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Genetic constitution of germ cells in intervarietal and interspecific chimeras of *Brassica* induced by in-vitro grafting

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Abstract The characteristics of intervarietal and interspecific chimeras synthesized by the graft-culture method were determined by morphology, anthocyanin pigmentation pattern, and crossing. In an intervarietal chimera between 'YR-ranpou' (green cabbage) and 'Ruby ball' (red cabbage) in *Brassica oleracea*, a segregation phenomenon was noted in which seeds giving rise to purple and green plants were both produced in a single capsule in F_1 progeny from crosses of chimeras with YR ranpou, the anthocyanin-free graft partner type. The degrees of segregation varied, reflecting the structure of the chimeras. YR ranpou-dominant chimeras produced capsules in which seeds gave rise to green plants at a high frequency, while Ruby ball-dominant chimeras produced capsules in which seeds in one capsule gave rise to purple plants at a high frequency. Mixed chimeras produced capsules with green plants or purple plants more regularly than did other chimeral types. Furthermore, a chimeral type in which seeds gave rise to green and purple plants was found in 3.2% of the total crosses. Segregation patterns in the progenies corresponded with the chimeral types. Chlorophyll-deficient variation (resulting in variegation or the production of albino plants) was found at a frequency of 2.6%. These results show that chimeric tissues are actually in a mixed state and that either the ovary develops from more than two cells or else that variation occurs in the germ-cell layer. In interspecific chimeras between Ruby ball and Komatsuna (*B. campestris*) various types of chimeras generally showed low pollen fertility, few capsules, and low seed-setting. Progenies from selves (geitonogamy), open crosses and crosses with the two parental species produce a predominantly homogeneous genotype showing either the Ruby ball or the Komatsuna type. Only two crosses produced four interspecific hybrids which expressed variations in their morphological and isozymic characters.

Key words *Brassica* · Chimera · Segregation · Tissue culture

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Introduction

We have shown the possibility of a DNA transfer process in the vascular system as a mechanism of graft-induced genetic change (Hirata 1979, 1980; Hirata and Yagishita 1986, 1987; Yagishita and Hirata 1987; Hirata et al. 1989; Yagishita et al. 1990). Further, to discover the relationship between the graft-induced genetic changes and the plant chimera, we synthesized intervarietal and interspecific chimeras by in-vivo and in-vitro grafting (Noguchi et al. 1989, 1992; Hirata et al. 1990, 1992) and have studied whether genetic interaction between genetically-distinct cells (tissues) could exist in chimeras of *Brassica*. Genotypic chimericity of germ cells in one flower (capsule) was seen to be about 3% in a cross experiment in the intervarietal in-vivo and in-vitro graft-chimeras (Hirata et al. 1990). In the case of the in-vivo interspecific chimera, low pollen fertility, little seed setting, and an extremely low frequency of interspecific hybrid formation were distinctive (Hirata et al. 1992). In the present paper, the characteristics of the intervarietal and interspecific chimeras produced by in-vitro grafting in *Brassica* are described. The genetic constitution of the germ cells in each capsule (flower) is also analyzed based on crosses between chimeras and the two parental materials.

Materials and methods

Cabbage cultivars 'Ruby ball' (red cabbage, abbreviated as R) and 'YR ranpou' (green cabbage, YR) of *B. oleracea* were used for inter-

varietal chimera formation. The *B. campestris* cultivar 'Bansei-Komatsuna' (Komatsuna, K) was also used for interspecific chimera formation with Ruby ball. Our in-vitro graft method (Noguchi et al. 1992) was applied to synthesize original chimeras (V_0). The vegetative progenies (V_1) from the young V_0 buds or from leaves of the original chimeras were used for this experiment.

In cross experiments for the production of intervarietal chimeras, chimeras (♀) were crossed only with YR-ranpou (♂) to identify the genotypic composition of the germ cells based on the segregation pattern in the progeny. The genetic nature of anthocyanin pigmentation was preliminarily determined to be single gene dominance of the purple color of Ruby ball against that of YR ranpou. In the interspecific chimeras, crosses were performed with *B. oleracea* (Ruby ball) and *B. campestris* (Komatsuna).

Results and discussion

Characteristics of chimeras produced by in-vitro grafting

The intervarietal chimeras were classified into Ruby ball-dominant mericlinal chimeras (R), YR ranpou-dominant mericlinal chimeras (YR), and mixed-chimeras (MX) in which the two different tissues contributed equally. These chimeral types were classified by appearance from the contributed tissue composition of each chimera. Interspecific chimeras (Fig. 1A–F) were classified into the Komatsuna (revertant) type (K, the constitution of apical layers was assumed to be LI-LII-LIII = KKK), the Ruby ball (revertant) type (R, RRR), a peripheral chimera with a Komatsuna green color (KP, KKR), the Komatsuna-dominant mericlinal chimera (KM, mainly KKK and KKR), the Ruby ball-dominant mericlinal chimera (RM, mainly KRR and RRR), a peripheral chimera with Ruby ball-purple color (RP, KRR) and a mixed chimera (MX) in which chimeric tissues were complexly mixed. Correctly-sectorial chimeras were not found in the course of this experiment.

Morphological features of the vegetative and reproductive organs were generally intermediate between the two parental materials used for the synthesis of both intervarietal (Hirata et al. 1990) and interspecific chimeras (Noguchi and Hirata 1994). Especially in the interspecific chimeras various morphological changes were observed. For example, the plant type was distributed from the rosette to the heading type at the vegetative stage according to the constitution of the two different specific tissues (Fig. 2; Noguchi and Hirata 1994). The inflorescence form ranged from the Komatsuna type (Fig. 2 far left) to the Ruby-ball type (Fig. 2 far right). These inflorescence types corresponded to the flowering-time spectrum from the early- flowering Komatsuna type to the late-flowering Ruby-ball type (Noguchi and Hirata 1994).

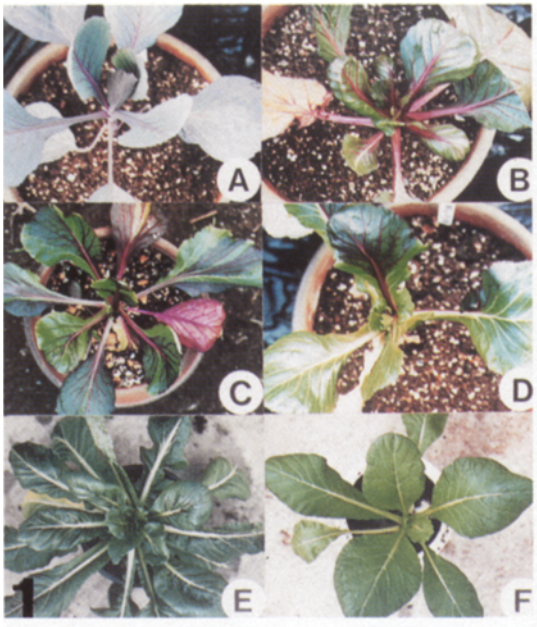
Flowers also varied in shape, color and size: Komatsuna has small flowers with round and yellowish petals (Fig. 3, right in the middle) as compared with the large flowers of Ruby ball which have spatulate and cream-colored petals (Fig. 3, left in the middle). Interspecific hybrids from the cross of the KM type (KKR + KRR) of chimera (chimera plant 102) with Komatsuna showed intermediate characters between the two species (Fig. 3 center).

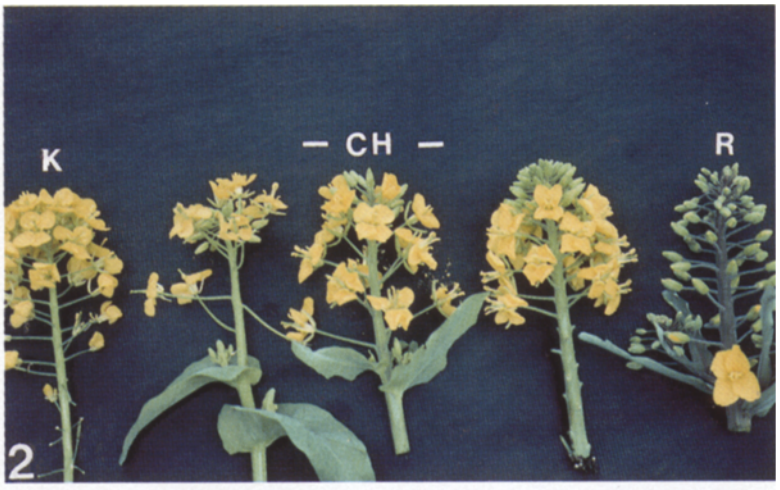
Fig. 1A–F Interspecific chimeras between Ruby ball (*B. oleracea*) and Komatsuna (*B. campestris*) induced by in-vitro grafting. Six typical chimeric types are shown: **A** Ruby ball (revertant) type (R, RRR). **B** Peripheral chimera with a Ruby ball-type purple color (RP, KRR). **C** Mixed chimera (MX) in which both plant types contributed evenly to form the chimera. **D** Komatsuna-dominant mericlinal chimera (KM, KKR + KKK). **E** Peripheral chimera with a Komatsuna-type green color (KP, KKR). **F** Komatsuna (revertant) type (K, KKK)

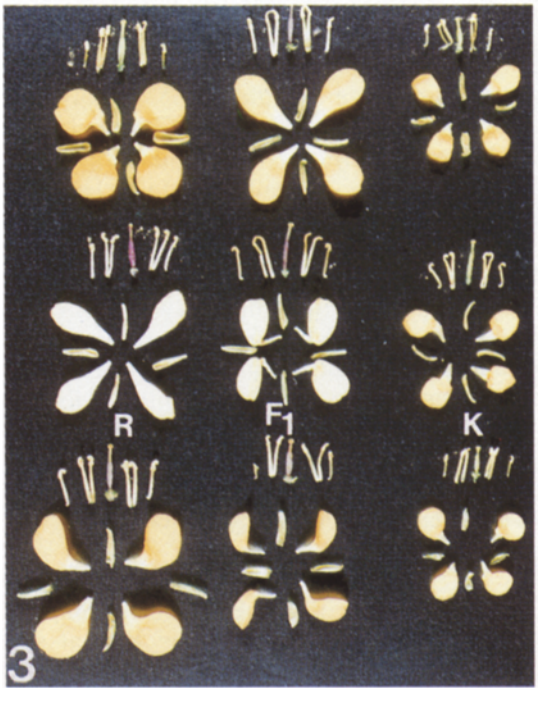
Figs. 2–5 Inflorescence types of interspecific chimeras. From left to right: far left, the corymb type of the Komatsuna inflorescence; middle three, intermediate types between corymb and raceme; far right, the raceme type of the Ruby ball inflorescence. **Fig. 3** Various flower types in interspecific chimeras. The upper three show flower types from Komatsuna-dominant mericlinal chimeras in which flower color is a typical Komatsuna-like yellow, but petal size and shape are varied. Middle three from left to right: Ruby ball, interspecific hybrid (F_1) between mixed chimera (KP 102) × Komatsuna, and Komatsuna. The Ruby ball type (*R*) has slender, cream colored petals while Komatsuna (*K*) has small round, yellow petals; the F_1 flower was intermediate between the two parental species. Lower two from left show the flowers of Ruby ball-dominant mericlinal chimeras. Petal color and shape also varied in chimeral plants, as shown here. **Fig. 4** 'Interspecific hybrid' derived from crosses of a mixed chimera (102) with Komatsuna. Four hybrid plants were obtained from two crosses (capsules), but two grown plants varied in anthocyanin pigmentation and leaf shape. **Fig. 5** Acid-phosphatase isozyme band patterns in three hybrids and chimeric tissues. The fourth to sixth from the left show the patterns of isozymes from the Komatsuna portion (*CHK*), the mixed part (*MX*), and the Ruby ball part (*CHR*). The third hybrid (F_1-3) was different from the other two (F_1-1, -2). In addition, isozyme-band patterns from chimeric tissues also varied depending on the constitution of the two specific tissues. ★ and * indicate the Ruby ball (*R*)-specific bands and the Komatsuna (*K*)-specific band, respectively

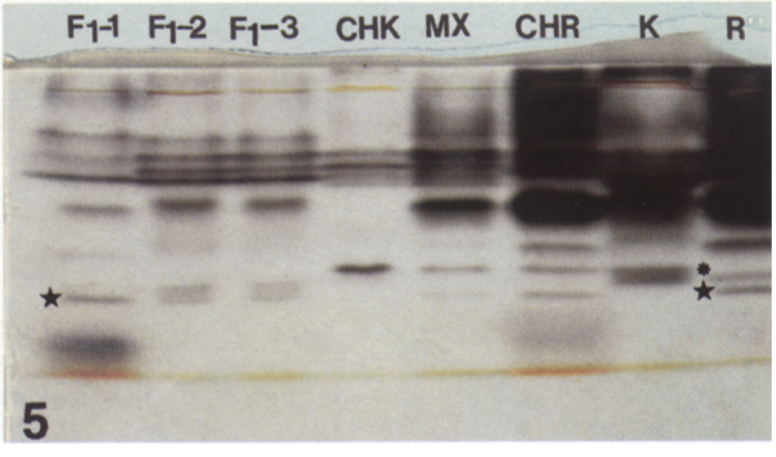
Characteristics of the progenies from the crosses of intervarietal chimeras with 'YR-ranpou'

The genotypic constitution of the germ cells of chimeras was determined by the crossing of intervarietal chimeras with YR ranpou which has a recessive homozygous genotype (*pp*. non-purple, green plant) for anthocyanin pigmentation in contrast with the purple Ruby-ball plant (*PP*). Segregation patterns of the F_1 progenies in the crosses are shown based on the genotypes for anthocyanin pigmentation given in Table 1. In the YR ranpou-dominant chimera, the 84.5% (414 of 490) of the capsules in which progeny seeds produced only green plants show that those chimeras generally had YR-ranpou genotypic germ cells (p). In Ruby ball-dominant chimeras, 88.3% (497 of 563) of the crosses in which seeds produced only purple progeny plants show the general correspondence of germ-cell genotype with that of external appearance. In the mixed type of chimeras, the crosses in which seeds produced only green plants or only purple plants accounted for 59.3% (73 of 123) and 35.0% (43 of 123), of the crosses respectively. Mixed









chimeras more evenly produced both types of progenies than did the YR-dominant and Ruby ball-dominant types.

Segregation phenomena in which seeds produced both green and purple plants in each cross were observed at 4.1% (20 of 490 crosses) in YR-dominant chimeras, 1.9% (11 of 563 crosses) in Ruby ball-dominant chimeras and 5.7% (7 of 123 crosses) in mixed chimeras, giving a total of 3.2% (38 of 1 176) (Table 1).

The chimeras in which progeny seeds produced green and purple plants occurred at 64.3% (9 chimeras/ 14 chimeras) in YR ranpou-dominant chimeras, at 54.5%

Table 1 Segregation of F_1 progeny plants from crosses of intervarietal chimeras with 'YR-ranpou'. The intervarietal V_1-chimeras used in this cross experiment were vegetatively propagated from the chimeras (V_0) induced by the graft-culture method. Data are shown as the number of crosses (capsules). YR, R and MX types show the YR ranpou-dominant type, the Ruby ball-dominant type and the evenly-mixed chimera of YR and R types, respectively. Genotypes, *P* and *p* indicate purple and non-purple green for anthocyanin pigmentation, respectively

V_1-chimera (♀) crossed with YR ranpou (♂)	No. of crosses (capsules)	Segregation types of F_1 progenies from the crosses			Assumed genotype of egg in each ovary of chimera
		Purple plant production capsule (%)	Green plant production capsule (%)	Type segregated into green and purple plants (%)	
YR-dominant type					
2 chimeras	36	0	35	1(2.8)	*P*
2 chimeras	3	3	0	0	*p*
2 chimeras	63	3	60	0	*P*, *p*
4 chimeras	164	0	156	8(4.9)	*P* + *p*, *p*
4 chimeras	224	50	163	11(4.9)	*P* + *p*, *P*, *p*
(Subtotal)	490	56(11.4)	414(84.5)	20(4.1)	
R-dominant type					
5 chimeras	156	156	0	0(0.0)	*P*
1 chimera	19	18	0	1(5.3)	*P* + *p*, *P*
4 chimeras	388	323	55	10(2.6)	*P* + *p*, *P*, *p*
(Subtotal)	563	497(88.3)	55(9.8)	11 (1.9)	
Mixed type					
2 chimeras	123	43	73	7(5.7)	*P* + *p*, *P*, *p*
(Subtotal)	123	43(35.0)	73(59.3)	7(5.7)	
Total	1176	596(50.7)	542(46.1)	38(3.2)	

(6/11) in Ruby ball-dominant chimeras and at 100% (2 of 2) in mixed chimeras. In YR ranpou-dominant chimeras, the crosses in which seeds produced only green plants or only purple plants accounted for 14.3% (2 of 14) and 7.1% (1 of 14), respectively, indicating that these chimeras had a uniform germ-cell-layer genotype. However, these segregation phenomena showed the existence of both genotypic *P* and *p* female germ cells in the ovary of one chimera, suggesting that either germ cells can originate from more than two germ-cell initials in a chimeric state or else that heritable changes can be induced by transformation or by mutation.

Similar segregation phenomena and a similar frequency in the F_1s from the crosses between chimeric plants and the original materials were also observed in the intervarietal in-vivo graft-chimeras in *B. oleracea* (Hirata 1990). These facts show that both in-vitro and in-vivo chimera formation is controlled by essentially the same mechanism. However, the precise basis of the segregation phenomena and relevant details of the developmental process of germ cells and ovary are still not clear; thus, a more thorough developmental study of chimeric plants is necessary at the cellular level. The segregation phenomena involving progeny derived from each cross and the variations in chlorophyll deficiency (albino or variegation production) are shown in Table 2. No parallelism between the segregation pattern and the chimeric type was found. Frequent variation of chlorophyll deficiency was observed in Ruby ball-dominant chimeras and in the Ruby-ball part of mixed chimeras and YR dominant-chimeras: for example, 7.8% (6/77 crosses) in one YR dominant-chimera, 9.5% (2 of 21) in one Ruby ball-dominant chimera, and 10.3% (7 of 68) in one Ruby-ball type of chimera. Almost all chlorophyll-deficient plants died at the cotyledonary stage. This may correspond with the phenomenon in which chlorophyll-deficient variation was observed in the progeny form the Ruby-ball parts in the interspecific chimeras between Ruby ball and Komatsuna (data not shown). The cause of such a high frequency of chlorophyll variation is not clear, but may be due to an irregularity in chloroplast formation or in gene expression during chimera formation.

Cross experiment in interspecific chimeras with two parental plants

Morphological features in interspecific chimeras showed considerable diversity from the Komatsuna type to the Ruby-ball type and with various combinations of the two parental characteristics, as was also observed in in-vivo graft-chimeras. Typical in-vivo and in-vitro interspecific chimeras generally have low pollen fertility, few capsules and low seed setting (Hirata et al. 1992, 1993), as has also been found in some remotely-related plant chimeras; namely, *Cititus adamii* (Neilson-Jones 1969; Tilney-Bassett 1986), tomato-nightshade (Masubuchi 1961; Kumagai and Usami 1964), *Solanum nigrum-S. tuberosum* (Binding et al. 1987), *Nicotiana-*

Table 2 Segregation of plant color and variation in the progenies from the crosses between chimeric plants and one recessive parent, YR-ranpou, used for chimera induction. Abbreviations are the same as in Table 1. Chlorophyll-deficient plants died at the cotyledonary stage; a% indicates the percentage of crosses which produced chlorophyll-deficient plants per total crosses of a chimera

Chimera plant crossed with YR-ranpou	No. of capsules (crosses)	Total no. of plants in capsule	No. of plants in segregation of progenies from the cross			No. of chlorophyll-deficient plants (a% per crosses)
			Purple	:	Green	
YR-dominant type						
3 (YR)	1	13	3	:	10	
5 (YR)	5	31	13	:	18	
	6	38	26	:	0	12 (7.8% = 6/77)
7 (YR)	3	37	4	:	33	
8 (YR)	3	19	8	:	11	
10 (YR)	1	9	4	:	5	
12 (YR)	3	22	4	:	18	
	1	8	7	:	1	1 (1.4% = 1/69)
21 (YR)	1	8	3	:	5	
22 (YR)	1	14	1	:	13	
28 (YR)	1	3	1	:	2	
(Subtotal)						13 (1.4% = 7/490)
R-dominant type						
4 (R)	1	8	5	:	0	3 (5.3% = 1/19)
	1	11	10	:	1	
6 (R)	3	42	10	:	32	
13 (R)	2	20	14	:	0	6 (2.7% = 2/75)
	2	16	8	:	8	
15 (R)	2	6	4	:	0	2 (9.5% = 2/21)
17 (R)	2	7	5	:	0	2 (2.0% = 2/101)
	1	4	3	:	1	
24 (R)	3	18	13	:	0	5 (4.2% = 3/71)
25 (R)	7	48	29	:	0	19 (10.3% = 7/68)
	1	5	4	:	1	
26 (R)	3	35	10	:	25	
(Subtotal)						37 (3.0% = 17/563)
Mixed type						
9 (MX)	5	26	21	:	0	5 (5.8% = 5/86)
	5	46	15	:	31	
23 (MX)	1	5	4	:	0	1 (2.7% = 1/37)
	2	11	6	:	5	
(Subtotal)						6 (4.9% = 6/123)
						Total 56 (2.6% = 30/1176)

Solanum (Kaddoura and Manthell 1991), *Lycopersicon esculentum-peruvianum* (Szymkomiak and Sussex 1992) and *B. oleracea-campestris* (Hirata et al. 1992). The different composition of the outermost layer (LI) and the two other apical layers (LII, LIII) in remotely-related plant chimeras, in particular, results in strong seed sterility (Binding et al. 1987; Kaddoura and Mantell 1991; Szymkoiak and Sussex 1992). In the present experiment the total percentage of capsule setting was very low in the crosses between chimeras and the two species used for chimera formation (2.2% = 30 of 1 355 crosses) (Table 3).

The genotype of the progenies from the crosses was nearly identical to that of the pollen species used. Only two crosses of a chimera with Komatsuna (chimera 102 × Komatsuna) produced interspecific hybrids. Three out of the four of these hybrids grew normally, and showed varied plant color, shape (Fig. 4), and isozyme pattern (Fig. 5). Some variation in the progenies from chimeras was also observed (data not shown). These data suggest two possible occurrences in chimeric plants: one is in-situ chimericity and a multicellular origin of the germ-cell layer, and the second is the presence of transformation or mutation. However, a precise determination of the origin of the germ cells is extremely difficult in apical cells. We are now studying the developmental process using transgenic plants with the GUS gene (Hirata et al. 1993).

From the viewpoint of plant breeding it is also of interest to determine whether or not there could be an increasing effect on hybrid formation in a remotely-related cross supplemented by grafting (Hosoda 1961; Hosoda et al. 1963; Namai 1971) in plant chimeras.

In conclusion, strong physiological interactions were found between different chimeric tissues induced by in-vitro and in-vivo grafting. Genetic interaction, as shown by Glushchenko (1974), was also suggested in chimeric tissues, although this was not as clear. To

Table 3 F_1 progenies from crosses of interspecific chimeric plants with Komatsuna (*B. campestris*) and 'Ruby ball' (*B. oleracea*)

Cross combination		No. of crosses (capsules)	No. of capsules setting (%)	No. of capsules (no. of plants) in F_1 progeny		
Chimera[a] type (♀)	Pollenizer (♂)			Ruby ball type	Komatsuna type	Hybrid type
K (1 chimera)	Open	64	0	0	0	0
	Komatsuna	54	2 (3.7)	0	2 (8)	0
	Ruby ball	–	–	–	–	–
KM (4 chimeras)	Open	49	0	0	0	0
	Komatsuna	522	0	0	0	0
	Ruby ball	41	0	0	0	0
MX (1 chimera)	Open	15	3 (20.0)	0	3 (10)	0
	Komatsuna	127	2 (1.6)	0	0	2 (4)
	Ruby ball	8	0	0	0	0
RM (1 chimera)	Open	75	0	0	0	0
	Komatsuna	173	3 (1.7)	0	3 (12)	0
	Ruby ball	–	–	–	–	–
RM (1 chimera)	117 (R)	22	5 (22.7)	0	2 (5)	0
R (2 chimeras)	Open	25	12 (48.0)	0	0	0
	Komatsuna	132	0	12 (300)	0	0
	Ruby ball	48	6 (12.5)	6 (30)	0	0
Total		1355	33 (2.4%)	18 (330) = 1.3%	10 (35) = 0.7%	2 (4) = 0.1%

[a] K, R, RM and MX indicate the Komatsuna type, the Ruby ball type, the Komatsuna-dominant mericlinal type, the Ruby ball-dominant mericlinal type, and a mixed-chimera, respectively

determine the physiological and genetical interactions between cells and tissues, the detailed developmental process of germ cells and sexual organs in *Brassica* must be studied at both cellular and molecular levels.

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