
The Use of Genome Information for Intergeneric Hybridization Breeding

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Abstract

Radish is an important vegetable crop and has several agriculturally useful traits to improve the breeding of *Brassica* crops, which include cytoplasmic male sterility, resistance to pod shattering, tolerance to saline soils, and resistance to nematode and diseases. To transfer these traits into *Brassica*, a number of intergeneric hybridization have been performed using intergeneric cross and/or protoplast fusion, and it was shown that some of them were introduced successfully into and expressed in *Brassica* or hybrids with *Brassica*. The various kinds of hybrid progenies such as synthetic amphidiploid line, alloplasmic line, alien gene(s) introgression line, and monosomic alien chromosome addition line would be valuable genetic resources not only to breed more useful cultivars but also to analyze each chromosome and gene concerned. Recently, the development of numerous molecular markers for genetic study has been accelerated in radish, and a high-density linkage map has been constructed. These molecular tools have been used for the identification of QTLs associated with important agronomic traits of radish, genetic analysis of hybridization barrier between radish and *Brassica* crops, and comparative genomics of Brassicaceae. The further accumulation of genome information will facilitate the cloning of candidate genes in QTLs and the marker-assisted breeding in radish, in addition to the intergenomic transfer of useful radish genes into *Brassica* crops through intergeneric hybridization.

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14.1 Introduction

Radish (*Raphanus sativus* L., $2n = 18$, RR) belongs to the family Brassicaceae and is an economically important vegetable crop that is grown and consumed around the world,

especially in East Asia. The genus *Raphanus* originates from coastal regions along the Mediterranean and Black Seas, and *R. sativus* is ecologically classified into five varieties: var. *radicula* DC. (var. *sativus* Saz., European small radish), var. *niger* (Mill.) Pers. (var. *major* A. Voss., black radish), var. *raphanistroides* Makino (var. *hortensis* Backer, Asian big radish including north Chinese radish, south Chinese radish, and Japanese radish), var. *mougri* Helm (var. *caudatus* (L.) Hooker & Anderson, rat's tail radish), and var. *olerifer* Netz. (var. *oleiformis* Pers. oil radish) (Banga 1976; Hida 1990; Kumazawa 1965). European small radish with smaller root and early maturity is a popular garden vegetable all over the world. The north Chinese radish, which is characterized by smaller, colored, and starchy root, is used as pickles, fresh vegetables, and condiments. The south Chinese radish with pubescent leaf and low starchy and juicy long root is used for salted vegetables and cooking (Kaneko et al. 2007).

The agricultural traits of the genus *Raphanus* were summarized by Warwick (1993). These include cytoplasmic male sterility (CMS) (Ogura 1968; Yamagishi and Terachi 1996; Tanaka et al. 2012), resistance to pod shattering (Agnihotri et al. 1990), tolerance to saline soils (Warwick 1993), and resistance to blackleg (Salisbury 1987), beet cyst nematode (Lelivelt and Krens 1992; Lelivelt et al. 1993a), and clubroot (Ashizawa et al. 1980). Other *Brassica* crops often lack these traits and are susceptible to these pathogens. Therefore, radish is an important genetic resource for the breeding of *Brassica* crops. A number of intergeneric hybridizations have been performed to transfer these agronomically useful traits into *Brassica* crops, since *Raphanobrassica*, an intergeneric hybrid between *R. sativus* and *B. oleracea*, was reported by Karpechenko (1924) (Kaneko et al. 2009). For nearly a century, cytogenetic research based on their hybridization has been conducted, resulting in the discovery of excellent evidence for partial homologous relations and speciation of these species. Recently, several genetic maps of radish have been constructed using RFLP, AFLP, RAPD, and EST-SSR markers, and these

have been applied to QTL identification of beet cyst nematode and clubroot resistance, the shape and pigmentation of root, and flowering time, and comparative genomics of the Brassicaceae (Kaneko et al. 2007; Tsuru et al. 2008; Budahn et al. 2009; Kamei et al. 2010; Shirasawa et al. 2011). Kitashiba et al. (2014) determined the *R. sativus* draft genome sequences by a next-generation sequencer (NGS) along with bacterial artificial chromosome (BAC)-end sequences. They constructed a high-density linkage map by adding new DNA markers and combining two different linkage maps. These genetic markers and high-density linkage maps could be used for the identification of candidate genes in QTLs, and for the marker-assisted breeding in radish, in addition to intergenomic transfer of radish genes into the *Brassica* crops through intergeneric hybridization followed by successive backcrossings.

Intergenic gene transfer has been carried out for over a century in an attempt to transfer valuable genes into crop species from wild relatives. Recently, intergeneric hybridization has been extensively performed between cultivated species, and between cultivated species and wild relatives, to develop more useful cultivars with improved biotic and abiotic stress tolerance (Harberd and McArthur 1980; Kaneko and Bang 2014; Kaneko et al. 2009; Prakash et al. 1999). The prospects of intergeneric hybridization for genetics and plant breeding in Brassicaceae were evaluated by Matsuzawa et al. (1996) who suggested a system for the development of the five hybrid progenies: synthetic amphidiploid line, alien gene(s) introgression line, alloplasmic line, monosomic alien chromosome addition line (MAL), and monosomic alien chromosome substitution line. These hybrid lines would be valuable genetic resources not only to breed more useful cultivars with novel agronomic traits but also to analyze each chromosome and gene concerned. In these kinds of research projects, it is prerequisite to grow true F₁ hybrids and as many of their progenies as possible, although the reproductive isolation in the intergeneric hybridization hampers the development of these hybrids and progenies (Kaneko and Bang 2014).

In this chapter, we will outline the intergeneric hybridization between radish and the *Brassica* crops, their development, preservation and improvement of hybrid lines, and application of molecular markers in order to expedite the progress of breeding.

14.2 Intergenic Hybridizations Between Radish and *Brassica* Crops

Intergenic hybridizations between radish and *Brassica* crops have been made to produce different types of *Raphanobrassica* and *Brassicoraphanus* hybrids for cytological and evolutionary studies, and for investigation of their agronomic potential. The amphidiploid *Raphanobrassica* (RRCC genomes), originating from pollinations of radish (RR) with *Brassica oleracea* (CC) pollen, was first obtained by Karpechenko (1924). Its reciprocal crossing, however, was not successful (Karpechenko 1924). Sarashima et al. (1980) obtained the same intergeneric hybrids using embryo rescue. When McNaughton (1973) compared differences between diploid and tetraploid *B. oleracea* plants as the pollen parent in crossings with radish, he found that the crossings with the tetraploid were more successful. Although the amphidiploid *Raphanobrassica* (RRAA), a hybrid between radish (maternal parent) and *B. rapa* (paternal parent), is reportedly much more difficult to produce, a few have been developed (U et al. 1937; Sarashima and Matsuzawa 1989), and several hybrids (RRAA) were obtained using embryo rescue (Matsuzawa et al. 2000). *Brassicoraphanus* (AARR), i.e., a reciprocal amphidiploid hybrid, has been developed using *B. rapa* as a maternal parent with embryo rescue (Tokumasu 1976; Sarashima and Matsuzawa 1989).

A number of intergeneric hybridizations between radish and *B. napus* have been performed in order to transfer agronomically useful traits from radish into *B. napus*, but only a few

hybrid plants have been obtained owing to their poor crossability. The use of *Raphanobrassica* and *Brassicoraphanus* hybrids as the bridge plants in the crossings with *B. napus* has been much more successful than the use of intergeneric hybridization between radish and *B. napus* (Agnihotri et al. 1990; Lelivelt et al. 1993a; Peterka et al. 2004). Several biotechnological methods have been performed to overcome the barrier existing in intergeneric hybridization between *B. napus* and radish: ovary culture (Luo et al. 1989), ovule culture (Dolstra 1982), flower culture (Metz et al. 1995), in vitro organ culture (Luo et al. 2000), and protoplast fusion (Lelivelt and Krens 1992; Pelletier et al. 1983; Sakai and Imamura 1990; Sakai et al. 1996; Wang et al. 2006). Agnihotri et al. (1990) successfully used ovary culture followed by embryo rescue to obtain plantlets by the hybridization between *B. napus* and *Raphanobrassica*. Hybridizations between two different *Raphanobrassica* hybrids (RRAA and RRCC) and *B. napus* (AACC) were performed in order to develop *B. napus*-radish monosomic addition lines (MALs) (Akaba et al. 2009a; Budahn et al. 2008). Ohtsu et al. (2015) obtained intergeneric hybrid seeds between several *B. napus* lines and *R. sativus* cv. 'Shogoin-daikon' without any embryo rescue, and they developed true intergeneric hybrids (AACRR) subsequent to chromosome doubling, and then generated *B. napus*-radish chromosome addition lines through successive backcrossing to *B. napus*.

A complete series of chromosome addition lines enables markers and genes to be physically allocated to chromosomes without a need for detectable morphological polymorphisms. The series could serve as a bridge plant between genetic maps in radish and physical location on single radish chromosomes by deletion strains. Such a series could be helpful for a more directed and stable introgression of interesting genes into *Brassica* genomes and contribute to the study of the evolutionary relationship of the genus *Raphanus* with other genera in the tribe *Brassicaceae*.

14.3 Homoeologous Chromosomes Between Radish and *Brassica* Crop Genomes

The existence of homoeologous chromosomes between radish and *Brassica* genomes has been reported by many researchers. Namai (1976) and Sarashima (1991) observed that the intergeneric hybrids between radish and *B. oleracea* showed a number of bivalent chromosomes at metaphase I, six being the maximum number, and also trivalent and tetravalent chromosomes in very rare occasions. Sarashima (1991) also observed four bivalent chromosomes in F₁ hybrids between *B. rapa* and radish, and up to five bivalent chromosomes in the reciprocal F₁ hybrids. Mizushima (1980) indicated the number of allopairs between R and A genomes is more than three after considering autopairst in an identical genome. Pollen mother cells (PMCs) in the RACC hybrids had varying numbers of univalent and bivalents, but always more than nine bivalents, suggesting that there are homoeologous chromosomes between the R and A genomes (Paulmann and Röbbelen 1988; Agnihotri et al. 1990). A cytological examination of the RCAA plants showed that the majority of PMCs had the expected 38 chromosomes, but there was a higher level of chromosome pairing than the expected one (10 bivalents) between just the A genome chromosomes, suggesting that the R and C chromosomes also make pairing (Lelivelt et al. 1993a). These results indicate that the introgression and gene transfer are possible by homoeologous chromosome pairing between radish and *Brassica* crops. Voss et al. (1999) also observed a translocation based on GISH analysis of BC₃ progenies between radish and *B. napus*, resulting from natural recombination events between the R genome and the A and C genomes, respectively. Recently, a comparison of the genome structure of radish with those of the A, B, and C genomes of *Brassica* based on high-density linkage maps using EST-based SNP markers revealed extensive chromosome homoeology among their genomes, resulting in facilitated transfer of genes from radish to *Brassica* crops (Li et al. 2011).

14.4 Intergeneric Gene Transfer from Radish to the *Brassica* Crops

In practical breeding of *Brassica* crops, desirable traits of radish have been introduced successfully into *Brassica* crops, for example, cytoplasmic male sterility, its fertility restorer, and resistance to diseases and pests such as Turnip mosaic virus (TuMV), clubroot, and beet cyst nematode. Molecular methods including GISH and several polymorphic DNA markers are being widely used in breeding by marker-assisted selection, and in identifying integrated alien chromosomes, chromosome segments, and genes (Table 14.1).

14.4.1 Introgression of the CMS Restorer Gene(s)

One of the CMS systems used by rapeseed (*B. napus*) breeders is the alloplasmic system derived from the radish male sterile cytoplasm such as Ogura CMS (Ogura 1968; Delourme and Budar 1999) and Kosena CMS (Sakai and Imamura 1990; Sakai et al. 1996; Koizuka et al. 2000). Although restored fertility is essential for production of F₁ seeds using the radish CMS, no restorer genes for this system are known to exist in rapeseed. In order to transfer the restorer fertility (*Rf*) genes from radish to the rapeseed recipient, intergeneric crossing or protoplast fusion between them was performed (Paulmann and Röbbelen 1988; Sakai et al. 1996). The Ogura CMS restorer gene (*Rfo*) was transferred into the C genome of *B. napus* from radish (Delourme and Eber 1992; Delourme et al. 1998). In situ hybridization and genetic mapping further confirmed that the radish fragment with the *Rfo* gene was introgressed into the distal region of linkage group 19, corresponding to chromosome 9 of *B. napus* C genome (Hu et al. 2008). Recently, the *Rfo* gene was introgressed from radish to *B. juncea*, and mapped on chromosome 9 of *B. juncea* A genome (Tian et al. 2014). Based on molecular markers tightly linked to the *Rfo* gene, the radish introgressed fragment

Table 14.1 Agronomic traits of radish introduced successfully into and expressed in *Brassica* crops or hybrids with *Brassica*

| Traits | Gene(s) | <i>Brassica</i> crops | Means to transfer | References |
|---------------------|---|--------------------------------|------------------------------------|--|
| CMS restorer | <i>Rfo</i> | <i>Brassica napus</i> | Intergenic cross/protoplast fusion | Delourme and Eber (1992) |
| | | <i>Brassica juncea</i> | Unknown | Delourme et al. (1998) INRA (cited by Tian et al. 2014) Tian et al. (2014) |
| | <i>Rfk1</i> | <i>B. napus</i> | Protoplast fusion | Sakai et al. (1996) Koizuka et al. (2000) |
| TuMV resistance | Unknown | <i>Raphanobrassica</i> | Intergenic cross | Krämer et al. (2003) |
| Nematode resistance | QTL <i>Hs1^{Rph}</i> | <i>B. napus-R. satius</i> MALs | Intergenic cross | Budahn et al. (2009) |
| Clubroot resistance | 'c' chromosome | <i>B. napus-R. satius</i> MALs | Intergenic cross | Akaba et al. (2009a, b) |
| | A major gene and two minor genes on 'c,' 'b,' and 'f' chromosomes, respectively | <i>B. napus-R. satius</i> | Intergenic cross | Ohtsu et al. (2015) |

in the *B. napus* Ogura CMS Rf lines has been shortened (Delourme et al. 1998; Giancola et al. 2003; Primard-Brisset et al. 2005). The unimproved *B. napus* Rf line carried a large radish introgression of at least 50 cM with 21 markers, whereas the improved *B. napus* Rf line lost 9 radish markers including Sch03 and PGIint, and showed improved growth vigor and fertility (Primard-Brisset et al. 2005). Although *Rfo* was transferred into *B. juncea* in INRA, the original restorer line exhibited poor vigor and low fertility and was black-seeded. Tian et al. (2014) improved the agronomic performance of the restorer line by marker-assisted breeding.

To establish a Kosena CMS-Rf system for F₁ hybrid seed production in rapeseed, a nuclear-encoded radish Rf gene was introduced into *B. napus* by protoplast fusion (Sakai et al. 1996). Two restorer loci were identified in Kosena CMS radish, and of these, *Rfk1* was

reported to be essential to restore fertility of the *B. napus* Kosena CMS cybrid (Koizuka et al. 2000). *Rfk1* was cloned by map-based cloning and shown to encode a pentatricopeptide repeat protein (Koizuka et al. 2003). Molecular characterization of the Rf genes will not only provide us insight into the interaction between mitochondrial and nuclear genes, but it will also contribute to the improvement of the F₁ seed production system using cytoplasmic male-sterility.

14.4.2 Introgression of the Resistance to Nematode

The beet cyst nematode (BCN), *Heterodera schachtii* Schm., is a widespread and serious parasite in beet and oilseed rape cultivating areas of the world. *H. schachtii* is a bisexual nematode,

and its multiplication is dependent on the development of the male and female nematodes (Mousa and Budahn 2012). Radish and white mustard (*Sinapis alba*) are useful sources of the resistance to BCN, and some attempts to transfer these resistances to *B. napus* crops by sexual and somatic hybridization have been carried out (Lelivelt and Krens 1992; Lelivelt et al. 1993a, b; Budahn et al. 2009). Voss et al. (1999) tried to introduce the resistance to nematodes from radish to rapeseed through the radish chromosome addition line of rapeseed by successive backcrossing. They were able to identify two radish chromosomes added to the rapeseed genome using the GISH analysis and confirmed that one of them had a close relation to nematode resistance. Peterka et al. (2004) found that the BCN resistance gene(s) was located on the 'd' chromosome of radish using *B. napus*-*R. sativus*-monosomic addition lines (MALs), and the resistance mediated by the 'd' chromosome acted in a dominant manner in the genetic background of susceptible rapeseed. The chromosome specified by Voss et al. (1999) might have corresponded to the 'd' chromosome of radish reported by Peterka et al. (2004). Budahn et al. (2009) clarified that the resistance to BCN is controlled by a major QTL *HsI^{Rph}* (quantitative trait locus for resistance against *Heterodera schachtii* from *Raphanus sativus*), which was found by the log-likelihood (LOD) plot for BCN resistance reaching the maximum score in the interval between markers E41M59-297 and OPI19-471 at 71.0 cm on the radish 'd' chromosome. Radish chromosome-specific anchor markers including the desired genes, such as *HsI^{Rph}*, with known map positions were made available for future recombination experiments to incorporate chromosome segments carrying desired genes from radish into rapeseed by means of the intergeneric recombination between rapeseed genome and the 'd' chromosome of radish. The anchor markers allow us to follow the dissection of the 'd' chromosome or other radish chromosomes in such studies and could assist in selection of introgression lines containing QTL *HsI^{Rph}* (Budahn et al. 2009).

14.4.3 Introgression of the Resistance to Clubroot

Clubroot disease is caused by an obligate pathogen *Plasmodiophora brassicae* Wor., and it is one of the most serious diseases of cruciferous crops. The pathogen survives as resting spores for long periods in the soil, and it is hard to control the disease using cultural practices or agrochemicals. The breeding of resistant cultivars is an effective approach to eliminate the use of fungicides and minimize crop losses caused by infection with this pathogen. Most radish cultivars and lines are highly resistant to the clubroot pathogen, but some are susceptible to it (Yoshikawa 1993). Kamei et al. (2010) performed a QTL analysis for clubroot resistance (CR) of radish using an F₂ population derived from a crossing of a CR Japanese radish and a clubroot susceptible Chinese radish, and proposed that a large part of radish CR is controlled by a single gene or closely linked genes, although minor effects of other genomic regions cannot be ruled out. The CR locus was named *Crs1*, of which linked markers should be useful for breeding CR cultivars of radish.

Many Japanese radish cultivars possess clubroot resistance, especially cv. 'Shogoin-daikon' is known to be immune against clubroot (Ashizawa et al. 1980). Akaba et al. (2009b) evaluated CR due to each single chromosome of radish cv. 'Shogoin-daikon' using all nine *R. sativus*-monosomic addition lines (MALs) of *B. napus* (*B. napus*-*R. sativus* MALs, a-i types which are not unified with those of Peterka et al. (2004) and Budahn et al. (2008) mentioned above), and identified that the CR against Ano-01 isolate is largely controlled by the 'c' chromosome of radish. However, there might be other minor and/or complementary gene(s) for resistance to clubroot in radish, because many plants carrying the 'c' chromosome showed slight disease symptoms. Ohtsu et al. (2015) reported that the CR of radish (cv. 'Shogoin-daikon') against field isolate pathogen is controlled by a single major gene and two minor genes using *B. napus*-*R. sativus* chromosome addition lines, and that the CR major

gene is located on the ‘c’ chromosome, and two minor genes are located on the ‘b’ and ‘f’ chromosomes, respectively, using RAPD markers developed by Akaba et al. (2009a). Ohtsu et al. (2015) developed the CR progenies having AAC genomes ($2n = 29$) without any radish chromosome from the *B. napus*–*R. sativus* MALs through successive backcrossing to *B. rapa*, suggesting that the CR genes of radish might have been transferred into the A and/or C genomes of *Brassica*. These backcrossing progenies should be useful as the bridge plants to introduce the CR genes into *Brassica* crops. In addition, the ‘c,’ ‘b,’ and ‘f’ chromosome-specific RAPD markers of radish proposed by Akaba et al (2009a) could be a helpful tool for the development of DNA markers linked to the CR loci.

As another disease resistance, resistance to Turnip mosaic virus (TuMV) was found in *R. sativus*, and a high level of resistance to various TuMV pathotypes was also detected in sexually developed *Raphanobrassica* hybrids (Krämer et al. 2003).

14.5 Genetic Analysis of Hybridization Barrier Between Radish and *Brassica* Crops

Although radish is useful as a gene source for disease and pest resistance and cytoplasmic male sterility as mentioned above, the hybridization barrier between radish and *Brassica* crops hampers the production of hybrids. In many cases of intergeneric crossing between *B. rapa* as a maternal parent and radish as a paternal parent, embryo rescues are essential for obtaining the hybrid plants, but successful hybridization has been known to depend on combinations of cultivars or lines (Karpechenko 1924). Although most *B. rapa* lines cannot produce intergeneric hybrids, a ‘Shogoin-kabu’ line has shown high ability to set seeds of intergeneric hybrids (Kaneko et al. 1993). Tonosaki et al. (2013) conducted genetic analysis of hybrid seed formation ability of *B. rapa* in intergeneric crossings with radish and

demonstrated that unsuccessful hybrid seed formation was due to post-zygotic, i.e., an arrest of embryo development at an early stage. QTL analysis using an F_2 population derived from a cross between a ‘Shogoin-kabu’ having relatively high ability to set seeds on intergeneric crossing with radish and an inbred line of ‘Chiifu’ with no seed sets revealed that two reproducibly detected QTLs had contrary effects on the number of hybrid seeds, and epistatic interaction between them existed. Genes located in these two QTLs were suggested to be involved in the hybrid formation ability of *B. rapa* in intergeneric crossing between *B. rapa* and radish. Dissection of the molecular mechanism controlling the intergeneric hybrid formation ability will be able to contribute to developing the means to overcome crossing barriers and improve the breeding of new cultivars having novel genetic traits from distantly related species.

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