4	0	0	3	1	1.40 1.44
Santaki No.2	4	0	0	3	3	1.73
Nagasakiaka	10	0		1:		1.10
Hakatasuwari	13		0		2	1.62
Total or mean	37	0	0	30	7	1.44
B. o leracea		0	0	4	1	
Miikechusei	5	0	2	8	2	1.20
Hakushin	12					1.10
kale (W)	12	0	0	9	3	1.16
kale (Y)	11	0	0	11	0	1.05
Total or mean	40	0	2	32	6	1.16
R. sativus						
Shijunichi	4	0	0	2	2	1.40
Minowase	8	0	0	1	7	1.49
Total or mean	12	0	0	3	9	1.43
B. napus						
Shoren	6	0	0	1	5	1.48
K-11	47	0	0	22	25	2.05

[z]G; globular stage, H;heart stage, T; torpedo stage, NM; nearly mature stage.

Table 6. Results of in *vitro* culture of *Brassicoraphanus* 'K-11' ovaries on MS medium.

Pollen parent	Days after polli-nation	Initial length of ovaries (mm)	No. of ovaries cultured	No. of seeds obtained	No. of seeds per explant
B. campestris					
Kyoto *No.3*	4	16.1	30	0	0
	8	23.9	30	12	0.40
	12	40.1	30	5	0.17
Hakatasuwari	4	14.3	30	0	0
	8	18.6	30	0	0
	12	45.4	30	32	*1.07*
B. oleracea					
Miikechusei	4	14.8	30	*0*	0
	8	28.8	30	*15*	0.50
	12	40.9	30	0	*0'*
kale (W)	8	24.9	30	8	*0.27*
kale (Y)	8	21.4	30	*7*	*0.23*
R. sativus					
Minowase	4	12.6	30	0	0
	8	24.5	30	*43*	1.43
	12	45.4	30	*4*	0.13

Table 7. Effect of various culture media on seed development in *Brassicoraphanus* 'K-11' ovaries.

Pollen parent	Medium[z]	Days after pollination	No. of ovaries cultured	No. of seeds obtained	No. of seeds per explant
Kyoto No.3	W	10	20	0	0
	W+C	10		1	
	MS	10	20	4	0.05 0.20
	1/2MS	10	20	6	0.30
	1 W	18	20	3	0.15
Hakushin	W+C	8	30	3	0.10
			30	1	0.03
	MS	8	30	6	0.20

[z]W; White medium (1963), W+C; White medium + 300 mgl^{-1} casein hydrolysate, MS; Murashige and Skoog medium (1962).

Table 8. Effect of growth regulators on hybrid embryo development in *Brassicoraphanus* 'K-11' ovaries collected 4 days after pollination with ***B. oleracea*** var. *capitata* 'Miikewase'.

Medium[z]	No. of ovaries cultured	No. of embryos obtained					No. of embryos per explant
		Embryo stage[y]				Total	
		G	H	T	NM		
	18	1	3		34	40	
W	11	1	4	2	24	30	2.27 2.22
W+C				1			
W+G	9	4	6	3		14	1.56
W+I	12	1	1		1	7	
W+C+G	13	4	3	0	5	10	0.58 0.77
W+G+I	16			1	2	0	
W+I+C	14	1	0.5	1	8	15	0 1.07
W+C+G+I	13	1	1	0	0	2	0.15

[z]W; White medium, C; Casein hydrolysate 300 mgl^{-1}, G; GA, 1 mgl^{-1}, I; IAA 1 mgl^{-1}.
[y]G; globular stage, H; heart stage, T; torpedo stage, NM; nearly mature stage.

Table 9. Results of ***in vitro*** culture of embryos taken from *Brassicoraphanus* 'K-11' 27 days after pollination with cruciferous crops.

Pollen parent	Embryo length (mm)	No. of embryos cultured (A)	No. of plantlets obtained (B)	B/A
B. campestris				
Santo	1.44	4	4	1.00
Kyoto No.3	1.40	4	4	1.00
Nozaki No.2	1.73	4	4	1.00
Nagasakiaka	1.10	10	3	0.30
Hakatasuwari	1.62	8	3	0.38
B. oleracea				
Miikechusei	1.20	5	4	0.80
Hakushin	1.10	12	12	1.00
kale (W)	1.16	12	7	0.58
kale (Y)	1.05	10	7	0.70
R. sativus				
Shijunichi	1.40	4	4	1.00
Minowase	1.49	10	7	0.70
B. napus				
Shoren	1.48	4	3	0.75
Brassicoraphanus				
K-11	2.05	15	14	0.93

intergeneric hybrid between radish and cabbage, was successfully backcrossed to radish and that vigorous and radish-like reversional plants were obtained. There are a few reports available that the crossability between another *Brassicoraphanus (B. campestris* × *R. sativus)* and *B. campestris* is low (Terasawa, 1933; Kato and Tokumasu, 1979; Sarashima, 1984). In the present study, it was demonstrated that F_1 hybrids between *Brassicoraphanus* and *R. sativus* can be easily obtained, whereas those between *Brassicoraphunus* and *Brassica* species are hardly.

The results that 'Miyashige' was more compatible with *Brassicoraphunus* than other cultivars in *R. sativus,* that pekinensis group was more than rapifera group in *B. campestris,* and that kales were more than other varieties of *B. oleracea* suggest that selection of certain species, subspecies, varieties or cultivars as pollen parents is important and it may improve the cross compatibility between them. Genomic difference may also limit the compatibility. The lower cross compatibility between 'K-11' and *B. campestris* with "aa" genome than that between 'K-11' and *R. sativus* with "rr" genome may be related to the difference in their genome constitution, since the genome contitution 'K - 11' is "rrcc". It is, however, difficult to explain the reason why the cross compatibility between 'K-11' and *B. oleracea* was lower than that between 'K-11' and *B. campestris,* in spite that the genome "cc" is common in both 'K-11' and *B. oleracea.*

Another reason of low seed fertility in the present crosses between 'K-11' and cruciferous crops may be attributed to difference in ploidy level. Generally, modifying the ploidy level of one species to match that of another may improve the success of obtaining an interspecific hybrid (Fehr, 1987). Karpechenko (1937) reported that the crossability between *Raphanobrassica* and tetraploid *B. oleracea* was higher than that between *Raphanobrassica* and the diploid. When *Brassicoraphunus* was crossed with tetraploid *B. oleracea,* Sarashima (1982) obtained 166 seeds or 2.25 seeds per silique from 254 pollinations. Thus, tetraploid cruciferous crops as pollen parents may improve the cross compatibility with 'K-11'.

Ovary and embryo cultures

In Cruciferae, rescuing of interspecific hybrids through ovary culture has been reported (Inomata, 1977). Our results also indicate that the hybrids between *Brassicoraphanus* and cruciferous crops are efficiently obtainable through ovary culture. The ovaries collected about 8 DAP will give successful development of them. The medium consisting of a half or full strength MS salts seems to be the best for hybrid seed development and White's medium supplemented with 300 mgl^{-1} casein hydrolysate is considered to be the best for the development of hybrid embryos. In accordance with our results, it has been reported that the development of hybrid embryos was promoted in the medium supplemented with casein hydrolysate (Inomata, 1975, 1977) and the seed formation in excised ovary was not influenced by auxin and gibberellin (Inomata, 1968).

Our results prove that the hybrids between *Brassicoraphanus* and cruciferous crops are effectively obtained through embryo culture as compared with *in vivo* and ovary cultures.

All the F_1 hybrids obtained by the crosses of *Brassicoraphunus* 'K-11' with cruciferous crops showed strong resistance to Williams' races 2 and 4 (Xing *et al.,* 1989).

Report on backcross compatibility of the F_1 hybrids and clubroot resistance of the progenies will follow.

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