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Intervarietal and interspecific chimera formation by in vitro graft-culture method in *Brassica*

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Summary. An efficient chimera formation method by tissue culture combined with grafting was studied in *Brassica*. Cabbage cultivars "Ruby-ball" and "YR-ranpou" (*Brassica oleracea*) were used for intervarietal chimera formation. Seven-day-old seedlings leaving one of two cotyledons were approach-grafted and cultured in vitro (AGSC method). Chimeric shoots were obtained by the subculture of directly growing chimeric leaves (DG-CL) from grafted part and cross-cut section of the united part after the graft culture. These were rooted and grown to complete chimera. An approach-grafted culture method was also available for interspecific chimera formation between "Komatsuna" (*B. campestris*) and "Ruby-ball" (*B. oleracea*), resulting in 20% formation per culture. Interspecific chimeras were identified as complex and peripheral-sectorial type by microscopic observation and the electrophoretic analysis of acid phosphatase isozyme pattern. The AGSC method was more effective than the usual Winkler's graft method and a simple mixed culture of heterogeneous cells or tissues.

Key words: *Brassica* – Chimera – Tissue culture – Graft

Introduction

Genetic interactions between chimeric tissues in a plant were described (Gluschchenko 1974, 1975); however, only a few reports have appeared since then. We studied intervarietal and interspecific graft chimeras from the viewpoint that they might undergo transformational events such as graft-induced genetic changes (Hirata et al. 1990).

We were unable to obtain stable plant chimeras at high frequency using Winkler's graft method (1907), especially between distantly related species (Hirata et al. 1990). In order to overcome the problem and to utilize chimeras as breeding material, we attempted to obtain stable plant chimera by applying a tissue culture method combined with a grafting method (Noguchi et al. 1989). In the previous experiments, the approach-grafted hypocotyl culture (AGHC) method was more efficient than simple mixed culture method of different cells or tissues (Carlson and Chaleff 1974; Zatyko et al. 1982; Marcotrigiano and Gouin 1984a, b). By this method we obtained several intergeneric chimeras between tomato and eggplant. However, these plants lost their chimeric structure with growth, showing that these were partial chimera.

In this paper, a modified AGHC method is described, which is novel and available to efficiently obtain chimeric plants in *Brassica*. Some characteristics of the chimeras obtained are also presented.

Materials and methods

Preparation of seedlings

Two cabbage cultivars, "Ruby-ball" (red cabbage) and "YR-ranpou" (green cabbage) (*Brassica oleracea*), were used for this graft-culture experiment to obtain intervarietal chimera. Seeds were sterilized and sown on the half-strength Murashige and Skoog (1962) basal medium (MS) containing 1% sucrose and 0.2% Gellan gum, with the combination of indole butylic acid (IBA) and 6-benzyl aminopurine (BA).

Grafting and tissue culture

Two grafting methods were applied using 7-day-old seedlings: the first method was an approach-grafted hypocotyl culture method (abbreviated as AGHC by Noguchi et al. 1989), and the second was modification of the first grafting, leaving one of the two cotyledons of each seedling (AGSC; see detail in Fig. 1). Four detailed treatments comprised the AGSC method: S-1, grafting with a half-cut apical part; S-2, grafting with an intact

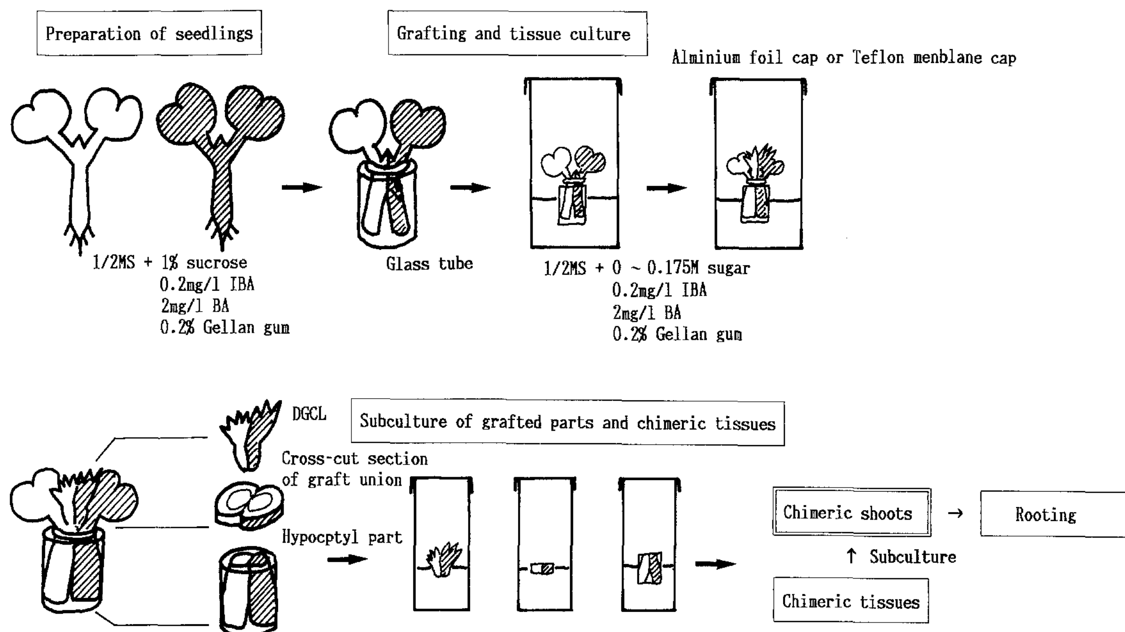

Fig. 1. Schematic presentation of approach-grafted seedling culture (AGSC) method

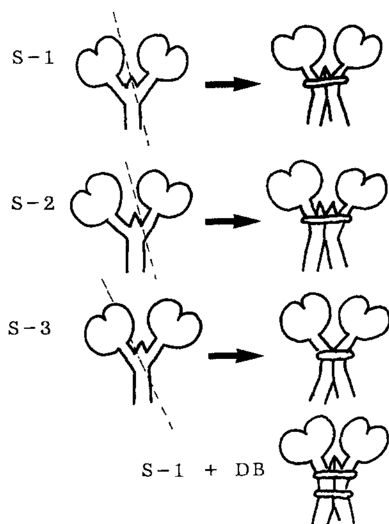

Fig. 2. Four preparations for the AGSC method: S-1, S-2, S-3, and S-1 + DB show the grafting and binding of two young seedlings at one point with a half-cut apical part, with an intact apical part, and without an apical part, and the binding of the two seedlings at two points with surgery fiber under the treatment of the S-1, respectively

apical part; S-3, grafting without an apical part; and S-1 + DB, binding at two points with surgery fiber under the treatment of S-1 (Fig. 2). These graftings were performed under a binocular microscope to assure symmetrical fit of the two materials used. The grafted seedlings were inserted in a small glass tube (3 mm in diameter and 15 mm in height) for support and then were cultured in half-strength MS medium containing 3% sucrose, 0.2 mg/l IBA, 2 mg/l BA, and 0.2% Gellan gum (Fig. 3a). The culture tubes were capped with aluminum foil or Teflon membrane. They were kept at 22 °C under 4,000 lux light for 16 h a day. Effect of sugars on graft union and chimera formation was tested in AGSC by supplying various concentrations of sucrose, glucose, fructose, and mannitol.

Subculture of grafted parts and chimeric tissues

Two weeks after the culture, directly growing chimeric leaves (DGCL) from the fused apical part, a cross-cut section of the graft union, and the united hypocotyl part remaining on a small glass tube in AGSC were subcultured in MS medium containing 3% sucrose, 0.2 mg/l IBA, 2 mg/l BA, and 0.2% Gellan gum. In AGHC, the united hypocotyl part was subcultured in the same medium. Chimeric tissues were further subcultured every 3 or 4 weeks. During the course of these cultures, regenerated shoots that appeared to be chimeras were transferred to half-strength MS medium containing 3% sucrose and 0.2% Gellan gum for rooting. After the acclimatization, chimeras were transplanted to soil pots in the greenhouse.

Interspecific chimera formation and characterization

For the interspecific chimera formation between "Komatsuna" (*B. campestris*) and "Ruby-ball" (*B. oleracea*), the same methods as described above were applied. Chimeric plants obtained by these methods served for histological observation of leaf section and electrophoretic analysis of acid phosphatase isozyme.

Results

Grafting method and culture condition for intervarietal chimera formation

The addition of growth regulators in seed germination medium, grafting methods, and material covering the

Fig. 3. **a** Planting stage of AGSC method. Two seedlings were united and bound with surgery fiber. **b** Chimeric shoots formation by the culture of cross-cut section of graft union. **c** Chimeric shoot formation by the culture of DGCL. **d** Root formation of interspecific chimera. **e** A sectorial-peripheral type of the chimera. **f** Microscopic observation of transversal leaf section of interspecific chimera. Externally sectorial chimeric part was served. A complex anthocyanin pigmentation pattern was obseved in subdermal cell layer(s). **g** In this case externally sectorial-peripheral chimeric tissue was served

culture tube affected the formation of both graft union and chimera. As shown in Table 1, the highest percentage of graft union formation (75%) and chimera formation (53%) was obtained by simultaneous application of germination medium containing 0.2 mg/l IBA and 2 mg/l BA, approach-grafting method, and Teflon membrane cap for culture tube. With a Teflon cap, which allowed for a non-airtight condition, the rate of vitrification was reduced and regenerated shoots were normally grown. The difference in the formation of graft union was not clear among the three detailed preparations on AGSC methods (S-1, S-2, and S-3) used in the present experiment. Binding with surgery fiber at two points for grafting also had no distinctive effect.

Chimeric shoots were regenerated mainly from the cross-cut section of the graft union near the apical meristem (12 out of 14 cases), partially from the united hypocotyl part (1 out of 14 cases), and from the DGCL (1 out of 14 cases) (Table 1 and Fig. 3b).

The type and concentration of sugars in the medium also affected the formation of graft union and regeneration of chimeric shoots (Table 2). The percentage of graft union formation was relatively high in almost all of the media, except for those containing high concentration of mannitol, while the percentage of DGCL formation was high at sugar concentrations lower than 0.022 *M*. Addition of mannitol had a negative effect on the shoot regeneration from the cross-cut section of graft union. In general, chimeric shoots were stably regenerated on media with less than 0.088 *M* sugars, but sugarless medium had a tendency to produce vitreous shoots.

Chimeric plants were propagated by the subculture of chimeric tissues. From 52 culture lines, 35 chimeras were selected and acclimatized. However, several regenerants lost their chimeric structure with age.

Interspecific chimera formation

Chimeric shoots in combination of interspecies were regenerated from the DGCL and from the section of graft union by the S-1 method combined with a Teflon membrane cap. The DGCL mainly arose in the medium of 0.022 *M* sucrose, slightly in that of 0.088 *M* (Table 3).

Two chimeral culture lines from the DGCL and two lines from the cross-cut sections were established (Fig. 3c). Two chimeras were acclimatized and transferred to the greenhouse (Fig. 3d–e).

Characterization of interspecific chimera

By microscopic observation of the transversal section of leaves and petioles in interspecific chimera, cells containing anthocyanin pigments were observed in a mixed state in "Komatsuna" green tissues, indicating that the chimeric plants were complex or sectorial-peripheral type

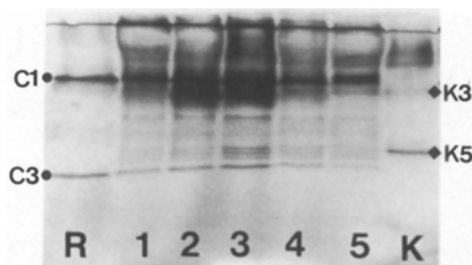

Fig. 4. Acid phosphatase isozyme band patterns of interspecific chimera and two cultivars used for chimera production. "Ruby-ball" (R) has its specific C1 and C3 bands. "Komatsuna" has its specific K3 and K5 bands. *Lanes 1–2*, *3*, and *4–5* are "Ruby-ball" parts, chimeral part, and "Komatsuna" parts in appearance, respectively. All parts (*lanes 1–5*) had both "Ruby-ball" and "Komatsuna" specific bands, suggesting that the chimera was a complex type

(Fig. 3f–g). The electrophoretic isozyme band pattern proved to be a complex chimera type, because the specific isozyme bands of both "Komatsuna" and "Ruby-ball" appeared in the extract from the green tissue, which was morphologically equal to the "Komatsuna" part of the chimeral plant (Fig. 4).

The results clearly show that chimeric plants obtained by the AGSC method have a more or less complex nature.

Discussion

An efficient chimera formation method was established by combining the tissue culture method with the approach-grafting of young seedlings.

In this method, it is important first to establish an appropriate medium to induce multiple shoot regeneration, because chimeric shoots arise from the portion that regenerate many adventitious shoots. For example, in this experiment, addition of 0.2 mg/l IBA and 2 mg/l BA to the germination medium was adequate to maintain the high regeneration ability, and the section of the graft union near the apical meristem could retain it; therefore, graft of the apical part (AGSC) is more effective than that of hypocotyls (AGHC: Noguchi et al. 1989).

By using a Teflon membrane cap, the percentage of graft union formation was increased and chimeric shoots were obtained more frequently than with an aluminum foil cap. This may be due to the reduction of relative humidity in the culture tube, because high relative humidity causes vitrification (Bottcher et al. 1988), which would prevent the formation of graft union by excessive cell growth without cell division.

The medium with a sugar content below 0.022 *M* promoted the development of leaves from the grafted apex, resulting in the DGCL formation, and many

Table 1. Influence of hormone constitutions of germination medium and graft culture methods on chimera formation in cabbage

Germination medium IBA-BA (mg/l)	Culture method		No. of graft cultures[a]	No. of graft unions[b] (%b/a)	No. of cultures with DGCL[c]	No. of cultures with chimera(s) from			Total no. of cultures with chimera(s)[d+e+f] [% = (d+e+f)/b]
	Grafting method	Cap of culture tube				DGCL[d]	cross-cut section of graft union[e]	united hypocotyl part[f]	
0 –0	H	AF	24	6 (25)	–	–	0	0	0 (0)
0 –1	H	AF	21	14 (67)	–	–	0	1	1 (7)
0.2–2	S-1	AF	10	5 (50)	0	–	1	0	1 (20)
0.2–2	S-2	AF	12	7 (58)	0	–	0	0	0 (0)
0.2–2	S-3	AF	12	6 (50)	0	–	2	0	2 (33)
0.2–2	S-1+DB	AF	25	16 (64)	1	1	1	0	2 (13)
0.2–2	S-1	TM	20	15 (75)	1	0	8	0	8 (53)

H, AGHC method; S-1, AGSC method with half-cut apical part; S-2, AGSC method with intact apical part; S-3, AGSC method without apical part; DB, binding at two points at grafting; AF, aluminum foil; TM, Teflon membrane; DGCL, directly growing chimeric leaves from apical part

Table 2. Influence of sugar constitution on chimera formation by AGSC method in cabbage

Sugar constitution of culture medium	No. of graft cultures[a]	No. of unions[b] (% = b/a)	No. of cultures with DGL[c] (% = c/b)	No. of cultures with chimera(s) from		Total no. of cultures with chimera(s)[d+e] [% = (d+e)/b]
				DGCL[d] (% = d/c)	cross-cut section of graft union (% = e/b)	
Sugar free	33	33 (100)	8 (24)	1 (13)	10 (30)	11 (33)
0.011 *M* Mannitol	19	17 (90)	7 (41)	3 (43)	3 (18)	6 (35)
0.011 *M* Sucrose	20	20 (100)	5 (25)	2 (40)	6 (30)	8 (40)
0.022 *M* Mannitol	25	21 (84)	9 (43)	2 (22)	2 (10)	4 (19)
0.011 *M* Glucose + 0.011 *M* Fructose	5	5 (100)	1 (20)	0 (0)	2 (40)	2 (40)
0.011 *M* Sucrose + 0.011 *M* Mannitol	20	20 (100)	11 (55)	2 (18)	2 (10)	4 (20)
0.022 *M* Sucrose	34	34 (100)	13 (38)	3 (23)	8 (24)	11 (32)
0.044 *M* Mannitol	10	8 (80)	0 (0)	–	1 (13)	1 (13)
0.044 *M* Sucrose	9	9 (100)	0 (0)	–	1 (11)	1 (11)
0.088 *M* Sucrose	9	8 (89)	1 (13)	1 (100)	2 (25)	3 (38)
0.175 *M* Mannitol	5	0 (0)	0 (0)	–	–	–
0.022 *M* Sucrose + 0.153 *M* Mannitol	5	0 (0)	0 (0)	–	–	–
0.175 *M* Sucrose	14	12 (86)	1 (8)	0 (0)	1 (8)	1 (8)
Total	208	187 (90)				52 (28)

Abbreviations are the same as in Table 1

Table 3. Frequency of interspecific chimera formation by AGSC method between "Komatsuna" and "Ruby-ball"

Culture method			No. of graft cultures[a]	No. of graft unions[b] (% = b/a)	No. of cultures with DGCL[c]	No. of cultures with chimera(s) from		Total no. of cultures with chimera(s)[d+e] [% = (d+e)/b]
Grafting method	Cap of culture tube	Sucrose content				DGCL[d]	cross-cut section of graft union[e]	
H	AF	0.088	10	9 (90)	–	–	0	0 (0)
S-1	AF	0.088	14	10 (71)	0	–	0	0 (0)
S-1+DB	AF	0.088	15	11 (73)	1	0	0	0 (0)
S-1	TM	0.088	10	10 (100)	0	–	1	1 (10)
S-1	TM	0.022	16	15 (94)	3	2	1	3 (20)

Abbreviations are the same as in Table 1

chimeric shoots were obtained by the successive subculute of DGCL. The cross-cut section also regenerated many chimeric shoots in the subculture.

This culture method was also applied to interspecific chimera formation, resulting in 20% formation per culture (Table 3). We were not able to obtain complete intergeneric chimera between tomato and eggplant combination (Noguchi et al. 1989). This may be a sympton of graft incompatibility as shown by Parkinson et al. (1987). This experiment suggests that chimera formation is also related to the regenerative ability of coherent cells of two kinds. As for interspecific chimera formation by the tissue culture method, Carlson and Chaleff (1974) were able to raise 28 chimeral plants from 237 regenerants selected from the culture of approximately 300 chimeric calli, which were obtained by placing two different interspecific pith slices of tobacco, whereas Marcotorigiano and Gouin (1984a, b) were unable to obtain chimeric plants from mixed callus cultures, although four chimeras arose in the intervarietal combination in tobacco. The efficiency of chimera formation in this experiment was higher than that of a simple mixed culture of cells or tissues and than so-called Winkler's method of graft chimera formation in *Brassica* (Hirata et al. 1990). Furthermore, if graft preparation is done in such a way that the shoot apex or leaf primordia of the two materials is symmetrically fit together, the percentage of chimera formation by the present method will be improved. Recently, a new technique for the production of plant chimeras by co-culture of isolated protplasts was offered (Binding et al. 1987, 1989, 1990; Lindsay et al. 1990). The method for chimera synthesis should be applied between remote species such as intergeneric combinations.

Carlson and Chaleff (1974) confirmed that most chimeras (25/28) were of the peripheral type, and Marcotrigiano and Gouin (1984a) indicated that three of four chimeras were of the peripheral type, suggesting that peripheral chimeras might be induced more frequently than other types. On the other hand, it was confirmed by external and histological observation and by electrophoretic isozyme analysis that interspecific and intervarietal chimeras obtained in this experiment were of the complex or sectorial-peripheral type. The cause of this difference is unknown. Preparation of repeated cutting-back of chimeric segments followed by subculture was one of the probable factors in producing complex or sectorial-peripheral chimeras in this culture method. Vegetative propagation of sectorial chimeras actually produced various complicated types of chimera (Hirata et al. 1990). If the chimera formation is more easily controlled, the chimeric plant itself will be utilizable in horticulture, as well as being a good tool to study plant morphogenesis and histocompatibility.

As existence of genetic interaction was suggested between heterogeneous tissues in an intervarietal graft chimera in cabbage (Hirata et al. 1990), further testcrosses are being performed to clarify the genetic constitution of the germinal tissue of the chimeral plants.

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References

Binding H, Witt D, Monzer J, Mordhorst G, Kollmann R (1987) Plant cell graft chimeras obtained by co-culture of isolated protoplasts. Protoplasma 141:64–73

Binding H, Bünning D, Gorshen E, Hansen U-P, Ling HQ, Röhl S, Zuba M (1989) Protoplast plating and fusion in agarose lenses applied to potato. In: Vorträge für Pflanzenzüchtung 15-1 (Göttingen), Poster Abstract 8–10

Binding H, Mordhorst G, Glockmann C, Kollmann R (1990) Cell graft chimeras *Tropaeolum* (+) *Solanum* of two subclasses of the *Magnoliatae*. Eur J Cell Biol 51 [Suppl. 30]:29

Bottcher I, Zoglauer K, Goring H (1988) Induction and reversion of vitrification of plants cultured in vitro. Physiol Plant 72:560–564

Carlson PS, Chaleff RS (1974) Heterogeneous association of cells formed in vitro. In: Ledoux L (ed) Genetic manipulations with plant materials. Plenum Press, New York, pp 245–261

Gluschchenko IE (1974) Problem on the nature of plant chimera. Part I. Jpn J Michurin Biol 10:54–67

Gluschchenko IE (1975) Problem on the nature of plant chimera. Part II. Jpn J Michurin Biol 11:97–107

Hirata Y, Yagishita N, Sugimoto M, Yamamoto K (1990) Intervarietal chimera formation in cabbage (*Brassica oleracea* L.). Japan J Breed 40:419–428

Lindsay GC, Mordhorst G, Glockmann C, Kollmann R, Binding H (1990) Cell grafting and somatic hybridization of tomato cultivars with a *Solanum hybrid*. In: Abstr 7th Int Congr Plant Tiss Cell Cult (Amsterdam), Poster A8–41

Marcotrigiano M, Gouin FR (1984a) Experimentally synthesized plant chimeras. I. In vitro recovery of *Nicotiana tabacum* L. chimeras from mixed callus cultures. Ann Bot 54:503–511

Marcotrigiano M, Gouin FR (1984b) Experimentally synthesized plant chimeras. II. A comparison of in vitro and in vivo techniques for the production of interspecific *Nicotiana* chimeras. Ann Bot 54:513–521

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Noguchi T, Hirata Y, Yagishita N (1989) Intergeneric plant chimera formation by tissue culture methods in tomato-eggplant and mulberry-paper mulberry. Bull Gen Educ, Tokyo Univ Agric Tech 25:339–352

Parkinson M, Jeffree CE, Yeoman MM (1987) Incompatibility in cultured explant-grafts between members of the Solanaceae. New Phytol 107:489–498

Winkler H (1907) Über Propfbastarde und pflanzliche Chimaeren. Ber Dtsch Bot Ges 25:568–576

Zatyko JM, Simon I, Szalay I, Molnar I (1982) Mixed culture of callus homogenates of different plant species in vitro. In: Proc 5th Int Congr Plant Tiss Cell Cult (Tokyo), pp 61–62