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Research Paper

Screening of clubroot-resistant varieties and transfer of clubroot resistance genes to *Brassica napus* using distant hybridization

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Clubroot is an economically important disease affecting plants in the family *Cruciferae* worldwide. In this study, a collection of 50 *Cruciferae* accessions was screened using *Plasmodiophora brassicae* pathotype 4 in China. Eight of these demonstrated resistance, including three Chinese cabbages, two cabbages, one radish, one kale, and one *Brassica juncea*. The three clubroot-resistant Chinese cabbages (1003, 1007 and 1008) were then used to transfer the clubroot resistance genes to *B. napus* by distant hybridization combined with embryo rescue. Three methods including morphological identification, cytology identification, and molecular marker-assisted selection were used to determine hybrid authenticity, and 0, 2, and 4 false hybrids were identified by these three methods, respectively. In total, 297 true hybrids were identified. Clubroot resistance markers and artificial inoculation were utilized to determine the source of clubroot resistance in the true hybrids. As a result, two simple sequence repeat (SSR) and two intron polymorphic (IP) markers linked to clubroot resistance genes were identified, the clubroot resistance genes of 1007 and 1008 were mapped to A03. At last, 159 clubroot-resistant hybrids were obtained by clubroot resistance markers and artificial inoculation. These intermediate varieties will be used as the 'bridge material' of clubroot resistance for further *B. napus* breeding.

Key Words: clubroot, distant hybridization, embryo rescue, molecular markers.

Introduction

Clubroot is a soil-borne disease caused by *Plasmodiophora brassicae* (Woronin) that has been spreading rapidly worldwide and has been reported in 60 countries (Dixon 2009). In China, the area affected by clubroot disease currently accounts for 1/3 of the total area of *Cruciferous* crops, causing losses of 20%–30% yield, with more than 60% loss in the most seriously damaged regions, resulting in significant production constraints (Wang *et al.* 2012). It is difficult to control clubroot disease using traditional methods such as cultivation methods, chemical agents, biological control, because the pathogen can persist in the soil as resting spores for more than eight years, and can even survive up to 15 years in infected fields when conditions are suitable (Jubault *et al.* 2008).

In amphidiploid *Brassica* species, there are limited resistant sources available in *B. napus*, and no resistant genotypes were identified for the mustard species *B. juncea* and *B. carinata* (Peng *et al.* 2014). Germplasms resistant to a broad range of pathotypes of *P. brassicae* have been identified in the progenitor diploid *Brassica* species *B. rapa*, *B. nigra*, and *B. oleracea* (Hasan 2012, Peng *et al.* 2014), which could possibly be used for developing *B. napus* and mustard species with resistance to clubroot by re-synthesizing the *Brassica* amphidiploids. Therefore some researchers routinely use *B. rapa* and *B. oleracea* as sources for clubroot resistance genes. Clubroot resistance genes currently identified in Chinese cabbage primarily originated from European turnips, and at least eight resistance-related genes have been identified (Crute *et al.* 1980, Diederichsen *et al.* 2009, Hirai 2006, Piao *et al.* 2009). It have been reported that the clubroot resistance in Chinese cabbages is controlled by one or two major genes (Cho *et al.* 2016, Gao *et al.* 2014, Piao *et al.* 2004, Suwabe *et al.* 2003, Yoshikawa 1981). The clubroot resistance of *B. oleracea* was controlled

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by one or more recessive genes (Jichuan and Wang 1989, Voorrips and Visser 1993), while that of kale is controlled by one recessive or many major dominant genes (Laurens and Thomas 1993, Voorrips and Visser 1993).

In view of the current intensification of clubroot disease worldwide, traditional methods cannot effectively control the spread of clubroot. Therefore, clubroot resistance breeding is considered to be one of the most effective ways to control this disease. Studies have been reported from Japan, South Korea, Europe, North America and other countries, and many clubroot resistant vegetable varieties have been cultivated (Jichuan and Wang 1989). However, few resistant varieties of *B. napus* have been reported; therefore it is more feasible to transfer the clubroot resistance genes from vegetables to *B. napus* by interspecific hybridization. Using this method, a number of synthetic species have been reported, including *B. napus*, *B. carinata*, and *B. juncea* (Liu 1985). Initially, researchers utilized artificial crossing and natural fruiting to synthesize interspecific hybrids, but the rate of success was low (Liu 1992). With the emergence of embryo rescue technology, researchers began to use this method combined with conventional hybrid breeding to synthesize a range of new rapeseed varieties, concentrating primarily on oil content, quality, yield, and resistance (Zhang *et al.* 2001, Zhou *et al.* 2005). However, few of studies on clubroot-resistant germplasm synthesis of rapeseed were reported. Thus it is necessary to use resistant resources from the close relatives of rapeseed to cultivate clubroot-resistant varieties of *B. napus*. Therefore the purpose of this study is three folds: 1) Screening of clubroot-resistant *Cruciferous* varieties, 2) Transferring the clubroot resistance genes from Chinese cabbage to *B. napus* using distant hybridization, and 3) Verification of hybrid authenticity and clubroot resistance. It is hoped that this study will provide the 'bridge material' for clubroot resistance breeding in *B. napus*.

Materials and Methods

Sources of Brassica germplasm

A collection of 50 *Brassica* accessions including 42 inbred lines and eight hybrid varieties were obtained from Northwest A&F University (Yangling, Shaanxi, China) and Shanghai Academy of Agricultural Sciences (Shanghai, China) (**Table 1**). Species included *B. rapa* (13), *B. juncea* (3), Chinese cabbage (5, hybrid varieties), *B. napus* (4), cabbage (3, hybrid varieties), broccoli (7), cauliflower (8), kale (6) and radish (1).

Inoculation and resistance test

A single-spore isolate, defined as pathotype 4 (P4) of *P. brassicae* (Williams 1966), was used to test clubroot resistance of each accession. Ten individuals per accession were selected to be inoculated, and two replicates were assessed. The plants were grown in 50-well multipots. The resistance tests were carried out at Northwest A&F University (Yangling, Shaanxi, China) and Shenyang Agricultural University (Shenyang, Liaoning, China).

The *P. brassicae* isolate was propagated and isolated from infected root tissues of susceptible plants as described by Piao *et al.* (2004). Seedlings were inoculated 3 days after germination by injecting 10 ml *P. brassicae* resting spore suspension (1×10^7 spores/ml) into each well, and seedlings were then maintained in a greenhouse under a 16 hL/8 hD photoperiod at an average temperature of 20–25°C. The soil was kept moist during the treatment period. Infection was checked after 35 days by pulling out the plants. Roots of each accession were assessed for clubroot disease severity at 5 weeks after inoculation using a standard 0 to 3 scale where: 0 = no clubbing; 1 = small clubs only; 2 = moderate clubs; and 3 = severe clubbing. For statistical analysis, two indicators, disease incidence and disease index (DI) were used. The disease incidence of each accession was calculated according to this formula: disease incidence=Number of susceptible plants/total number of investigated plants. The DI was calculated using the following formula (Strelkov *et al.* 2006):

$$DI = \frac{\sum (\text{rating class}) \times (\#\ \text{plants in rating class})}{(\text{total}\ \#\ \text{plants in treatment}) \times 3} \times 100$$

The criteria for resistance classification according to the DI were as follows: DI = 0, highly resistant (HR); DI < 10, resistant (R); $10 \le DI \le 20$, moderately susceptible (MS); $20 \le DI \le 50$, susceptible (S); DI > 50, highly susceptible (HS). The plants with DI values of 0–10 were identified as resistant.

Analysis of resistance

The SPSS Statistics v20.0.0 software was used to analyze the standard deviation and the significance of 50 test accessions. The Euclidean cluster average method was utilized to study the cluster analysis of DI for the test materials (Huang *et al.* 2008). The linear regression method was used to analyze the correlation between the disease incidence and the DI of 50 accessions (Yang *et al.* 2011).

Distant hybridization

The resistant Chinese cabbage varieties (1003, 1007, and 1008) that were identified from the 50 accessions were used as the donors of the clubroot resistance genes, and the four susceptible *B. napus* varieties (833, 2348, 2523 and 2541) were used as receptors. These four *B. napus* varieties were used as the female parent and crossed with the three resistant Chinese cabbages. The method of distant hybridization combined with embryo rescue was used to transfer the clubroot resistance genes from Chinese cabbages to *B. napus*. The procedure was as follows: ten pods per combination were selected to isolate the embryos. The embryos (at 15 days after pollination) were cultured in B5 liquid medium with 2% sucrose, placed on a shaking platform with a rotating speed of 50 r/min, and a 16 hL/8 hD photoperiod at an average temperature of 25°C. When cotyledons appeared, they were transferred into B5 solid medium supplemented

Table 1. Information of 50 *Cruciferae* accessions

Accessions	Species	Type of plant	Source of materials
2941	*B. rapa* subsp. *sylvestris*	*B. rapa*	Northwest A&F University, Yangling
2944	*B. rapa* subsp. *sylvestris*	*B. rapa*	Northwest A&F University, Yangling
2948	*B. rapa* subsp. *sylvestris*	*B. rapa*	Northwest A&F University, Yangling
2952	*B. rapa* subsp. *sylvestris*	*B. rapa*	Northwest A&F University, Yangling
2957	*B. rapa* subsp. *sylvestris*	*B. rapa*	Northwest A&F University, Yangling
2968	*B. rapa* subsp. *sylvestris*	*B. rapa*	Northwest A&F University, Yangling
2927	*B. rapa* subsp. *sylvestris*	*B. rapa*	Northwest A&F University, Yangling
2972	*B. rapa* subsp. *sylvestris*	*B. rapa*	Northwest A&F University, Yangling
2985	*B. rapa* subsp. *sylvestris*	*B. rapa*	Northwest A&F University, Yangling
2990	*B. rapa* subsp. *sylvestris*	*B. rapa*	Northwest A&F University, Yangling
2998	*B. rapa* subsp. *sylvestris*	*B. rapa*	Northwest A&F University, Yangling
3002	*B. rapa* subsp. *sylvestris*	*B. rapa*	Northwest A&F University, Yangling
3009	*B. rapa* subsp. *sylvestris*	*B. rapa*	Northwest A&F University, Yangling
833	*B. napus*	*B. napus*	Northwest A&F University, Yangling
2348	*B. napus*	*B. napus*	Northwest A&F University, Yangling
2523	*B. napus*	*B. napus*	Northwest A&F University, Yangling
2541	*B. napus*	*B. napus*	Northwest A&F University, Yangling
1010	*B. juncea* subsp. *juncea*	*B. juncea*	Northwest A&F University, Yangling
1011	*B. juncea* subsp. *juncea*	*B. juncea*	Northwest A&F University, Yangling
1012	*B. juncea* subsp. *juncea*	*B. juncea*	Northwest A&F University, Yangling
1003	*B. rapa* subsp. *pekinensis*	Chinese cabbage	Northwest A&F University, Yangling
1004	*B. rapa* subsp. *pekinensis*	Chinese cabbage	Northwest A&F University, Yangling
1007	*B. rapa* subsp. *pekinensis*	Chinese cabbage	Northwest A&F University, Yangling
1008	*B. rapa* subsp. *pekinensis*	Chinese cabbage	Northwest A&F University, Yangling
1009	*B. rapa* subsp. *pekinensis*	Chinese cabbage	Northwest A&F University, Yangling
1001	*B. oleracea* var. *capitata*	Cabbage	Northwest A&F University, Yangling
1005	*B. oleracea* var. *capitata*	Cabbage	Northwest A&F University, Yangling
1006	*B. oleracea* var. *capitata*	Cabbage	Northwest A&F University, Yangling
1002	*R. raphanistrum* subsp. *sativus*	Radish	Northwest A&F University, Yangling
JL1	*B. oleracea* var. *acephala*	Kale	Shanghai Academy of Agricultural Sciences, Shanghai
JL2	*B. oleracea* var. *acephala*	Kale	Shanghai Academy of Agricultural Sciences, Shanghai
JL3	*B. oleracea* var. *acephala*	Kale	Shanghai Academy of Agricultural Sciences, Shanghai
JL4	*B. oleracea* var. *acephala*	Kale	Shanghai Academy of Agricultural Sciences, Shanghai
JL5	*B. oleracea* var. *acephala*	Kale	Shanghai Academy of Agricultural Sciences, Shanghai
JL6	*B. oleracea* var. *acephala*	Kale	Shanghai Academy of Agricultural Sciences, Shanghai
QH1	*B. oleracea* var. *italica*	Broccoli	Shanghai Academy of Agricultural Sciences, Shanghai
QH2	*B. oleracea* var. *italica*	Broccoli	Shanghai Academy of Agricultural Sciences, Shanghai
QH3	*B. oleracea* var. *italica*	Broccoli	Shanghai Academy of Agricultural Sciences, Shanghai
QH4	*B. oleracea* var. *italica*	Broccoli	Shanghai Academy of Agricultural Sciences, Shanghai
QH5	*B. oleracea* var. *italica*	Broccoli	Shanghai Academy of Agricultural Sciences, Shanghai
QH6	*B. oleracea* var. *italica*	Broccoli	Shanghai Academy of Agricultural Sciences, Shanghai
QH7	*B. oleracea* var. *italica*	Broccoli	Shanghai Academy of Agricultural Sciences, Shanghai
BH1	*B. oleracea* var. *botrytis*	Cauliflower	Shanghai Academy of Agricultural Sciences, Shanghai
BH2	*B. oleracea* var. *botrytis*	Cauliflower	Shanghai Academy of Agricultural Sciences, Shanghai
BH3	*B. oleracea* var. *botrytis*	Cauliflower	Shanghai Academy of Agricultural Sciences, Shanghai
BH4	*B. oleracea* var. *botrytis*	Cauliflower	Shanghai Academy of Agricultural Sciences, Shanghai
BH5	*B. oleracea* var. *botrytis*	Cauliflower	Shanghai Academy of Agricultural Sciences, Shanghai
BH6	*B. oleracea* var. *botrytis*	Cauliflower	Shanghai Academy of Agricultural Sciences, Shanghai
BH7	*B. oleracea* var. *botrytis*	Cauliflower	Shanghai Academy of Agricultural Sciences, Shanghai
BH8	*B. oleracea* var. *botrytis*	Cauliflower	Shanghai Academy of Agricultural Sciences, Shanghai

with 2% sucrose. When the seedlings grew main roots, they were inoculated with P4 (10 ml, 1×10^7 spores/ml), using the procedure as described by Piao *et al.* (2004).

Identification of F_1 hybrid authenticity

In this study, morphological, cytological and molecular markers were used to identify the authenticity of F_1 hybrids derived from Chinese cabbages and *B. napus*.

Morphological identification

The morphological characteristics at the seedling and flowering stages of F_1 hybrids and their parents were compared to determine whether the hybrids had similar characteristics to their parents. These characteristics included leaf shape, leaf margin, bud, flower size and flower color.

Cytological identification

Flower buds of 2–3 mm diameter were picked for chromosome count. These were then placed in 0.002 M 8-hydroxyquinoline under darkness at room temperature for 3–4 hours. The pistil was then transferred to Kano fixed liquid (ethanol: glacial acetic acid = 3:1) overnight, and then

placed at –20°C for long-term storage. Chromosomes were visualized using the method reported by Li *et al.* (1995). In brief, the pistils were first placed in 1 M HCl for 6–8 minutes at 60°C and then soaked in distilled water for 1 minute, followed by addition of one drop of magenta dye for 30 seconds. Chromosome preparations were then observed under an optical microscope.

Molecular marker identification

Genomic DNA was extracted from young leaves of parents and F_1 individuals using the CTAB method (Doyle 1990). The final DNA concentration was adjusted to 50 ng/μl. The SSR amplification was performed as described by Lowe *et al.* (2002). Sequences of all SSR markers were obtained from public sources including the databases on http://ukcrop.net/perl/ace/search/BrassicaDB (Lowe *et al.* 2004) and http://www.brassica.info/resource/markers.php (for those with the prefixes: Ra, Na, BN, and BRMS), as well as the electronic supplementary material of Piquemal *et al.* (2005) (for those primer pairs with the prefixes 'BRAS' and 'CB'). Silver staining was performed according to the procedures described by Lu *et al.* (2001). Eighty one pairs of SSR primers that amplified multiply bands in the previous studies were used to amplify both parents and F_1 individuals. The F_1 individuals that were found to be consistent with the parental male bands after amplification were identified as true hybrids.

Clubroot resistance identification

Clubroot resistant markers assisted selection and artificial inoculation using P4 were used to identify the resistance of F_1. Initially, 25 SSR markers linked to seven clubroot resistance genes, such as *Crr1*, *Crr2*, *Crr3*, *Crr4*, *CRk*, *CRc* and *CRb* (Hirai *et al.* 2004, Piao *et al.* 2003, Sakamoto *et al.* 2008, Suwabe *et al.* 2003, 2006) were selected and 12 pairs of IP primers around *CRa* were designed to amplify the parents and F_1 individuals. The individuals that were identified as resistant by both methods were selected for future study.

Results

Analysis of disease resistance for test materials

The results showed that the disease incidence of the 50 accessions was between 5.00% and 100.00%, with an average incidence of 76.93%. The DI was between 1.67 and 83.34, with an average of 46.58. The disease incidence of these materials is significantly different, and the DI also has a significant difference (**Table 2**). Among these, DI of eight accessions were less than 10 (**Fig. 1**), including three Chinese cabbages (1003, 1007 and 1008), two cabbages (1001, 1005), one radish (1002), one *B. juncea* (1012) and one kale (JL6). These accessions were confirmed as resistant materials according to the criteria for resistance classification, accounting for 16% of the test materials. The DI of other test materials was greater than 10, showing different degrees of disease; among these, two were moderately susceptible to disease, 14 were susceptible to disease and 26 showed high sensitivity, accounting for 4%, 28% and 52% of the test materials, respectively.

Table 2. Significance analysis of 50 accessions on clubroot resistance

Names	Disease incidence	Disease index
1012	5.00 ± 0.07aA	1.67 ± 2.35aA
1002	5.00 ± 0.07aA	1.67 ± 2.35aA
JL6	17.50 ± 0.06abA	6.67 ± 4.72aA
1003	21.00 ± 0.01abA	7.04 ± 0.52aAB
1005	15.50 ± 0.06abA	7.04 ± 0.52aAB
1008	16.50 ± 0.05abA	7.50 ± 1.17aAB
1001	15.00 ± 0.07abA	8.34 ± 2.35aAB
1007	26.00 ± 0.06bA	8.71 ± 1.83aAB
BH2	59.50 ± 0.05cdBC	19.68 ± 1.63bBC
BH6	52.00 ± 0.11ghijB	19.91 ± 3.40bCD
QH2	66.00 ± 0.13cdefBCD	26.49 ± 3.79bcCDE
QH5	69.00 ± 0.03defgBCDE	27.32 ± 3.27bcCDE
JL5	82.00 ± 0.10efghijCDEFG	29.17 ± 5.89bcdCDEF
QH7	69.00 ± 0.27defgBCDE	29.17 ± 5.89bcdCDEF
BH5	75.00 ± 0.07defghCDEF	31.67 ± 2.35cdCDEF
QH1	82.00 ± 0.10efghijCDEFG	32.92 ± 5.30cdeDEF
QH3	94.50 ± 0.08jFG	38.34 ± 2.35defEFG
1006	65.00 ± 0.07cdeBCD	41.67 ± 2.35efgFGH
BH7	84.00 ± 0.08ghijDEFG	41.86 ± 6.81efgFGH
BH1	82.00 ± 0.10efghijCDEFG	46.99 ± 1.64fghGHI
QH4	84.50 ± 0.06ghijDEFG	47.22 ± 3.93fghGHI
1004	75.00 ± 0.07defghCDEF	48.15 ± 5.24ghiGHIJ
2998	95.00 ± 0.07jFG	48.34 ± 2.35ghiGHIJ
3009	95.00 ± 0.07jFG	49.08 ± 1.31ghiGHIJ
BH3	83.00 ± 0.07fghijDEFG	50.70 ± 6.88ghijGHIJk
QH6	89.50 ± 0.01hijEFG	50.74 ± 3.66ghijGHIJk
1009	76.00 ± 0.08defghiCDEF	53.71 ± 2.62hijkHIJkL
BH4	95.00 ± 0.07jFG	55.00 ± 2.36hijklHIJkLM
2927	100.00 ± 0.00jG	56.30 ± 4.19hijklIJkLM
JL4	84.50 ± 0.06ghijDEFG	56.67 ± 4.72hijklmIJkLMN
2968	100.00 ± 0.00jG	57.78 ± 3.14ijklmIJkLMN
1011	95.00 ± 0.07jFG	58.15 ± 6.81ijklmnIJkLMN
3002	100.00 ± 0.00jG	58.34 ± 2.35ijklmnIJkLMN
JL3	94.50 ± 0.08jFG	59.63 ± 0.52jklmnIJkLMN
1010	100.00 ± 0.00jG	60.00 ± 4.71klmnoIJkLMN
2948	95.00 ± 0.07jFG	61.67 ± 7.07klmnoJkLMN
2957	95.00 ± 0.07jFG	63.34 ± 9.43klmnoKLMNO
JL1	93.00 ± 0.10ijFG	64.59 ± 2.95lmnoLMNO
2972	100.00 ± 0.00jG	65.00 ± 2.36lmnoLMNO
2944	100.00 ± 0.00jG	66.67 ± 4.72mnopLMNO
2541	100.00 ± 0.00jG	68.15 ± 7.33nopMNOP
BH8	95.00 ± 0.07jFG	68.34 ± 2.35nopMNOP
2990	100.00 ± 0.00jG	70.00 ± 4.71opNOPQ
2941	100.00 ± 0.00jG	75.00 ± 2.36pqOPQR
2952	100.00 ± 0.00jG	75.00 ± 2.36pqOPQR
2348	100.00 ± 0.00jG	79.63 ± 2.62qPQR
JL2	100.00 ± 0.00jG	80.00 ± 9.43qPQR
2985	100.00 ± 0.00jG	81.67 ± 2.35qQR
2523	100.00 ± 0.00jG	82.23 ± 6.29qQR
833	100.00 ± 0.00jG	83.34 ± 4.72qR

Euclidean cluster analysis of the DI of test materials

The cluster analysis of the DI of the 50 accessions showed that when the Euclidean distance was 9.12, the 50 accessions could be classified into 4 groups: resistant, moderately susceptible, susceptible and highly susceptible. Eight resistant materials (1001, 1002, 1003, 1005, 1007,

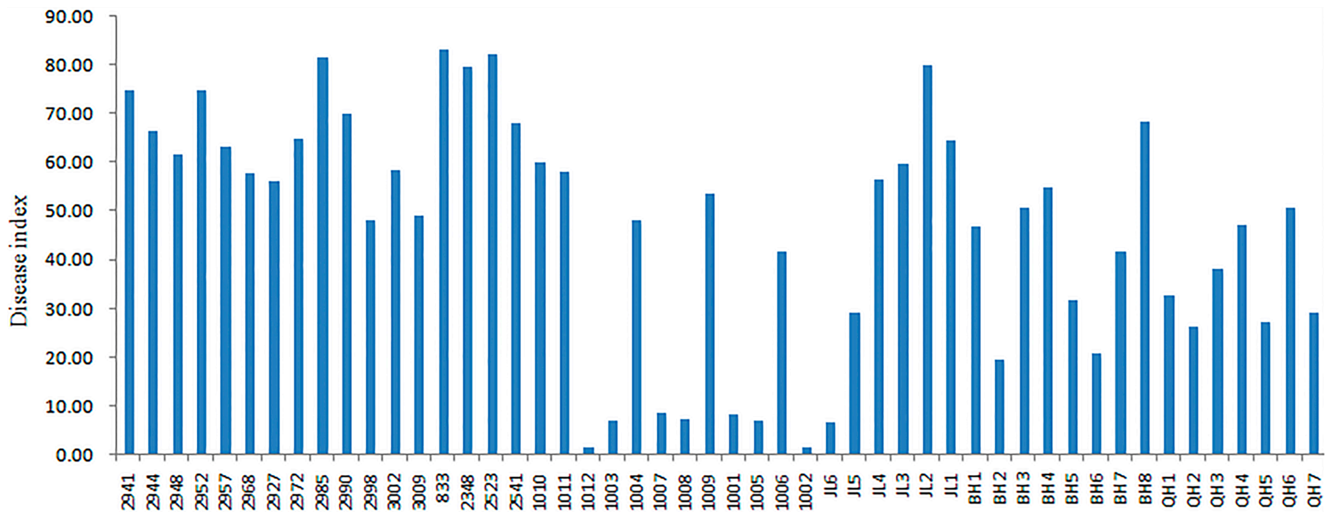

Fig. 1. Comparison of disease index (DI) of 50 *Cruciferae* accessions inoculated with pathotype 4 of *Plasmodiophora brassicae*. The horizontal axis represents the accessions name, and the vertical axis is DI.

1008, 1012 and JL6) clustered into one group with a Euclidean distance of 3.98. The moderately susceptible materials BH2 and BH6 clustered together with a Euclidean distance of 0.97; six susceptible materials (JL5, QH7, QH2, and others) clustered with a Euclidean distance of 3.11. Eight susceptible materials (BH1, QH3, QH4, and others) clustered together with a Euclidean distance of 5.00 (**Fig. 2**).

Correlation analysis between disease incidence and DI

The correlation between the disease incidence and the DI of different accessions showed that the DI increased with the increase of disease incidence, showing a significant correlation between them ($P = 0.05$, $r = 0.906$). The linear regression equation was $Y = -9.603x + 72.953$ (**Fig. 3**). The DI of the eight disease-resistant accessions in the rectangle was lower than 10, and the incidence was lower than 30%. The DI of 26 highly-sensitive accessions in the triangle frame was greater than 50, and the disease incidence was also higher than 75%. In the dotted rectangular box, the DI of 14 susceptible accessions was between 20 and 50, and the disease incidence was higher than 60%. The other two were moderately susceptible (**Fig. 3**).

Comparison of embryo rescue for different hybridizations

The results showed that the average number of embryos per 10 pods was 53.88. Using the embryo rescue method, the average number of embryos that survived (per 10 pods) was 37.63. The germination rate of embryos in eight hybridizations ranged from 50% to 84.48%, with an average of 69.29%. A total of 112 embryos derived from two cross-hybridizations, 2348 × 1003 and 2348 × 1008, were selected, and the number of surviving embryos from these two hybridizations was 49 and 44, respectively, and the embryo germination rates were 84.48% and 81.48%, respectively. When the female parent was *B. napus* (2348), the rate of

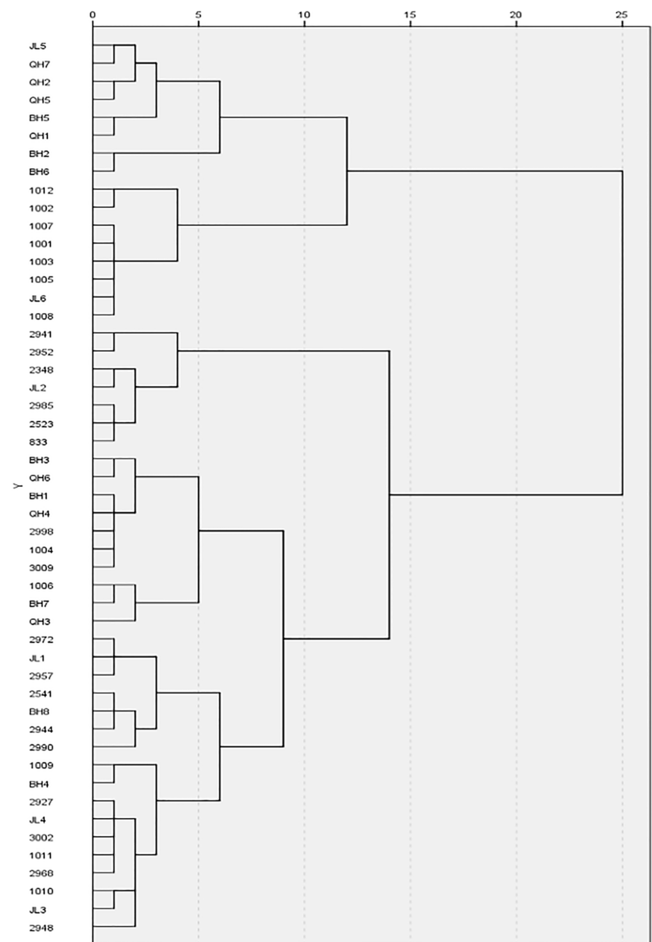

Fig. 2. Cluster analysis with Euclidean distance of 50 *Cruciferae* accessions. The horizontal axis and vertical axis represent Euclidean distance and the accessions name, respectively.

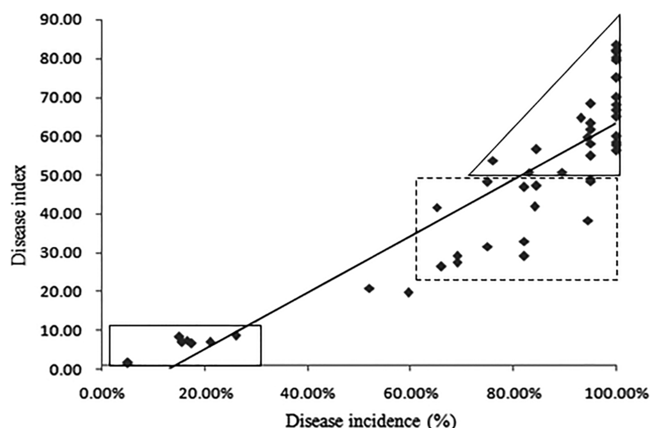

Fig. 3. Relation analysis between disease incidence and disease index of 50 *Cruciferae* accessions. Rectangles and triangle represent coverage similar individuals together.

embryo germination per 10 pods was higher than those of other hybridizations (**Table 3**).

Table 3. Comparison of embryo rescue for different cross combinations

Cross combination	No. of siliqua	No. of embryo culture	No. of developing embryo	Rate of embryo germination
833 (S) × 1007-5 (R)	10	52	26	50.00%
833 (S) × 1008-2 (R)	10	48	24	50.00%
2523 (S) × 1007-4 (R)	10	55	39	70.91%
2523 (S) × 1008-1 (R)	10	56	44	78.57%
2348 (S) × 1003-7 (R)	10	58	49	84.48%
2348 (S) × 1008-1 (R)	10	54	44	81.48%
2541 (S) × 1007-5 (R)	10	53	37	69.81%
2541 (S) × 1008-1 (R)	10	55	38	69.09%
Mean	10	53.88	37.63	69.29%

Identification of F_1 hybrid authenticity

After preliminary evaluation, the distant hybrids were found to have similar characteristics to both parents, with phenotypes between the two parents, though some characteristics derived from only one parent. For example, for the cross-hybridization 2348 × 1003, the leave margin of the female parent 2348 is sharp, while the leaves of F_1 hybrids and male parent 1003 were round. Thus, the leaf characteristics of the hybrid derived wholly from the male parent, but the long petiole traits were similar to the female parent. For the buds, the hybrids were yellow-green and similar to the male parent. The plant height and branch number of this hybrid were similar to those of the male parent (**Fig. 4**). Finally, morphological identification of all hybrids revealed no false hybrids (**Table 4**).

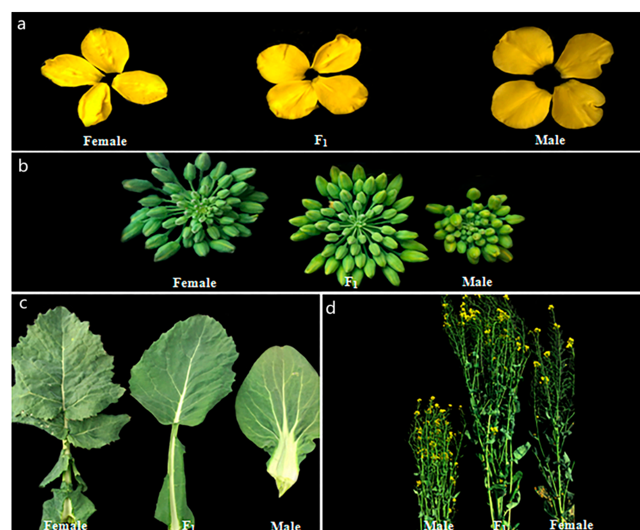

Fig. 4. Morphological identification of F_1, a, b, c and d represent the flowers, leaves, buds and mature plants of F_1 and two parents (2348 and 1003).

Since Chinese cabbage has 20 chromosomes and *B. napus* has 38 chromosomes, the interspecific hybrids should have 29 chromosomes. Cytological identification of 301 hybrid seedlings showed that there were two individuals without the 29 chromosomes; therefore they were identified as false hybrids, while the plants with the 29 chromosomes were regarded as true hybrids (**Fig. 5**). The two false hybrids were from the hybrid combinations 2523 × 1007 and 2348 × 1003 (**Table 4**).

Eighty pairs of SSR primers were used to amplify distant hybrids and their parents. Seven pairs of SSR primers showed polymorphism between F_1 hybrids and their parents (**Table 5**), accounting for 9% of the total primers. These polymorphic primers were used to screen F_1 individuals,

Table 4. Identification of F_1 authenticity using three methods

Cross combination	No. of developing embryo	No. of false hybrids using morphological identification	No. of false hybrids using cytological identification	No. of false hybrids using molecular markers identification
833 (S) × 1007-5 (R)	26	0	0	0
833 (S) × 1008-2 (R)	24	0	0	0
2523 (S) × 1007-4 (R)	39	0	1	1
2523 (S) × 1008-1 (R)	44	0	0	0
2348 (S) × 1003-7 (R)	49	0	1	2
2348 (S) × 1008-1 (R)	44	0	0	1
2541 (S) × 1007-5 (R)	37	0	0	0
2541 (S) × 1008-1 (R)	38	0	0	0
Total	301	0	2	4

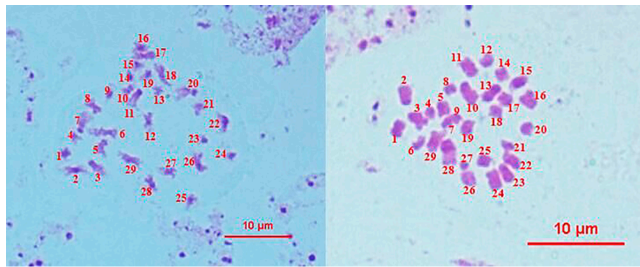

Fig. 5. Cytological identification of F_1 hybridization 2541 × 1008, both right and left ones has 29 chromosomes, indicating that they were the true hybrids. Each chromosome is numbered by 1, 2, 3, ... 29.

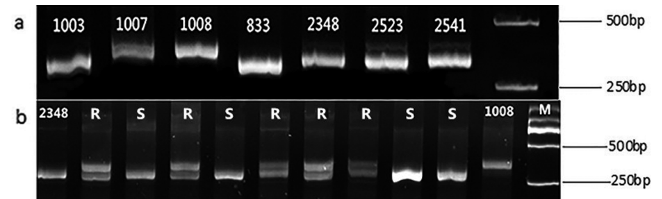

Fig. 6. a: PCR amplification of seven parents used in distant hybridization using MS1marker. b: Partial PCR amplification of cross hybridization 2348 × 1008 with MS1marker, 1–9 represent F_1 hybrids, R and S are the resistant and susceptible plants respectively, M is DNA2000 marker.

Table 5. Sequences of SSR markers used in markers identification

Markers	Sequences (5′ to 3′)
BrgMS225	CGGCAGAAAGAAAGAAAGAGAG ACCAAACCAAAAGGAGAGTCAA
BrgMS321	CCTCTGTCCTCTGTAGTCCCAT GCTTACTCTAATCAGGCCCATC
BnGMS43	TTTGATGGGTCTTCATCTTC GAGGTTAAGGGTTTGGAGTT
CB10504	GGTGTCCCAACTGTTGAA CATTGGCATAGGAACAGG
CB10347	ATCTGAACACTTTCGGCA GGAAGCACCATGTCAGC
CB10524	ATGGAAGGCAACGATTCT TTCTGTGCTAGGTCTGCC
Na10-E08	TCGGGGTTTGTTGTGAGG GAGGAGGATGCTAAGAGTGAGC

which revealed that four individuals did not have the same banding pattern as those of the male parents. These were from three combinations (2348 × 1003, 2348 × 1008 and 2523 × 1007). Thus, the numbers of false hybrids were two, one and one, respectively (**Table 4**), and the remaining 297 true hybrids were used for clubroot resistance identification.

Clubroot resistance identification

Thirty seven pairs of primers around eight clubroot resistance genes of *B. rapa* were selected to screen the parents, and as a result, two SSR primers (TCR108 and MS1) and two IP primers (IPr1 and IPr2) close to *CRa* and *CRb* were found to have the ability to amplify the polymorphic clubroot resistant fragment between the resistant parents 1007 and 1008 (**Fig. 6**, **Table 6**). TCR108 and MS1 were in 23.77 Mb and 24.05 Mb on A03, respectively. IPr1 and IPr2 were in 25.36 Mb and 25.56 Mb on A03, respectively. However, the susceptible bands were also amplified in the resistant parent 1003 and susceptible parents, which suggested that the resistance genes of 1007 and 1008 are linked to each other or the same, and the resistance gene of 1003 was different. The two molecular markers TCR108 and MS1 were used to screen all combinations except 2348 × 1003, and the results indicated that 140 clubroot resistant individuals derived from seven combinations (**Fig. 6**). The percentage of resistant plants per combination was generally between 36.36% and 72.97% (**Table 7**). Two hundred and ninety-seven individuals from eight combinations were inoculated using P4 (**Fig. 7**), with the results indicating that 165 plants were resistant to P4. The percentage of resistant

Table 6. Information of molecular markers linked to the clubroot resistance

Markers	Sequences (5′ to 3′)	Location on A03 (Mb)
MS1	AAAACAAATATCCACCACG/CTCAATCCCACAAACCTG	24.05
TCR108	CGGATATTCGATCTGTGTTCA/AAAATGTATGTGTTTATGTGTTTCTGG	23.77
IPr1	GAGGCCTCCTTTTCTGGTTT/CCGGAGAAGTTTGATTCGAG	25.36
IPr2	TGGAAGCATTGGGAGGATAG/TGGGGGTTTTCACATTCATT	25.56

Table 7. Clubroot resistance identification of F_1 using molecular marker

Cross combination	No. of hybrids seedlings	No. of susceptible materials	No. of resistant materials	Rate of susceptible materials	Rate of resistant materials
833 (S) × 1007-5 (R)	26	8	18	30.77%	69.23%
833 (S) × 1008-2 (R)	24	12	12	50.00%	50.00%
2523 (S) × 1007-4 (R)	38	15	23	39.47%	60.53%
2523 (S) × 1008-1 (R)	44	28	16	63.64%	36.36%
2348 (S) × 1008-1 (R)	43	20	23	46.51%	53.49%
2541 (S) × 1007-5 (R)	37	10	27	27.03%	72.97%
2541 (S) × 1008-1 (R)	38	17	21	44.74%	55.26%
Total	250	110	140		

Fig. 7. Performance of cross hybridization 2523 × 1008 six weeks later after inoculation using pathtype 4.

Table 8. Clubroot resistance identification of F_1 using inoculation

Cross combination	No. of hybrids seedlings	Class of disease				Disease incidence
		0	1	2	3	
833 (S) × 1007-5 (R)	26	18	2	0	6	30.77%
833 (S) × 1008-2 (R)	24	12	3	0	9	50.00%
2523 (S) × 1007-4 (R)	38	25	1	2	10	34.21%
2523 (S) × 1008-1 (R)	44	16	2	2	24	63.63%
2348 (S) × 1003-7 (R)	47	22	1	0	24	53.19%
2348 (S) × 1008-1 (R)	43	24	0	0	19	44.19%
2541 (S) × 1007-5 (R)	37	27	0	0	10	27.03%
2541 (S) × 1008-1 (R)	38	21	2	0	15	44.74%
Total	297	165	11	4	117	

plants per combination was between 30.77% and 63.63% (**Table 8**). Furthermore, the results of most combinations were in agreement regardless of which of the two identification methods were used. Two plants from combination 2523 × 1007 were classified as resistant using clubroot resistance markers but were susceptible to clubroot disease after inoculation with P4. One individual from combination 2348 × 1008 was identified as susceptible using clubroot resistance markers, but was resistant when inoculated with P4. After removal of these three individuals that were deemed inconsistent depending on the method used, 159 resistant plants from eight combinations were selected to be used for future cultivation of clubroot resistant *B. napus*.

Discussion

Synthesis of clubroot-resistant B. napus using embryo rescue

Clubroot disease is caused by the biotrophic soil-borne pathogen *P. brassicae*. In recent years, this disease has been rapidly spreading among the areas where rapeseed is grown in China, causing huge losses in rapeseed production. However, there are few clubroot-resistant strains of *B. napus* in China. Fortunately, many clubroot-resistant materials have been found in species that are closely related to rapeseed, such as *B. rapa*, *B. oleracea*, *B. nigra*, and others. In this study, 50 *Cruciferae* accessions were analyzed to identify the clubroot-resistant germplasms. Eight clubroot-resistant accessions were obtained, of which only one rapeseed (*B. juncea*) germplasm was identified as clubroot resistant, and no clubroot-resistant *B. napus* were identified. Similar results have been reported in other studies (Peng *et al.* 2014), where 955 *Brassica* accessions were screened using pathotype 3 in Canada, but only one resistant individual out of 94 *B. napus* sources was identified, and the other resistant materials were primarily from *B. rapa*, *B. oleracea*, and *B. nigra*. Therefore, it is difficult to obtain resistant materials from existing *B. napus* sources, and thus the only alternative is to obtain the resistance genes from closely-related species.

Since clubroot disease is spreading widely in many countries, rapeseed production has been affected worldwide. One of the most effective ways to prevent the spread of clubroot disease is to cultivate *B. napus* clubroot-resistant varieties. Therefore, the resistant genes of *B. rapa* or *B. olerecea* have been transferred to *B. napus* to cultivate clubroot-resistant *B. napus* varieties by means of distant hybridization. However, interspecific hybrids between *B. napus* and other species are difficult to obtain due to misogamy. Therefore, the embryo rescue technique has been used to overcome the incompatibility between interspecific hybridization. This method has been successfully applied in several studies; for example, interspecific hybridization between radish and Chinese Cabbage (Zhao 1983), distant hybridization between *B. napus* and kale (Chen *et al.* 2000), and interspecific hybridization between radish and cabbage (Fang *et al.* 1983). In this study, distant hybrid seedlings were also successfully obtained by the embryo rescue technique. We also compared the two methods of natural seed setting and embryo rescue, and it was found that embryo rescue technology can improve the embryo germination rate of cross combinations (data not show). In addition, we also found that when *B. napus* 2348 was used as a female parent, it was relatively easy to obtain hybrids, indicating that the success of distant hybridization is closely related to the selection of the female parent.

Identification of F_1 hybrid authenticity

Because false hybrids are likely to appear when using the distant hybridization technique, it is necessary to verify the authenticity of distant hybrids. Currently, morphological observation, cell observation, and molecular marker identification are commonly used for verification of hybrid authenticity. In this study, these three methods were used in combination, allowing identification of 297 true hybrids and four false hybrids. These results indicate that verification of distant hybrids is necessary as false hybrids will appear in the offspring. In addition, morphological identification is likely to be influenced by environmental and other factors. Therefore, a variety of identification methods should be utilized in combination to increase the reliability and authenticity of the identified hybrids for credible results.

Identification of F_1 resistance

Resistance identification is an integral part of clubroot resistance breeding, and currently, artificial inoculation at the seedling stage is the most commonly used method. However, artificial inoculation is easily influenced by temperature, light, pH and other factors, affecting identification of resistant individuals. Thus, molecular markers linked to resistance genes are a more sensitive method that can be used to screen for clubroot-resistant plants. In this study, two methods, artificial inoculation and markers assisted selection were used to identify the resistance of the F_1 generation. The results obtained from the two methods were similar; however, molecular marker-assisted selection is obviously much simpler. The artificial inoculation method is not suitable for screening a large number of disease-resistant materials, and the procedures are relatively complex. In addition, when inoculation conditions are modified, some individuals may not be inoculated successfully, resulting in an incorrect identification of the phenotype. Therefore, in large-scale identification of resistant individuals, the molecular marker method would be the preferred method to save labour and cost.

Analysis of clubroot resistant genes

In the current study, four molecular markers associated with clubroot resistance were identified, which were located in a region from 23.77 Mb to 25.56 Mb on A03 of *B. rapa*. In the adjacent area of this region, two clubroot resistant genes *CRa* (Ueno *et al.* 2012) and *CRb* (Hatakeyama *et al.* 2017) have been cloned, in which both genes encode the TIR-NBS-LRR (TNL) protein. Through analyzing the genes structure of the region surrounding the *CRa* and *CRb*, it was found that six genes *Bra012540*, *Bra012541*, *Bra019409*, *Bra019410*, *Bra019412* and *Bra019413* have the structure of TNL. It is difficult to determine which TNL gene is the candidate gene for this study based on the present results. Therefore, it is necessary to clone these six TNL genes in our materials to analyze the gene structures, and this work is ongoing.

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