Breeding Science 71: 528–537 (2021)
doi: 10.1270/jsbbs.21014

Research Paper

Development of novel clubroot resistant rapeseed lines (*Brassica napus* L.) effective against Japanese field isolates by marker assisted selection

Mitsuyo Kawasaki*[1,2,6], Takayoshi Ohara[3], Masahiko Ishida[3,7], Yoshihito Takahata[4,5] and Katsunori Hatakeyama[4]

[1] *Tohoku Agricultural Research Center, NARO*, 4 Akahira, Shimo-kuriyagawa, Morioka, Iwate 020-0198, Japan
[2] *The United Graduate School of Agricultural Sciences, Iwate University*, 3-18-8 Ueda, Morioka, Iwate 020-8550, Japan
[3] *Institute of Vegetable and Floriculture Science, NARO*, 360 Kusawa, Ano, Tsu, Mie 514-2392, Japan
[4] *Faculty of Agriculture, Iwate University*, 3-18-8 Ueda, Morioka, Iwate 020-8550, Japan
[5] *Iwate Biotechnology Research Center*, 22-174-4 Narita, Kitakami, Iwate 024-0003, Japan
[6] *Present address: Institute of Vegetable and Floriculture Science, NARO*, 360 Kusawa, Ano, Tsu, Mie 514-2392, Japan
[7] *Present address: Headquarters, NARO*, 3-1-1 Kannondai, Tsukuba, Ibaraki 305-8517, Japan

Clubroot is an important disease infectible to cruciferous plants and a major threat to rapeseed production in Japan. However, no clubroot resistant rapeseed cultivars have been released. We surveyed pathotype variation of six isolates collected from rapeseed fields and found they were classified as pathotype groups 2 and 4 using Japanese F_1 Chinese cabbage cultivars. We produced the resynthesized clubroot resistant *Brassica napus* harboring two resistant loci, *Crr1* and *Crr2*, by interspecific crossing and developed resistant rapeseed lines for southern and northern regions by marker-assisted selection and backcrossing. We improved the DNA marker for erucic acid content to remove linkage drag between *Crr1* and high erucic acid content and successfully selected lines with clubroot resistance and zero erucic acid for northern regions. A novel line, 'Tohoku No. 106', suitable for southern regions showed stable resistance against all six isolates and high performance in infested fields. We conclude that *Crr1* and *Crr2* are important genes for CR rapeseed breeding and marker-assisted selection is effective in improving clubroot resistance.

Key Words: clubroot, *Brassica napus*, rapeseed, disease resistance, erucic acid.

Introduction

Clubroot, caused by the obligate parasite *Plasmodiophora brassicae* Woronin, is an important soilborne disease of cruciferous plants. The disease is spread worldwide causing estimated 10–15% yield losses (Dixon 2009). Furthermore, intensive production of rapeseed (*Brassica napus*) for decades have caused rapid increases of infested areas and yield losses in Canada, China, and Europe (Chai *et al.* 2014, Diederichsen *et al.* 2014, Donald and Porter 2014, Rahman *et al.* 2014). The pathogen can propagate rapidly and survive for over 20 years in soil (Kuginuki *et al.* 1999). Clubroot is difficult to manage by agricultural practices such as crop rotation, increasing soil pH, and use of agrochemicals. Therefore, the breeding of clubroot resistant (CR) cultivars has been considered the most effective method to control this disease.

Communicated by Ryo Ohsawa
Received February 9, 2021. Accepted July 16, 2021.
First Published Online in J-STAGE on October 12, 2021.
*Corresponding author (e-mail: kmitsuyo@affrc.go.jp)

More than 10 CR genes have been identified in *B. rapa*, mainly in European fodder turnips, and have been utilized in breeding *Brassica* crops (Diederichsen *et al.* 2009, Hatakeyama *et al.* 2013, Hirai 2006, Kato *et al.* 2013, Suwabe *et al.* 2003, Ueno *et al.* 2012). *Brassica napus* (AACC, 2n = 38) is an amphidiploid including the A-genome from *B. rapa* (AA, 2n = 20) and C-genome from *B. oleracea* (CC, 2n = 18); the resynthesized *B. napus* has been used to transfer the CR genes in the *B. rapa* genome to *B. napus* (Bradshaw *et al.* 1997, Diederichsen and Sacristán 1996). On the other hand, CR genes in *B. oleracea* has continuous resistance profile involving various combination of both major and minor genes and have not been fully utilized in CR rapeseed breeding (Neik *et al.* 2017). In Japan, CR breeding has been focused mainly on Chinese cabbage (*B. rapa*). The CR Chinese cabbage, 'Parental Line No. 9 (PL9)', was developed using marker-assisted selection (MAS) of the two CR loci, *Crr1* and *Crr2*, and CR Chinese cabbage cultivars have been released using 'PL9' as a resistant parent (Matsumoto *et al.* 2017). *Crr1* and *Crr2* originating from the European turnip 'Siloga' are known to be necessary for resistance to the more virulent

isolate and levels of resistance are more stable when both loci are homozygous than those when they are heterozygous (Suwabe *et al.* 2003). However, it has not been confirmed whether *Crr1* and *Crr2* function in *Brassica napus* genome because these loci have not been utilized in CR rapeseed breeding in the world. In contrast, CR breeding has not progressed with respect to rapeseed in Japan. The rapeseed production area in Japan reached its peak in the 1950s and then decreased rapidly due to increase of imports of oil crops including rapeseed and soybean due to the import liberalization, (Nonaka and Ono 2015). However, rapeseed production area tends to increase after the 2000s and clubroot is now becoming a major threat in rapeseed production with an increase of repeated cropping. Although CR rapeseed cultivars harboring CR genes originating from *B. rapa* have been developed worldwide (Diederichsen and Sacristán 1996, Diederichsen *et al.* 2009, Zhan *et al.* 2020), there is no CR rapeseed cultivar for oil use in Japan. Some CR vegetable cultivars of *B. napus* have been developed using rutabaga cultivars as CR donors (Tanaka *et al.* 2009); however, their resistance is insufficient and needs improvement. Thus, the development of a CR oilseed rape cultivar with stable resistance to a wide range of pathogens is needed in Japan.

Understanding the relationship between pathogen virulence and host plants is indispensable for CR breeding. The differential set developed by Williams (1966), European clubroot differentials (ECDs) (Buczacki *et al.* 1975), and the differential set developed by Somé *et al.* (1996) have been commonly used to classify pathotypes of this pathogen. In Canada, Canadian clubroot differentials (CCD) set was developed based on three former differential sets for canola (Strelkov *et al.* 2018). In Japan, two Japanese F_1 Chinese cabbage cultivars, 'CR Ryutoki' and 'Super CR Hiroki' were proposed as clubroot differentials because of the uniformity of the genotype and four pathotypes were recognized in Chinese cabbage production fields (Hatakeyama *et al.* 2004). 'PL9', harboring *Crr1* and *Crr2*, showed resistance against isolates of pathotype groups 1, 2, 4, but not group 3 (Kato *et al.* 2012). However, pathotypes of isolates collected from *B. napus* production fields in Japan have not been investigated.

Erucic acid is a 22-carbon, mono-unsaturated fatty acid, which is found in the seeds of many cruciferous plants. Containing a high concentration of erucic acid is considered as a nutritionally undesirable trait for rapeseed. The first low erucic acid (LEA) cultivar, 'Oro' was developed using the LEA mutant found in a feed rape cultivar, 'Liho' (Harvey and Downey 1964), and many LEA cultivars have been released since. Erucic acid content in rapeseed oil is limited to no more than 5% or 2% of the component fatty acids in many countries. *FATTY ACID ELONGATION 1* (*FAE1*) gene encodes β-ketoacyl-CoA synthase (KCS) which regulates erucic acid biosynthesis (Millar and Kunst 1997). In *B. napus*, erucic acid content is controlled additively by two genes: *BnFAE1.1* on the A-genome and *BnFAE1.2* on the C-genome (Barret *et al.* 1998, Fourmann *et al.* 1998, Jourdren *et al.* 1996). An amino acid substitution from serine to phenylalanine at position 282 of FAE1 protein in LEA rapeseed caused by a transition in the coding region is considered responsible for the loss of function (Han *et al.* 2001, Katavic *et al.* 2002). Because Chinese cabbage cultivars generally contain erucic acid in seeds, selection of the LEA trait is needed in rapeseed breeding using Chinese cabbage as a parent. An existing dCAPS marker of *BnFAE1.1* for LEA selection in *B. rapa* requires laborious processes that include treatment with a restriction enzyme and electrophoresis using acrylamide gel to detect single nucleotide polymorphisms (SNP) (Karim *et al.* 2016). Therefore, simplification of MAS for LEA is needed for application in practical breeding programs.

Here, we attempted to develop CR rapeseed lines suitable for the southern and northern regions of Japan. We first surveyed the pathotype variation of field isolates on rapeseed production and specified *Crr1* and *Crr2* as effective CR genes. The CR resynthesized *B. napus* line harboring *Crr1* and *Crr2* was generated and used as a donor plant for marker-assisted backcrossing. There are roughly two types of rapeseed cultivars in Japan, southern and northern types, which are different in level of vernalization requirement. The latter requires a longer period of cold temperature to induce flowering than the former and is resistant to winter coldness. The new CR line for southern regions, named 'Tohoku No. 106', was confirmed to present superior performance in both healthy and infested field trials. For the development of lines for northern regions, we developed markers for LEA based on *BnFAE1.1* sequence and selected LEA lines harboring *Crr1* and *Crr2* using marker-assisted backcrossing.

Materials and Methods

Plant materials

The Chinese cabbage (*B. rapa*) line 'Parental Line No. 9 (PL9)' was used as the donor of the two CR loci, *Crr1* and *Crr2*, derived from European fodder turnip 'Siloga' (Suwabe *et al.* 2003). The cabbage (*B. oleracea*) F_1 cultivar, 'Umekichi' (Nippon Norin Seed Co., Tokyo, Japan) was used as a female parent to develop CR resynthesized *Brassica napus*. One vegetable rape cultivar of *B. napus*, 'Nagashima zairai No. 1' and two Japanese zero-erucic rapeseed cultivars, 'Kizakinonatane' and 'Nanashikibu', were used as crossing parents. We used the Japanese zero-erucic rapeseed cultivars, 'Nanashikibu' and 'Kizakinonatane', which are leading cultivars for southern and northern regions respectively, as recurrent parents to develop CR lines. The two zero-erucic cultivars for southern regions, 'Nanashikibu' and 'Nanaharuka', were used at field trials in Chikujo.

Clubroot resistance test

We collected clubs from 2011 to 2015 in the rapeseed

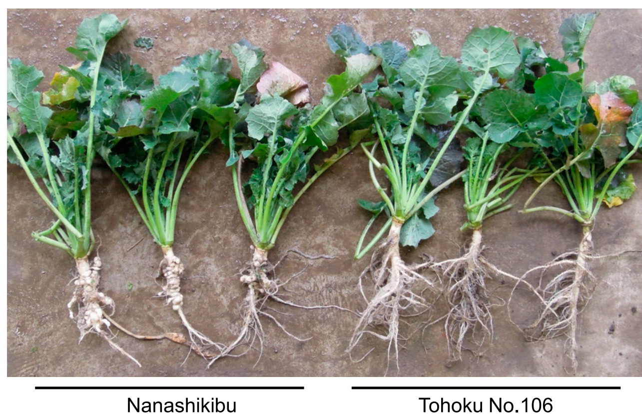

Fig. 1. Clubroot at infested field at Chikujo. Root symptom of susceptible rapeseed cultivar 'Nanashikibu' and CR rapeseed line 'Tohoku No. 106'.

fields of six growers in Japan (**Fig. 1**). The sampling sites were as follows, Yokohama town in Aomori prefecture, Yurihonjo city and Misato town in Akita prefecture, Yamagata city in Yamagata prefecture, and Chikujo town in Fukuoka prefecture. We collected clubs at two distant points only in Yamagata city. Six field isolates of purified *P. brassicae* spores were used for pathotype classification and the other CR tests. We performed pathotype classification of *P. brassicae* using 'Super CR Hiroki' (Sumika Agrotech Co., Osaka, Japan) and 'CR Ryutoku' (Watanebe Seed Co., Miyagi, Japan) which are Japanese F_1 Chinese cabbage differentials of Hatakeyama *et al.* (2004). We also performed using 13 differentials which include all hosts of Williams (1966) and selected hosts of ECDs (Buczacki *et al.* 1975). The disease index (DI) was scored according to Hatakeyama *et al.* (2004), with a slight modification, on a scale of 0 to 3: 0, no symptoms; 1, a few small galls, separate globular clubs on the lateral roots; 2, no clubs on the main root, but moderate clubbing of lateral roots or small clubs on the main root; 3, absence of normal roots and presence of large galls. Plants with a DI score of 0 to 1 were categorized as resistant and those with 2 to 3 were categorized as susceptible.

Production of CR resynthesized Brassica napus line

The cabbage cultivar, 'Umekichi', was crossed with 'PL9' harboring *Crr1* and *Crr2* in 2007. Interspecific cross-derived hybrid ovules extracted from pods were cultured in 1/2 MS medium (hormone-free) until roots and shoots were developed. The plantlets were then planted in 8 cm-jiffy pots and grown in a greenhouse. Hybridity of the plantlets was confirmed by PCR analysis using DNA markers, BRMS-088 for *Crr1* and BRMS-096 for *Crr2* (Suwabe *et al.* 2003).

Marker assisted selection of CR genes

In the breeding process, to select the plants with two CR loci, *Crr1* and *Crr2*, in each generation, we used SSR markers closely linked to these genes. BSA1 was used for MAS of *Crr1*, and BRMS-096 was used for *Crr2* (Suwabe *et al.* 2003, 2006). Each SSR locus was amplified with 60 ng of template DNA in a 20 µl reaction volume containing 400 nM of each primer, 1× Go Taq Green Master Mix (Promega, Wisconsin, USA). Thermal cycling conditions included denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 30 s, and a final extension at 72°C for 5 min. PCR products were mixed with DNA EZ Vision two (VWR Life Science Amresco, Ohio, USA), separated by electrophoresis on 2.0% agarose gel, and visualized under a UV imaging system.

Marker assisted selection of LEA plant

Based on *BnFAE1.1* sequence of zero erucic and low glucosinolate cultivar, 'Kirariboshi' (LEA) and HEA *B. rapa* cultivar, 'Tori-7' (Karim *et al.* 2016), we designed allele-specific forward primers to detect SNP at the 845th base; T for LEA and C for HEA. The base sequence forward primers were as follows: BnFAE1.1_kira827misF for LEA (5′-GGGCCGCTATTTTGCTATT-3′) and BnFAE1.1_tori827misF for HEA (5′-GGGCCGCTATTTTGCTATC-3′). BnFAE1.1-3UTR-cR (Karim *et al.* 2016) was used as the reverse primer (5′-GACGTCATAGTGTTAGGCGTTT-3′). PCR was performed using 60 ng of template DNA in a 20 µl reaction volume containing 100 nM of each primer, 0.5 U of *Ex Taq* polymerase HS, 2 µl of *Ex Taq* buffer, and 1.6 µL of 2.5 mM dNTP mixture (Takara Bio, Japan). Thermal cycling conditions comprised 35 cycles of denaturation at 98°C for 10 s, annealing for 30 s and extension at 72°C for 1 min. The annealing temperature was 57°C for SNP detection of LEA and 60°C for HEA. Polymorphism was detected as described above. We first confirmed the applicability of the developed allele-specific primers to the zero erucic acid cultivar, 'Kizakinonatane' (LEA) and high erucic acid CR Chinese cabbage cultivar, 'PL9' (HEA). We surveyed the genotype at *BnFAE1.1* after selection of plants heterozygous for both *Crr1* and *Crr2* from BC_7 plants bred by backcrossing using 'Kizakinonatane' as a recurrent parent (**Fig. 2**). Then, erucic acid contents in 5 BC_7S_1 seeds per BC_7 plant were surveyed by analysis of fatty acid methyl esters using capillary gas chromatography with flame ionization detection.

Field trials of a novel CR rapeseed line

Agronomic traits of a novel CR rapeseed line were evaluated at Chikujo town in Fukuoka, Japan. The cropping cycle was from October to June. Field trials were conducted in 2017–2018, and 2018–2019, and seeds were sown in a 70 cm row at a sowing density of 625,000 seeds per hectare with three replications in a randomized block design. We performed field trials in infested fields and healthy fields. DI scores of plants grown in the infested field were evaluated as described above.

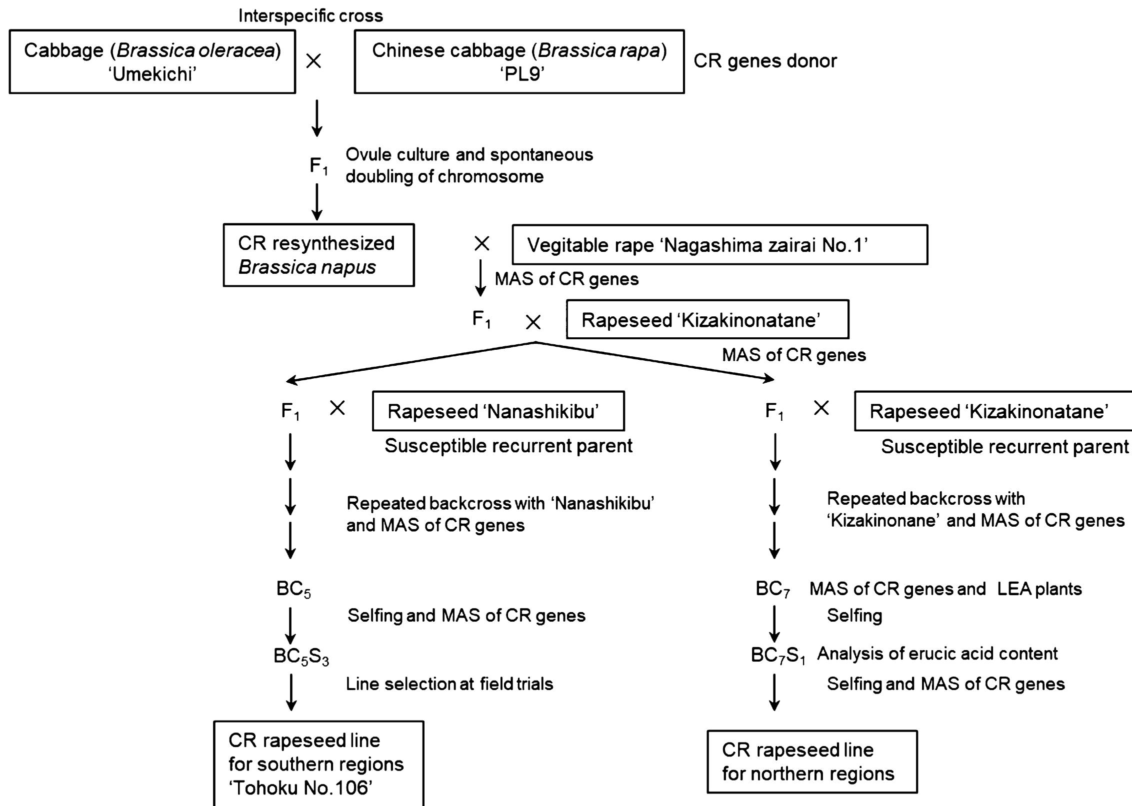

Fig. 2. Schematic process of development of CR rapeseed lines.

Results

Pathotypes of the collected isolates

We collected clubs from six rapeseed production fields that were severely infested with *P. brassicae* and performed inoculation tests using clubroot differentials, 'Super CR Hiroki' and 'CR Ryutoku' (Hatakeyama *et al.* 2004). Non-CR cultivar, 'Muso', showed completely susceptible against all six field isolates (**Table 1**). Both differentials showed resistance against 5 isolates, except for Yamagata2. 'Super CR Hiroki' showed resistance to Yamagata2, whereas 'CR Ryutoku' was susceptible. These results indicate that the six field isolates collected from rapeseed production fields in Japan were classified into groups 2 and 4 (**Table 1**).

We also conducted inoculation tests using differential hosts as mentioned by Williams (1966) and ECD hosts (Buczacki *et al.* 1975). Some hosts contained both susceptible and resistant individuals among tested plants and showed intermediate DI scores against isolates, unlike the differential hosts of Japanese CR F_1 Chinese cabbage cultivars (**Supplemental Table 1**). This result is consistent with the results obtained using isolates collected from Chinese cabbage production fields in Japan (Hatakeyama *et al.* 2004).

Table 1. Pathogen classification using resistance response of Chinese cabbage F_1 cultivars against six Japanese field isolates of *Plasmodiphora brassicae*

Field isolate	Pathogen classification	Chinese cabbage F_1 varieties						CR Chinese cabbage	
		Super CR Hiroki		CR Ryutoku		Muso		PL9	
		Mean DI[a]	Susceptibility	Mean DI	Susceptibility	Mean DI	Susceptibility	Mean DI	Susceptibility
Yokohama	Group 4	0.1	R	0.1	R	3.0	S	0.3	R
Yurihonjo	Group 4	0.1	R	0.0	R	3.0	S	0.0	R
Misatomachi	Group 4	0.0	R	0.1	R	3.0	S	0.0	R
Yamagata1	Group 4	0.0	R	0.1	R	3.0	S	0.0	R
Yamagata2	Group 2	0.0	R	2.9	S	2.9	S	0.0	R
Chikujo	Group 4	0.1	R	0.0	R	3.0	S	0.0	R

[a] DI denotes disease index.

Development of a novel CR rapeseed line 'Tohoku No. 106'

We obtained 20 interspecific hybrids between cabbage cultivar 'Umekichi' and Chinese cabbage 'PL9' by ovule culture. PCR analysis confirmed that all the hybrids possessing *Crr1* and *Crr2* loci originated from 'PL9'. Among them, selfed seeds were obtained from five hybrids in which doubling of chromosomes occurred spontaneously. We crossed one of the derived resynthesized *B. napus* (08AACC3-4) with the vegetable rape cultivar, 'Nagashima zairai No. 1', and then with the rapeseed cultivar, 'Kizakino-natane' (**Fig. 2**). The resultant plants were backcrossed with two different rapeseed cultivars, 'Nanashikibu' and 'Kizakinonatane' as recurrent parents for the development of CR rapeseed lines for southern and northern regions, respectively. During this process, genotyping was carried out with more than 72 plants per generation to select the plants heterozygous for both *Crr1* and *Crr2* by using two SSR markers, BSA1 and BRMS-096, respectively (**Fig. 3**, Suwabe *et al.* 2003, 2006). After backcrossing, we selected the plants homozygous for both loci from the selfed progeny using SSR genotyping and surveyed erucic acid content in their seeds using capillary gas chromatography.

One of the three zero-erucic lines selected by MAS of CR genes for southern regions was named 'Tohoku No. 106'. To evaluate clubroot resistance of 'Tohoku No. 106', inoculation test with the collected field isolates was performed (**Table 2**). 'Tohoku No. 106' showed resistant to all six isolates, with no symptom or only small clubs at lateral roots, whereas 'Nanashikibu' showed complete susceptibility to these isolates, with large clubs at main root. DI of 'Tohoku No. 106' was 0.0 to 0.3, quite lower than those of 'Nanashikibu' in all the isolates (**Table 2**).

The clubroot resistance of 'Tohoku No. 106' was also evaluated in the infested field in Chikujo. 'Nanashikibu' formed clubs at a later stage just before maturity in 2017–2018 and at an early stage two months after sowing in 2018–2019. 'Nanashikibu' includes 34.8% and 86.0% of susceptible individuals, respectively and zero erucic rapeseed cultivar 'Nanaharuka' includes 25.4% and 97.7%, respectively (**Table 3**). By contrast, all individuals of 'Tohoku No. 106' showed complete resistance in both 2017–2018 and 2018–2019 (**Fig. 1**, **Table 3**). Therefore, 'Tohoku No. 106' is considered to have useful resistance to clubroot pathogens in rapeseed production.

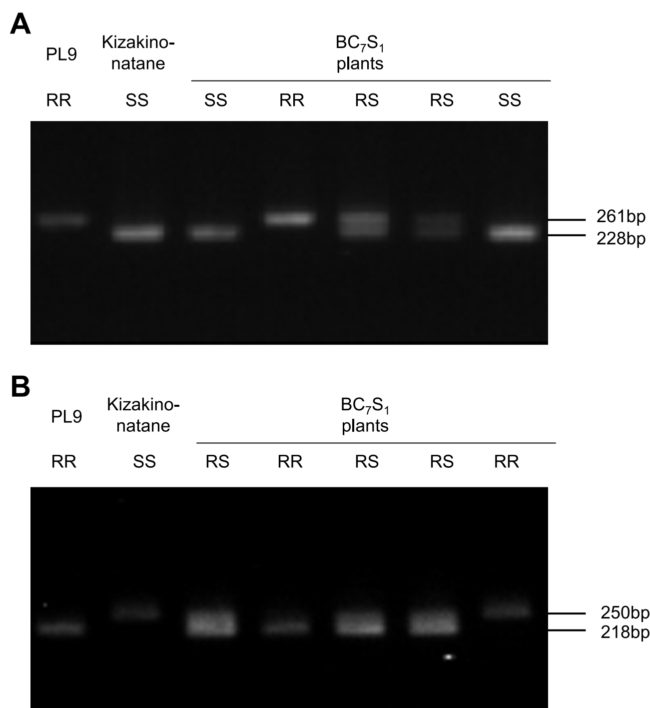

Fig. 3. Gel electrophoresis images of selection using SSR markers linked to *Crr1* and *Crr2*. Genotypes of Chinese cabbage line 'PL9', rapeseed cultivar 'Kizakinonatane', and five BC_7S_1 plants are shown above the gel. RR, homozygous resistant; RS, heterozygous; SS, homozygous susceptible. (A) Selection of individuals harboring *Crr1*. (B) Selection of individuals harboring *Crr2*.

Agronomic traits of 'Tohoku No. 106' were evaluated in cultivation in both healthy and infested fields. At healthy fields in Chikujo, 'Tohoku No. 106' showed similar flowering, maturity, plant height and 1000 seeds weight to those of 'Nanashikibu' which is the recurrent parent of backcrossing, while the yield of 'Tohoku No. 106' was slightly lower than that of 'Nanashikibu' and 'Nanaharuka' on average (**Table 4**). At infested fields in Chikujo, 'Tohoku No. 106' showed similar flowering, maturity, and 1000

Table 2. CR testing of CR rapeseed line 'Tohoku No. 106' against six Japanese field isolates of *Plasmodiphora brassicae*

Field isolate	Tohoku No. 106			Nanashikibu		
	No. of plants	IC[a]	Mean DI[b]	No. of plants	IC	Mean DI
Yokohama	29	0.0	0.2	29	100.0	3.0
Yurihonjo	32	0.0	0.2	27	100.0	3.0
Misatomachi	31	0.0	0.0	35	100.0	3.0
Yamagata1	37	0.0	0.0	36	100.0	3.0
Yamagata2	30	0.0	0.3	36	100.0	3.0
Chikujo	21	0.0	0.0	20	100.0	3.0

[a] IC refers to disease incidence (%).
[b] DI denotes disease index.

Table 3. CR testing of CR rapeseed line 'Tohoku No. 106' at infested field in Chikujo

Cultivar	Year	No. of plants	DI[a]				IC[b]	Mean DI
			0	1	2	3		
Tohoku No. 106	2017–2018	60	60	0	0	0	0.0	0.0
	2018–2019	84	84	0	0	0	0.0	0.0
	Average	72	72	0	0	0	0.0	0.0
Nanashikibu	2017–2018	66	42	1	6	17	34.8	1.0
	2018–2019	86	5	7	34	40	86.0	2.3
	Average	76	24	4	20	29	60.4	1.6
Nanaharuka	2017–2018	63	47	0	5	11	25.4	0.7
	2018–2019	86	0	2	38	46	97.7	2.5
	Average	75	24	1	22	29	61.5	1.6

[a] DI denotes disease index.
[b] IC refers to disease incidence (%).

Table 4. Agronomic traits of CR rapeseed line 'Tohoku No. 106' at healthy field in Chikujo

Cultivar	Year	Days to flowering	Days to maturity	Plant height (cm)	Yield (kg/a)	1000 seeds weight (kg/a)
Tohoku No. 106	2017–2018	153	211	141	19.6	4.2
	2018–2019	149	215	137	20.4	3.8
	Average	151	213	139	20.0	4.0
Nanashikibu	2017–2018	153	211	146	20.9	4.2
	2018–2019	147	214	136	23.0	4.0
	Average	150	213	141	21.9	4.1
Nanaharuka	2017–2018	151	208	145	17.4	4.3
	2018–2019	142	208	139	26.9	4.0
	Average	147	208	142	22.2	4.2

Table 5. Agronomic traits of CR rapeseed line 'Tohoku No. 106' at infested field in Chikujo

Cultivar	Year	Days to flowering	Days to maturity	Plant height (cm)	Yield (kg/a)	1000 seeds weight (g)
Tohoku No. 106	2017–2018	155	211	129	17.5	5.0
	2018–2019	148	215	142	20.4	3.9
	Average	152	213	136	19.0	4.5
Nanashikibu	2017–2018	155	211	128	16.2	4.9
	2018–2019	147	213	129	13.2	4.1
	Average	151	212	129	14.7	4.5
Nanaharuka	2017–2018	152	207	133	11.8	4.5
	2018–2019	142	209	135	12.5	4.3
	Average	147	208	134	12.2	4.4

seeds weight to those of 'Nanashikibu', however, plant height of 'Tohoku No. 106' was higher than that of 'Nanashikibu' and yield of 'Tohoku No. 106' was 8–55% higher than that of 'Nanashikibu' and 48–63% higher than that of 'Nanaharuka' (**Table 5**).

Development of marker linked to BnFAE1.1 and application

Although LEA cultivar 'Kizakinonatane' was used as recurrent parent for the development of CR rapeseed suitable for northern regions, all BC_6 lines selected for *Crr1* and *Crr2* were HEA. Erucic acid content is regulated additively *BnFAE1.1* on the A genome and *BnFAE1.2* on the C genome. Because we expected that the HEA allele of *BnFAE1.2* on the C genome was substituted by LEA allele during repeated backcross with a high probability, we focused on MAS for *BnFAE1.1* on the A genome to improve the erucic acid content using DNA markers. To simplify the selection of LEA plants instead of the known dCAPS marker, we designed allele-specific primers to detect the SNP at the 845th base of *BnFAE1.1*. The forward primers containing only SNP at the 3′ terminus did not discriminate between LEA and HEA alleles. To improve the specificity of the PCR reaction, we redesigned the forward

primer for LEA and HEA, following Hayashi *et al.* (2004). The cytosine residue (C) at the third base from the 3′ terminal in both forward primers was substituted with adenine (A), leading to an artificial mismatch base pair. When we adapted the redesigned allele-specific primers to rapeseed cultivar 'Kizakinonatane' (LEA) and CR Chinese cabbage cultivar 'PL9' (HEA), an expected band pattern was obtained by agarose gel electrophoresis. The PCR product of the LEA marker (BnFAE1.1_kira827misF/BnFAE1.1-3UTR-cR) was detected in 'Kizakinonatane' and not detected in 'PL9', whereas the PCR product of the HEA marker (BnFAE1.1_tori827misF/BnFAE1.1-3UTR-cR) was detected in 'PL9' and not detected in 'Kizakinonatane' (**Fig. 4**). We examined the developed HEA and LEA marker genotypes for the selected 32 BC_7 plants harboring *Crr1* and *Crr2* and found that 13 plants were LEA homozygotes and 19 plants were heterozygotes. Erucic acid contents of BC_7S_1 seeds of 32 BC_7 plants varied from 0.0 to 32.4%, whereas those of 'Kizakinonatane' and 'PL9' were 0.0% and 45.5%, respectively. In the BC_7 plant, all 13 LEA homozygous plants produced zero-erucic seeds (LEA) and 19 heterozygous plants produced both HEA and zero-erucic seeds (**Table 6**). We performed an inoculation test using BC_7S_2 seeds homozygous for *Crr1* and *Crr2* obtained by self-fertilization of the selected six LEA BC_7 plants. All lines showed resistance against the group 4 isolate of 'Yokohama' with no symptoms or only small clubs at lateral roots (**Table 7**).

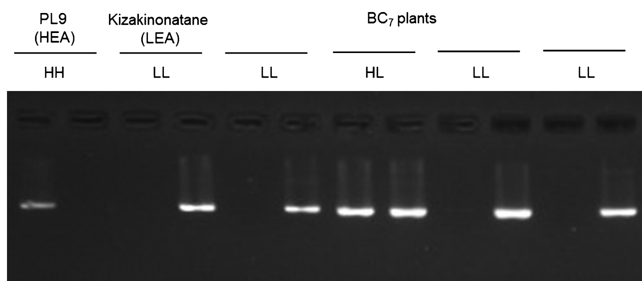

Fig. 4. Gel electrophoresis images of the allele-specific markers for *BnFAE1.1*. The left and right lanes of each sample indicate HEA and LEA markers, respectively. Genotypes of 'PL9', 'Kizakinonatane', and four BC_7 plants are shown above the gel. HH, homozygous HEA; HL, heterozygous; LL, homozygous LEA.

Table 6. Relationship between genotypes of BC_7 plants categorized by allele-specific markers of *BnFAE1.1* and erucic acid contents in BC_7S_1 seeds

BnFAE1.1 genotypes of BC_7 plants[a]	No. of BC_7 plants	
	Plants with both HEA and LEA seeds[b]	Plants with only LEA seeds
HL	19 (0–32.4%)	0
LL	0	13 (0%)

Values in parenthesis are erucic acid content in BC_7S_1 seeds.
[a] H denotes the allele of 'PL9' and L denotes that of 'Kizakinonatane'.
[b] HEA is the seed having erucic acid content more than 2% and LEA no more than 2%.

Table 7. Clubroot resistance of developed lines for northern regions against Yokohama field isolate of *Plasmodiophora brassicae*

Line	No. of plants	IC[a]	Mean DI[b]
CRB1-1	9	0.0	0.0
CRB1-2	7	0.0	0.0
CRB1-3	9	0.0	0.1
CRB1-4	8	0.0	0.0
CRB1-5	7	0.0	0.0
CRB1-6	9	0.0	0.0
Kizakinonatane	8	100.0	3.0

[a] IC refers to disease incidence (%).
[b] DI denotes disease index.

Discussion

In this study, we developed CR rapeseed lines for the southern and northern regions of Japan by using the CR resynthesized *B. napus* and DNA markers linked to two CR loci derived from *B. rapa*. European winter canola, CR cv. 'Mendel' originated from a resynthesized *B. napus* line (Diederichsen and Sacristán 1996). Backcrossing of the susceptible cultivar, production of DH lines from backcross progeny, and selection of resistant lines with *P. brassicae* isolates was repeated twice, and the resultant resistant line was used as a male parent of F_1 cv. 'Mendel' (Diederichsen *et al.* 2006). Although initially selected lines were expected to have three dominant CR genes, the genetic mapping study revealed that cv. 'Mendel' had only one dominant CR gene due to the loss of two dominant genes during breeding process. Similar loss of CR genes has also been reported in the breeding of rutabaga line (Bradshaw *et al.* 1997). Based on pathotype analysis, we specified *Crr1* and *Crr2* as effective CR genes. Because levels of resistance are less stable when the two loci are heterozygous and the effect of *Crr2* is visible only in combination with *Crr1* (Suwabe *et al.* 2003), selection of the plants harboring the two loci only by inoculation test is even more difficult. The rapeseed lines developed in this study conferred stable and strong resistance against pathogen isolates from rapeseed fields in both inoculation tests and field trials. This is a first report to demonstrate that the two *B. rapa* CR loci can be introduced efficiently and properly into the recurrent parental lines by using CR loci-linked markers, and the two CR loci function in *B. napus* genome as well as those in *B. rapa* genome.

We demonstrated that *P. brassicae* at rapeseed production fields in Japan were classified into group 2 and group 4 using Japanese CR F_1 Chinese cabbage cultivars 'Super CR Hiroki' and 'CR Ryutoku'. CR cultivars have not been utilized in rapeseed production in Japan. Most of the production area consists of paddy fields and hardly overlap with that of the other *Brassica* crops. This may be the reason for the lower differentiation of *P. brassicae* observed in

rapeseed fields. Because *Crr1* and *Crr2* confer resistance to pathotypes groups 1, 2, and 4 (Kato *et al.* 2012), the CR rapeseed lines developed in this study are considered relatively robust against the risk of overcoming resistance. In addition to pyramiding of pathotype-specific resistance genes, utilization of pathotype non-specific resistance genes is considered desirable to enhance resistance. One of the candidate genes is *PbBo*(*Anju*)*1*, a QTL found in CR cabbage cultivar 'Anju', which conferred resistance against broad ranges of pathotypes in Japan (Tomita *et al.* 2013). However, *PbBo*(*Anju*)*1* conferred moderate resistance even along with the other three minor CR genes. In addition, Diederichsen and Sacristán (1996) reported that CR from *B. oleracea* appeared to be strongly diluted due to the epistatic factors of the *B. rapa* genome in resynthesized *B. napus*. Understanding the details of these QTLs and further investigation on new CR resources are needed for application to CR rapeseed breeding.

BrFAE1.1, a gene responsible for HEA, is located on A08 of the *B. rapa* genome (Fourmann *et al.* 1998), and *Crr1* is also located on A08 (Suwabe *et al.* 2006). *Bra020861* is assumed to be an allele of *Crr1a* (Hatakeyama *et al.* 2013). The physical distance between *Bra020861* and *Bra034635.1*, corresponding to *BrFAE1.1*, is estimated to be approximately 743 kb in the *B. rapa* genome (Wang *et al.* 2011). Because Chinese cabbage 'PL9' as a CR donor parent is a HEA cultivar, HEA of the selected CR lines in this study is considered due to the close linkage of *Crr1* and *BrFAE1.1*. Recently, linkage drag between the HEA and CR locus *PbBa1.1*, which is possibly allelic to *Crr1a*, was reported in the Chinese CR rapeseed line 'ZHE 226' (Zhan *et al.* 2020). To remove linkage drag, the use of allele-specific markers is considered more efficient than a large population and many generations of backcrossing. We performed MAS using 204 BC_7 plants, which were about twice as many plants as we surveyed in the former generations (73~108 plants). The increase of population size and use of marker is probably one of the reasons why we could obtain the northern type LEA lines harboring *Crr1* and *Crr2* in the BC_7 generation. On the contrary, we obtained LEA lines for southern regions harboring *Crr1* and *Crr2* without MAS for LEA. In the past rapeseed breeding in Japan, researchers used *B. rapa* as a crossing parent to introduce its earliness to *B. napus*. Through this process, the current rapeseed cultivars adapted to southern region presumably have the region partially replaced with *B. rapa* genome. Therefore, it is possible that the breaking of the linkage drag occurred more easily in the breeding line for southern regions due to sequence variation in *BrFAE1.1* or nearby region between the two recurrent parents.

In this study, we improved the PCR-based marker for LEA *BnFAE1.1*, and successfully obtained the CR lines with LEA by marker-assisted selection. Sequence variation of *Brassica FAE1* genes has been reported. Twenty-eight bp deletion of the promoter region of *BrFAE1.1* was found to contribute to LEA traits of *B. rapa* (Yan *et al.* 2015) and four-nucleotide deletions in the *BnFAE1.1* gene from the A-genome and *BnFAE1.2* gene from the C-genome were found to contribute to the LEA traits of *B. napus* (Wu *et al.* 2008). Therefore, when a need arises to use other CR resources in breeding programs, markers for LEA may need modifications.

A temperature range of 20–24°C and wet soil is considered appropriate for clubroot pathogen infection (Karling 1968). In southern regions of Japan, the average air temperature during the sowing season is about 20°C and rapeseed production is mainly on paddy fields, which drain much more poorly than the field, leading to clubroot formation at an early stage and serious damage in yields. 'Tohoku No. 106' has stable resistance and superior agronomic traits at southern regions, therefore we applied for variety registration. The rapeseed lines developed in this study contribute significantly to rapeseed production by reducing economic loss due to clubroot disease.

Author Contribution Statement

KM designed this research and wrote the manuscript. KM, KH, OT and MI conducted the experiments and data analysis. YT and KH provided advice on the experimental implementation and helped to draft the manuscript.

Acknowledgments

The authors would like to thank Dr. S. Matsumoto for technical guidance and valuable advice with the experiments. We are grateful to Dr. T. Kakizaki for constructive suggestions on the development of CR rapeseed lines. We also thank agricultural extension officers of Fukuoka prefecture, officers of Chikujo town, and Mr. T. Ohta for their helpful support in conducting field tests at Chikujo town.

Literature Cited

Barret, P., R. Delourme, M. Renard, F. Domergue, R. Lessire, M. Delseny and T.J. Roscoe (1998) A rapeseed *FAE1* gene is linked to the E1 locus associated with variation in the content of erucic acid. Theor. Appl. Genet. 96: 177–186.

Bradshaw, J.E., D.J. Gemmell and R.N. Wilson (1997) Transfer of resistance to clubroot (*Plasmodiophora brassicae*) to swedes (*Brassica napus* L. var. *napobrassica* Peterm) from *B. rapa*. Ann. Appl. Biol. 130: 337–348.

Buczacki, S.T., H. Toxopeus, P. Mattusch, T.D. Johnston, G.R. Dixon and L.A. Hobolth (1975) Study of physiologic specialization in *Plasmodiophora brassicae*: proposals for attempted rationalization through an international approach. Trans. Br. Mycol. Soc. 65: 295–303.

Chai, A.L., X.W. Xie, Y.X. Shi and B.J. Li (2014) Research status of clubroot (*Plasmodiophora brassicae*) on cruciferous crops in China. Can. J. Plant Pathol. 36: 142–153.

Diederichsen, E. and M.D. Sacristán (1996) Disease response of resynthesized *Brassica napus* L. lines carrying different combinations of resistance to *Plasmodiophora brassicae* Wor. Plant Breed.

115: 5–10.
Diederichsen, E., J. Beckmann, J. Schondelmeier and F. Dreyer (2006) Genetics of clubroot resistance in *Brassica napus* ‘Mendel’. Acta Hortic. 706: 307–311.
Diederichsen, E., M. Frauen, E.G.A. Linders, K. Hatakeyama and M. Hirai (2009) Status and perspectives of clubroot resistance breeding in crucifer crops. J. Plant Growth Regul. 28: 265–281.
Diederichsen, E., M. Frauen and J. Ludwig-Mueller (2014) Clubroot disease management challenges from a German perspective. Can. J. Plant Pathol. 36: 85–98.
Dixon, G.R. (2009) The occurrence and economic impact of *Plasmodiophora brassicae* and clubroot disease. J. Plant Growth Regul. 28: 194–202.
Donald, E.C. and I.J. Porter (2014) Clubroot in Australia: the history and impact of *Plasmodiophora brassicae* in *Brassica* crops and research efforts directed towards its control. Can. J. Plant Pathol. 36: 66–84.
Fourmann, M., P. Barret, M. Renard, G. Pelletier, R. Delourme and D. Brunel (1998) The two genes homologous to *Arabidopsis FAE1* co-segregate with the two loci governing erucic acid content in *Brassica napus*. Theor. Appl. Genet. 96: 852–858.
Han, J.X., W. Luhs, K. Sonntag, U. Zahringer, D.S. Borchardt, F.P. Wolter, E. Heinz and M. Frentzen (2001) Functional characterization of β-ketoacyl-CoA synthase genes from *Brassica napus* L. Plant Mol. Biol. 46: 229–239.
Harvey, B.L. and R.K. Downey (1964) The inheritance of erucic acid content in rapeseed (*Brassica napus*). Can. J. Plant Sci. 44: 104–111.
Hatakeyama, K., M. Fujimura, M. Ishida and T. Suzuki (2004) New classification method for *Plasmodiophora brassicae* field isolates in Japan based on resistance of F_1 cultivars of Chinese cabbage (*Brassica rapa* L.) to clubroot. Breed. Sci. 54: 197–201.
Hatakeyama, K., K. Suwabe, R.N. Tomita, T. Kato, T. Nunome, H. Fukuoka and S. Matsumoto (2013) Identification and characterization of *Crr1a*, a gene for resistance to clubroot disease (*Plasmodiophora brassicae* Woronin) in *Brassica rapa* L. PLoS ONE 8: e54745.
Hayashi, K., N. Hashimoto, M. Daigen and I. Ashikawa (2004) Development of PCR-based SNP markers for rice blast resistance genes at the *Piz* locus. Theor. Appl. Genet. 108: 1212–1220.
Hirai, M. (2006) Genetic analysis of clubroot resistance in *Brassica* crops. Breed. Sci. 56: 223–229.
Jourdren, C., P. Barret, R. Horvais, N. Foisset, R. Delourme and M. Renard (1996) Identification of RAPD markers linked to the loci controlling erucic acid level in rapeseed. Mol. Breed. 2: 61–71.
Karim, M.M., N.N. Tonu, M.S. Hossain, T. Funaki, M.B. Meah, D.M. Hossain, M.A. ud-Doullah, E. Fukai and K. Okazaki (2016) Marker-assisted selection of low erucic acid quantity in short duration *Brassica rapa*. Euphytica 208: 535–544.
Karling, J. (1968) The Plasmodiophorales, 2nd edn. Hafter Publishing Co., New York and London, p. 256.
Katavic, V., E. Mietkiewska, D.L. Barton, E.M. Giblin, D.W. Reed and D.C. Taylor (2002) Restoring enzyme activity in nonfunctional low erucic acid *Brassica napus* fatty acid elongase l by a single amino acid substitution. Eur. J. Biochem. 269: 5625–5631.
Kato, T., K. Hatakeyama, N. Fukino and S. Matsumoto (2012) Identification of a clubroot resistance locus conferring resistance to a *Plasmodiophora brassicae* classified into pathotype group 3 in Chinese cabbage (*Brassica rapa* L.). Breed. Sci. 62: 282–287.
Kato, T., K. Hatakeyama, N. Fukino and S. Matsumoto (2013) Fine mapping of the clubroot resistance gene *CRb* and development of a useful selectable marker in *Brassica rapa*. Breed. Sci. 63: 116–124.
Kuginuki, Y., H. Yoshikawa and M. Hirai (1999) Variation in virulence of *Plasmodiophora brassicae* in Japan tested with clubroot-resistant cultivars of Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*). Eur. J. Plant Pathol. 105: 327–332.
Matsumoto, S., K. Hatakeyama, S. Takashita, T. Miyazaki and T. Kondo (2017) Development of a medium-late maturing Chinese cabbage (*Brassica rapa* L.) F_1 cultivar, ‘CR Kanjiro’ harboring two clubroot resistance genes, *Crr1* and *Crr2*. Bull. NARO, Veg. & Flor. Sci. 1: 23–34 (in Japanese with English summary).
Millar, A.A. and L. Kunst (1997) Very-long-chain fatty acid biosynthesis is controlled through the expression and specificity of the condensing enzyme. Plant J. 12: 121–131.
Neik, T.X., M.J. Barbetti and J. Batley (2017) Current status and challenges in identifying disease resistance genes in *Brassica napus*. Front. Plant Sci. 8: 1788.
Nonaka, A. and H. Ono (2015) Revitalization of rural economies though the restructuring the self-sufficient realm—Growth in small-scale rapeseed production in Japan. Jpn. Agric. Res. Q. 49: 383–390.
Rahman, H., G. Peng, F. Yu, K.C. Falk, M. Kulkarni and G. Selvaraj (2014) Genetics and breeding for clubroot resistance in Canadian spring canola (*Brassica napus* L.). Can. J. Plant Pathol. 36: 122–134.
Somé, A., M.J. Manzanares, F. Laurens, F. Baron, G. Thomas and F. Rouxel (1996) Variation for virulence on *Brassica napus* L. amongst *Plasmodiophora brassicae* collections from France and derived single–spore isolates. Plant Pathol. 45: 432–439.
Strelkov, S.E., S.F. Hwang, V.P. Manolii, T.S. Cao, R. Fredua-Agyeman, M.W. Harding, G. Peng, B.D. Gossen, M.R. McDonald and D. Feindel (2018) Virulence and pathotype classification of *Plasmodiophora brassicae* populations collected from clubroot resistant canola (*Brassica napus*) in Canada. Can. J. Plant Pathol. 40: 284–298.
Suwabe, K., H. Tsukazaki, H. Iketani, K. Hatakeyama, M. Fujimura, T. Nunome, H. Fukuoka, S. Matsumoto and M. Hirai (2003) Identification of two loci for resistance to clubroot (*Plasmodiophora brassicae* Woronin) in *Brassica rapa* L. Theor. Appl. Genet. 107: 997–1002.
Suwabe, K., H. Tsukazaki, H. Iketani, K. Hatakeyama, M. Kondo, M. Fujimura, T. Nunome, H. Fukuoka, M. Hirai and S. Matsumoto (2006) Simple sequence repeat-based comparative genomics between *Brassica rapa* and *Arabidopsis thaliana*: The genetic origin of clubroot resistance. Genetics 173: 309–319.
Tanaka, Y., T. Katayama, Y. Shibato, T. Hayashida and S. Himeno (2009) Characteristics of clubroot resistant Nabana ‘Harukana’. Bull. Fukuoka Agric. Res. Cent. 28: 84–88 (in Japanese with English summary).
Tomita, H., M. Shimizu, M. Asad-ud Doullah, R. Fujimoto and K. Okazaki (2013) Accumulation of quantitative trait loci conferring broad-spectrum clubroot resistance in *Brassica oleracea*. Mol. Breed. 32: 889–900.
Ueno, H., E. Matsumoto, D. Aruga, S. Kitagawa, H. Matsumura and N. Hayashida (2012) Molecular characterization of the *CRa* gene conferring clubroot resistance in *Brassica rapa*. Plant Mol. Biol. 80: 621–629.
Wang, X.W., H.Z. Wang, J. Wang, R.F. Sun, J. Wu, S.Y. Liu, Y.Q. Bai, J.H. Mun, I. Bancroft, F. Cheng *et al.* (2011) The genome of the mesopolyploid crop species *Brassica rapa*. Nat. Genet. 43: 1035–1039.

Williams, P.H. (1966) A system for determination of races of *Plasmodiophora brassicae* that infect cabbage and rutabaga. Phytopathology 56: 624–626.

Wu, G., Y.H. Wu, L. Xiao, X.D. Li and C.M. Lu (2008) Zero erucic acid trait of rapeseed (*Brassica napus* L.) results from a deletion of four base pairs in the *fatty acid elongase 1* gene. Theor. Appl. Genet. 116: 491–499.

Yan, G.X., D. Li, M.X. Cai, G.Z. Gao, B.Y. Chen, K. Xu, J. Li, F. Li, N. Wang, J.W. Qiao *et al.* (2015) Characterization of *FAE1* in the zero erucic acid germplasm of *Brassica rapa* L. Breed. Sci. 65: 257–264.

Zhan, Z.X., Y.F. Jiang, N.D. Shah, Z.K. Hou, Y.W. Zhou, B.C. Dun, S.S. Li, L. Zhu, Z.Y. Li, Z.Y. Piao *et al.* (2020) Association of clubroot resistance locus *PbBa8.1* with a linkage drag of high erucic acid content in the seed of the European turnip. Front. Plant Sci. 11: 810.