

# Synthesis and Utilization of *in Vitro* Artificially Synthesized Chimeras<sup>©</sup>

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## INTRODUCTION

A plant chimera consists of at least two genotypes in the same meristem, organ, or tissues in one plant. In the past, chimeras were used mainly for ornamental plants such as variegations (chlorophyll chimera) that were induced by natural and artificial mutations (Tilney-Basset, 1986).

Plant chimera utilization and research is a very old but innovative field. The idea to combining different cells, tissues, and organs is very mysterious and especially creative thought. In 1907, Winkler developed a graft-chimera using black nightshade and tomato (Winkler, 1907), obtaining "Burdo", a graft hybrid. However, application for breeding has not been performed although chimeras have been used for morphogenetical studies.

Little is known about potential graft-induced genetic change or gene transfer that occurs in graft chimeras. In our studies to examine the mechanism of interaction between different cells and tissues, Winkler's graft method (Winkler, 1907) was applied to obtain *Brassica* interspecific and intraspecific chimeras. However, the efficiency of chimera synthesis was low compared to applying *in vivo* grafting discussed below.

## NEW METHOD FOR ARTIFICIALLY SYNTHESIZED CHIMERAS

In the present work we developed a new *in vitro* method for synthesizing and propagating chimeras (Noguchi, et al., 1989, 1992), named "approach-grafted seedling culture (AGSC) method", which consists of seed sterilization, followed by culture and approach grafting of seedlings. Seeds of plant material were sterilized in 70% ethanol solution and in 1% sodium hypochlorite solution. The sterilized seeds were cultured on Murashige and Skoog (1962) basal medium (MS) containing 3% sucrose, 0.3% Gellan gum, and 0.1 ppm 6-benzylaminopurine (BA). Seven-day-old seedlings were cut into two pieces and the two halves of different plants were tightly fitted with each other by using surgery fiber. Approach-grafted seedlings are put into small tubes and then grown in test tubes. After fusion of the graft union, the grafted union and the remaining hypocotyls part are cut into thin discs and subcultured on MS medium containing 2 ppm BA and 0.2 ppm indolebutyric acid (IBA) (Fig. 1). Using this method chimeral plants were synthesized and the genetic interactions between different tissues via morphogenesis have been studied. In this paper we will present recent results on synthesized chimera using the AGSC method and utilization of interspecific and intergeneric chimeras.

## INTERSPECIFIC AND INTERGENERIC CHIMERAS

*Brassica rapa* Pervirides Group 'Komatsuna', *B. oleracea* Capitata Group 'Ruby Ball', and transgenic *B. napus* 'Westar' with GUS gene, and radish, *Raphanus sativus* 'Miryokuna' were used as plant materials.

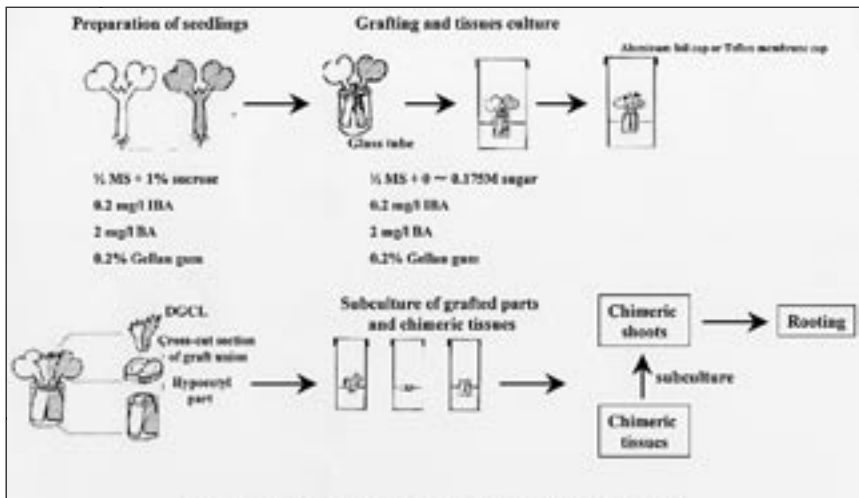
Using the cultivars above described, two interspecific chimeras were obtained. One is between ‘Komatsuna’ and ‘Ruby Ball’, named KR chimera and other is between ‘Westar’ and ‘Ruby Ball’, named WR chimera. In addition, one intergeneric chimera between ‘Miryokuna’ radish and ‘Ruby Ball’, named MR chimera, also was obtained.

The characterization of synthesized chimeric plants was done by observation of morphological characteristics like leaf shape, leaf margin form, presence or absence of trichome, observation of anthocyanin pigmentation (in case of ‘Ruby Ball’), GUS gene expression (in case of ‘Westar’) in the leaf tissue section, and electrophoretic analysis of acid phosphatase isozyme. The cell layer constitution of each chimera was designated according to “Tunica-Corpus” theory (Schmidt, 1924). According to this theory higher plant tissues and organs are originated from three layers in the apical meristem: LI, outer tunica; LII, inner tunica; and LIII, corpus. In this paper, we express the chimeric layer structure by three-letter codes where each layer represents the initial letter of parental plants. For example, in the interspecific chimera KRR obtained between ‘Komatsuna’ and ‘Ruby Ball’, the first letter K represent the LI layer derived from ‘Komatsuna’, the second R represent the LII layer, and the third letter also R represent LIII layer that derived from ‘Ruby Ball’.

**CHARACTERISTICS OF BRASSICA INTERSPECIFIC CHIMERA (KR CHIMERA) BETWEEN ‘KOMATSUNA’ AND RED CABBAGE.**

Interspecific chimera synthesized between ‘Komatsuna’ and ‘Ruby Ball’ resulted in a six types of chimeric plants (Oguni et al., 1996). We could classify the chimeric plants obtained, according to three letter codes. In revertant to red cabbage type we could be represented by LI-LII-LIII=RRR, periclinal chimera KRR, mixed type of chimera KRR+KKR, ‘Komatsuna’-dominant mericlinal type KRR+KKR+KKK, periclinal chimera KKR, and the revertant ‘Komatsuna’ type KKK.

Applying AGSC method, the number of chimeras obtained was higher in quantity than chimeras obtained by Winkler’s graft method. In the case of KR chimera, success of synthesis using AGSC method was 26%, while Winkler method result in



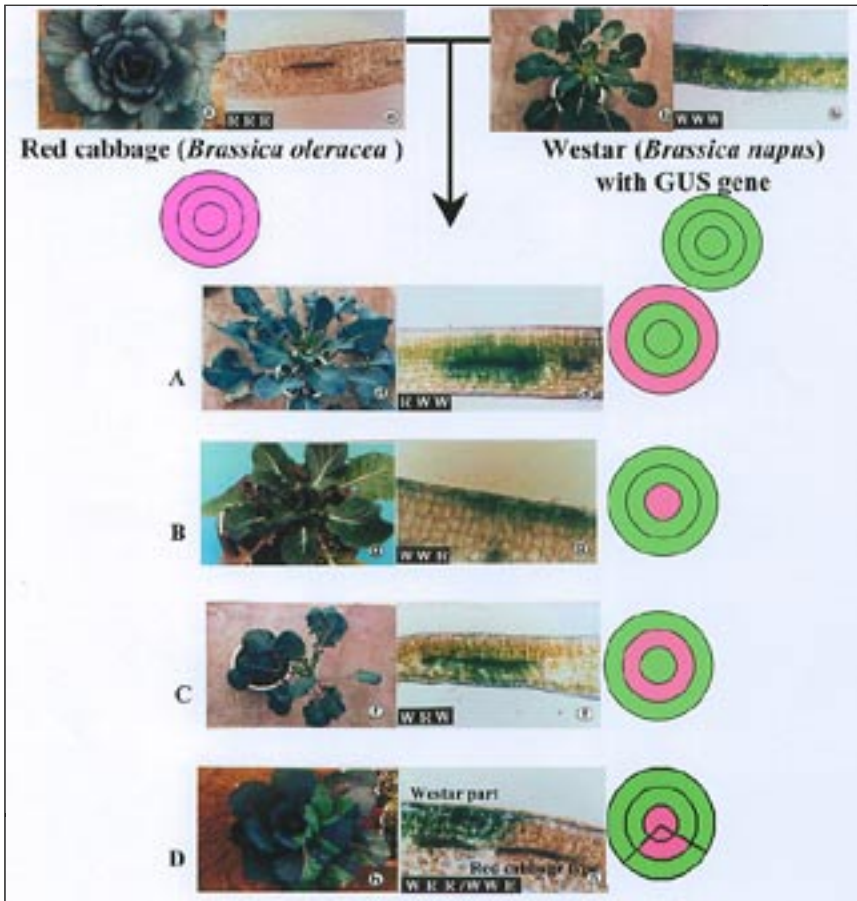
**Figure 1.** Schematic presentation of approach-grafted seedling culture (AGSC) method.

Table 1. Qualitative characteristics of parental plants and interspecific chimeras.

Characteristics	Komatsuna	Red cabbage	Periclinal chimera	
	KKK	RRR	KKR	KRR
Leaf color	fresh green	purple	green	red
Leaf shape	spatulate	oval with stipule	spatulate to oval	oval with stipule
Leaf thickness	thin	thick	thin	thick
Color of leaf vein	white	purple	white with red sectors	red
Life cycle	annual	perennial	annual	perennial
Petal color	yellow	cream	yellow	yellow
Petal shape	round	spatulate	round	spatulate
Flowering habit	early	late	intermediate	comparatively late
				green
				spatulate to cordate
				thick
				red
				annual
				-
				-
				-

1.3% in interspecific ones (Oguni et al., 1996). The comparison between qualitative characteristics of parental plants and KR chimera was summarized in Table 1.

Typical example of the useful change by in vitro synthesized chimera is the induction of cytoplasmic male sterility (CMS) in seed progeny (Hirata et al., 2001). By backcrossing of 'Komatsuna'-dominant mericlinal chimera (KRR+KKR+KKK) with the mother plant, 'Komatsuna', this resulted in a four CMS seed progeny. CMS was induced by chimera synthesis between normal 'Komatsuna' and normal 'Ruby Ball'. Obtained CMS variant lines basically maintain the phenotypes of 'Komatsuna' with slight morphological variations in flower organs, some other variations were also observed such as in leaf shape, petal color, capsule shape, and chlorotic expression in the progenies individuals. (Hirata et al., 2001)



**Figure 2.** Morphology (left), leaf tissue structure (middle) and apical meristem layer structure (left) of parental types and periclinal and mericlinal chimera. (A) periclinal chimera RWW, (B) periclinal chimera WWR, (C) periclinal chimera WRW and (D) GUS expression pattern in mericlinal chimera WRR/WWR. GUS activity shows Westar part (left) of inner structure in the leaf. Light area (right) is red cabbage part which are justified by anthocyanin-pigmentation.

To search the basis of CMS variation, analysis of 12 mitochondrial genes and 17S ribosomal RNA nuclear genes were performed by PCR and/or Southern blot analysis. These results showed interestingly that Ogura radish type of CMS appeared via chimera synthesis and propagation and that the mechanism was due to stoichiometric shift of responsible mitochondria genome from normal to Ogura type.

This drastic cytoplasmic genome change is a very new finding and importance to elucidate the mechanism for future CMS breeding. We are further exploring the mechanism and genome organization.

### **INTERSPECIFIC CHIMERA BETWEEN TRANSGENIC BRASSICA NAPUS WITH GUS GENE AND RED CABBAGE.**

At the next step, to search the inner structure of *Brassicaceae* leaf, 'Westar' with GUS gene and 'Ruby Ball' that express anthocyanin pigmentation were grafted to obtain a WR interspecific chimera (Oguni et al., 1996). It resulted in 7 types of periclinal chimeras (RRR, WWW, RRW, RWW, WRR, WRW, and WWR) and 1 type of mericlinal chimera (WRR/WWR). These chimeral types were investigated by observation of anthocyanin pigmentation and GUS expression pattern under a light microscope and confirmed by acid phosphatase isozyme analysis.

Anthocyanin pigmentation corresponds to 'Ruby Ball' cell. If a chimera has even one layer of 'Ruby Ball', anthocyanin is visible in the leaf. On the other hand, GUS expression pattern were useful markers to identify the presence of 'Westar' tissue(s) (Fig. 2). For example, in the leaf inner structure of mericlinal chimera WRR/WWR, GUS activity was found. The 'Ruby Ball' part was determined by anthocyanin pigmentation before GUS staining.

Leaf histochemical analysis by GUS expression and anthocyanin pigmentation clarify that the leaf structures were also composed of three layers: LI-epidermis, LII-outer palisade and spongy tissues, and LIII-inner palisade and spongy tissue and vascular bundle. These results were closely related with morphological characteristics. As it is well known, each cell layer contributes to the formation of specific organs of plant. However, precise study on apical cell layer constitutions would be necessary to explain more deeply the development of plants in *Brassica*.

### **Intergeneric Chimera Between *Brassica oleracea* and *Raphanus sativus*.**

Followed by interspecific chimeras, to investigate the morphogenetical, physiological nature of the intergeneric chimeras, Miryokuna radish (M) and red cabbage (R) were used as a material and synthesized by in vitro grafting method (Hirata et al., 2000). Chimeral structures were justified and totally classified based on the morphological characteristics, isozymatic band patterns, and PCR analysis. Physiological interactions were typically recognized such as in flowering date, pollen fertility, and pod and seed set. However, genetic changes were not found in the progeny derived from the crosses with both parents like in interspecific chimera.

General characterization of periclinal and mericlinal intergeneric chimeras obtained could be identified and classified based on the three layers in apical meristem. Red cabbage type could be represented by RRR, 'Komatsuna' radish type = KKK, two kinds of periclinal type = MRR and MMR, and four types of mericlinal types = MMR+MRR, MRR+MMR, MMR+MRR, and MMR+MRR+MMM.

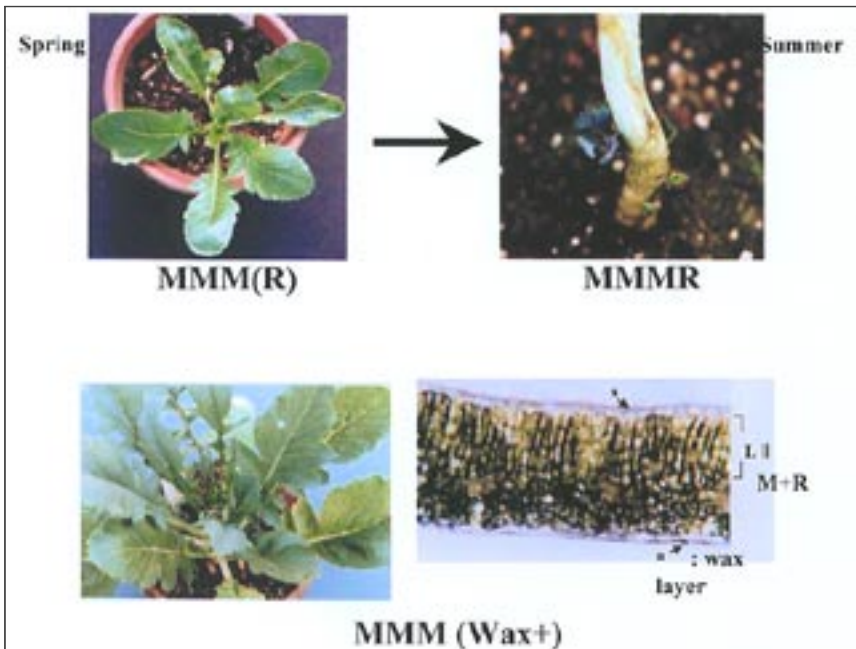
General characterization of those intergeneric chimeras could be identified based on the classification criteria of interspecific chimeras, but chimeral structures changed by the replacement of cell layer constitution along with developmental

stages and seasons. Every layer displacement could occur in both directions, from M to R and vice versa. During the summer season growth of cabbage seems to be predominant over that of radish. On the contrary, growth of radish seems to be dominant over that of cabbage in winter and cool seasons. A distinctive structure was discovered, that is a putative four-layered origin of apical meristem. In the revertant type of radish, red cabbage emerged from the basal part of stem, suggesting that in the more inner LIII layer exist another layer. This type would be identified as the MMMR type, one type of four-layer origin. Another probable four-layered type was found. This new type was named MMM (Wax+) because in the leaf surface layer of this type, a wax layer was confirmed. Compared with 'Miryokuna' radish or revertant type of radish (three layers in apical meristem), MMM (wax+) has an additional wax layer, on the surface (Fig. 3).

### CITRUS CHIMERA BETWEEN 'KAWANO-NATSUDAIDAI' AND FUKUHARA' ORANGE.

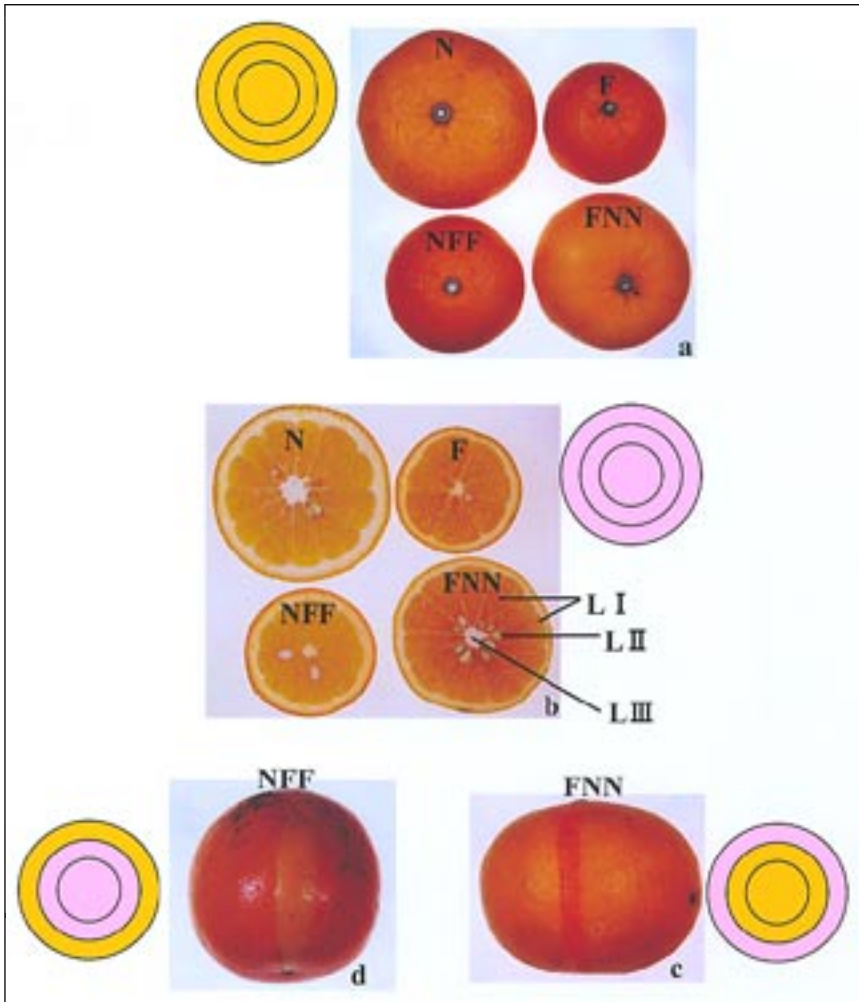
Utilizing the in vitro grafting method presented in this paper a new technique to breed fruit or vegetable will be expected. Also it can be used to induce a variation in progenies like cytoplasmic male sterility (CMS), described above.

Another example of in vitro grafting method in fruit tree is the synthesis of citrus chimera. Using *C. natsudaidai* Hayata 'Kawano-Natsudaidai' (abbreviated as N)



**Figure 3.** Chimera type, putative four layer structure origin in the intergeneric chimeras. Appearance of red cabbage shoot from putative MMMR type which was justified as revertant radish type in an earlier stage and included cabbage tissue in the more inner LIII layer (upper). MMM(wax+) is an another putative four layered type (bottom). Compared with Miryokuna radish and revertant type of radish with present three layers in apical meristem, this type has an additional layer, wax layer, on the leaf surface.

that has disease resistance and a good-tasting *C. sinensis* 'Fukuhara' orange (abbreviated as F) as material, citrus chimera with disease resistance and good taste could be produced (Kuhara, 1988, 1989, and Zhou et al., 2002). These periclinal chimeras also could be identified based on the three-layer structure in the apical meristem. Here we designate these chimeras as FNN= LI - LII - LIII and NFF= LI- LII- LIII. Morphological analysis of parental plants and two types of chimeras showed us that N differs from F in fruit color, size, and shape. The chimera NFF compared with its donor, increased the fruit size and weight, while FNN decreased. In addition, FNN has yellow epicarp in oblate shape as N and orange juice sac as F, while NFF has orange epicarp in spheroid shape as F and yellow juice sacs as N indicating that epicarp derived from LII and juice sacs from LI and fruit size is de-



**Figure 4.** Interaction between F and N tissues in chimeral fruits. Morphological analysis like fruit size, shape and color, in combination with genetical analysis, shows us that interaction of different tissues have resulted in DNA variation in the chimeral plants.

terminated by LII and/or LIII (Fig. 4). Southern hybridization in chimeras with FNN specific RAPD marker as a probe was performed to analyze genetical chimeras' type. As a result, genomic DNA of FNN revealed five distinct fragments, all different from either of its parental plants. This shows us that interactions of different tissues have resulted in DNA variation in the chimeral plants.

**Applicability Of Chimeras For Annual Crops And Perennial Trees Improvement.** As our chimeras and their distinctive natures are introduced, chimeral plants have some applicability for horticulture and breeding.

The first applicability is to use the chimera itself as an ornamental plant for characteristics such as for flower color and variegation by combining good characters into one plant as done in the citrus chimera. We are now trying to synthesize *Melaleuca*-resistant nature and *Eucalyptus* fast growing nature for new resistant tropical paper tree for severe conditions.

The second possibility is to apply this technology method to induce new types of genetic change in the progeny derived from a chimera such as *Brassica* CMS.

The third is to create a new plant science about cell-to-cell interactions at morphogenetical, physiological, and genetic levels.

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