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

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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 F₁ 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.

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. rapa* (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 F1 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.

Eruvic 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, ‘Lihō’ (Harvey and Downey 1964), and many LEA cultivars have been released since. Eruvic 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*) F1 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. rapa*, ‘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 Chikuyo.

#### Clubroot resistance test

We collected clubs from 2011 to 2015 in the rapeseed
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’ (Watanabe Seed Co., Miyagi, Japan) which are Japanese F1 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 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, ‘Kiraribishi’ (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’-GGGCCGCTATTTTGTATT-3’) and BnFAE1.1_tori827misF for HEA (5’-GGGCCGCTATTTTGTATCT-3’). BnFAE1.1-3UTR-cr (Karim et al. 2016) was used as the reverse primer (5’-GACGTCTAGTGTTAGGGCTT-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 BC7 plants bred by backcrossing using ‘Kizakinonatane’ as a recurrent parent (Fig. 2). Then, erucic acid contents in 5 BC5S1 seeds per BC7 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.
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 F1 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 F1 cultivars against six Japanese field isolates of *Plasmodiphora brassicae*

| Field isolate | Pathogen classification | Chinese cabbage F1 varieties | CR Chinese cabbage |
|---------------|-------------------------|------------------------------|-------------------|
|               |                         | Super CR Hiroki | CR Ryutoku | Muso | PL9 |
| Yokohama      | Group 4                 | 0.1               | 0.1        | 3.0  | S   | 0.3  | R   |
| Yurihonjo     | Group 4                 | 0.1               | 0.0        | 3.0  | S   | 0.0  | R   |
| Misatomachi   | Group 4                 | 0.0               | 0.1        | 3.0  | S   | 0.0  | R   |
| Yamagata1     | Group 4                 | 0.0               | 0.1        | 3.0  | S   | 0.0  | R   |
| Yamagata2     | Group 2                 | 0.0               | 2.9        | 2.9  | S   | 0.0  | R   |
| Chikujo       | Group 4                 | 0.1               | 0.0        | 3.0  | S   | 0.0  | R   |

* 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, ‘Nagashimazairai No. 1’, and then with the rapeseed cultivar, ‘Kizakinonatane’ (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.

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

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

| Field isolate | 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.*
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

| Cultivar      | Year     | No. of plants | DI$^0$ | 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                   |
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 BC₁ plants harboring Crr1 and Crr2 and found that 13 plants were LEA homozygotes and 19 plants were heterozygotes. Erucic acid contents of BC₅₁ seeds of 32 BC₁ plants varied from 0.0 to 32.4%, whereas those of ‘Kizakinonatane’ and ‘PL9’ were 0.0% and 45.5%, respectively. In the BC₁ zygotes and 19 plants were heterozygotes. Erucic acid contents of BC₁ seeds of 32 BC₁ plants varied from 0.0 to 32.4%, whereas those of ‘Kizakinonatane’ and ‘PL9’ were 0.0% and 45.5%, respectively. In the BC₁, 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 BC₅₁ seeds homoygous for Crr1 and Crr2 obtained by self-fertilization of the selected six LEA BC₁ plants. All lines showed resistance against the group 4 isolate of P. brassicae ‘Yokohama’ with no symptoms or only small clubs at lateral roots (Table 7).

**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 F₁ 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 F₁ 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

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

| Line     | No. of plants | ICᵃ | Mean DIᵇ |
|----------|---------------|-----|----------|
| 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.

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**Table 6. Relationship between genotypes of BC₁ plants categorized by allele-specific markers of BnFAE1.1 and erucic acid contents in BC₅₁ seeds**

| BnFAE1.1 genotypes of BC₁ plantsᵃ | No. of BC₁ plants | Plants with both HEA and LEA seedsᵇ | Plants with only LEA seedsᵇ |
|-----------------------------------|-------------------|-------------------------------------|----------------------------|
| HL                                | 19 (0–32.4%)      | 0                                   | 13 (0%)                    |
| LL                                | 0                 |                                     |                            |

Values in parenthesis are erucic acid content in BC₅₁ seeds.

ᵃ H denotes the allele of ‘PL9’, L denotes that of ‘Kizakinonatane’.
ᵇ HEA is the seed having erucic acid content more than 2% and LEA no more than 2%.

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**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 BC₁ plants are shown above the gel. HH, homozygous HEA; HL, heterozygous; LL, homozygous LEA.
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 combination of resistance. 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.

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