Germplasm Enhancement and Identification of Loci Conferring Resistance against *Plasmodiophora brassicae* in Broccoli

Qi Xie 1,†, Xiaochun Wei 2,†, Yumei Liu 1, Fengqing Han 1 and Zhanhong Li 1,*

1 Key Laboratory of Biology and Genetic Improvement of Horticultural Crops, Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing 100081, China
2 Institute of Horticulture, Henan Academy of Agricultural Sciences, Zhengzhou 450002, China
* Correspondence: lizhansheng@caas.cn
† These authors contributed equally to this work.

Abstract: In order to breed broccoli and other *Brassica* materials to be highly resistant to clubroot disease, 41 Brassicaceae varieties were developed and identified between 2020 and 2021. Seven known clubroot genes were used for screening these materials. In addition, the resistant and susceptible broccoli cultivars were designed for observing their differences in the infection process with *Plasmodiophora brassicae*. The results showed that 90% of total materials had carried more than two clubroot resistance genes: one material carried two disease resistance genes, four materials carried seven genes for clubroot resistance, two materials carried six genes for clubroot resistance, and in total 32% of these materials carried five genes for clubroot resistance. As a result, several new genotypes of Brassicaceae germplasm were firstly created and obtained based on distant hybridization and identification of loci conferring resistance against *Plasmodiophora brassicae* in this study. We found and revealed that similar infection models of *Plasmodiophora brassicae* occurred in susceptible and resistant cultivars of broccoli, but differences in infection efficiency of *Plasmodiophora brassicae* also existed in both materials. For resistant broccoli plants, a small number of conidia formed in the root hair, and only a few spores could enter the cortex without forming sporangia while sporangia could form in susceptible plants. Our study could provide critical *Brassica* materials for breeding resistant varieties and new insight into understanding the mechanism of plant resistance.

Keywords: *Plasmodiophora brassicae*; clubroot; broccoli; germplasm enhancement; breeding

1. Introduction

Clubroot, a major disease of Brassicaceae crops caused by *Plasmodiophora brassicae* (*P. brassicae*), is an economically important soil borne disease worldwide. It was first discovered in the Mediterranean and southern Europe but now occurs all over the world and has become one of the most serious diseases of cruciferous crops including cauliflower, cabbage, broccoli, Chinese cabbage, turnip, radish and rapeseed [1–3]. Due to the long survival time of its spores in soil, traditional agricultural control methods usually have little effect. Therefore, the effective ways to control the spores are germplasm enhancement and breeding of resistant varieties of *Brassica* plants [4,5]. Therefore, creating Brassicaceae germplasms resistant to clubroot for crop breeding was a fundamental method and essential to crop production.

Broccoli (*Brassica oleracea* L. var. *italica*) is an important member of the Brassicaceae family and widely cultivated as a popular vegetable crop worldwide. It is rich in vitamins, proteins and minerals, as well as some anticancer bioactive compounds, such as sulforaphane and indole-3-carbinol [6–8]. More than 80,000 ha of broccoli has been cultivated in China, which has become the largest producer of broccoli in the world [9]. In recent years, clubroot disease of broccoli has been noted in Zhejiang, Henan, Yunnan and Shandong provinces, which were the dominant broccoli planting areas in China. Therefore, it was necessary to create broccoli resistance materials for future breeding [10,11].
To date, most studies have focused on the location and mining of resistance loci and the verification of their function. So far, four clubroot resistance (CR) genes have been cloned, including Crr1, CRa, CRd and CRb*kato. Crr1 and CRa belong to the TIR-NB-LRR gene family and are found in mainly turnip and Chinese cabbage [1,12–14]. Due to the diversity of physiological strains and the strong strain specificity of single-gene disease-resistant materials, long-term planting of single-gene disease-resistant varieties will lead to a loss of resistance [13]. In the future, we will carry out multi-resistance site polymerization breeding and cultivate varieties with high resistance to multiple physiological strains of P. brassicae to address the continuous harm to cruciferous plant production.

There exist differences in diversity and pathogenicity among countries, regions and root disease seasons [15]. The purpose of the study was to screen clubroot resistant germplasms developed by distant hybridization from cruciferous materials in 2020 and 2021. These materials were collected based on previous reported crops (turnip, cabbage and oilseed rape). At the same time, some possible identifying molecular markers to rapidly detect CR genes among Brassicaceae resources were used for detecting all materials in the study. Therefore, our study would facilitate resource innovation and improvement of materials in the Brassica genus to develop varieties with multiple CR genes. The aim of this study was to provide essential materials and theoretical support for the breeding of new varieties of broccoli and the other Brassica crops with multiple CR genes, as well as to provide new insight into understanding the mechanism of clubroot infection in crops [16,17].

2. Materials and Methods
2.1. Plant Materials

Cruciferous resources from different regions of China, including the Yangtze River Basin and northwest, southeast and central regions, were collected and then improved. All the materials were preserved and planted at the experimental station of Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences (IVF-CAAS). The genetic information of the 41 cruciferous accessions is shown in Table 1.

| Accessions | Species | Generations | Source Origin | Disease Resistant | Year |
|------------|---------|-------------|---------------|------------------|------|
| B891       | Tuscan kale (Brassica oleracea L. var. acephala) | F12          | CAAS-IVF, Beijing, China | S       | 2020 |
| B991       | Broccoli (Brassica oleracea L. var. italica) | F6           | CAAS-IVF, Beijing, China | S       | 2020 |
| B994       | Broccoli (Brassica oleracea L. var. italica) | F1           | Syngenta, China     | S       | 2020 |
| B1007      | Chinese black moss (Brassica campestris L.var. purpurea Baileysh) | OP         | Hubei, China       | S       | 2020 |
| B1081      | Black mustard (Brassica nigra) | F12          | CAAS-IVF, Beijing, China | S       | 2020 |
| B1082      | Abyssinian mustard (Brassica carinata) | F12          | CAAS-IVF, Beijing, China | S       | 2020 |
| B1083      | Black mustard (Brassica nigra) | OP           | CAAS-IVF, Beijing, China | S       | 2020 |
| B1084      | Black mustard (Brassica nigra) | OP           | CAAS-IVF, Beijing, China | S       | 2020 |
| B1086      | Black mustard (Brassica nigra) | OP           | CAAS-IVF, Beijing, China | S       | 2020 |
| B359       | Broccoli (Brassica oleracea L. var. italica) | BC1         | CAAS-IVF, Beijing, China | S       | 2021 |
| B366       | Broccoli (Brassica oleracea L. var. italica) Wild cabbage (Brassica macrocarpa Guss.) | BC1        | CAAS-IVF, Beijing, China | S, R    | 2021 |
| B368       | Broccoli (Brassica oleracea L. var. italica) Wild cabbage (Brassica macrocarpa Guss.) Rape (Brassica napus L.) | BC1        | CAAS-IVF, Beijing, China | S, R, MR | 2021 |
| B369       | Broccoli (Brassica oleracea L. var. italica) Wild cabbage (Brassica macrocarpa Guss.) Rape (Brassica napus L.) | BC1        | CAAS-IVF, Beijing, China | S, R, MR | 2021 |
| B571       | Broccoli (Brassica oleracea L. var. italica) Turnip (Brassica rapa L. ssp. rapa) | F1          | CAAS-IVF, Beijing, China | HR      | 2021 |
| B578       | Rape (Brassica napus L.) (Oil rape) | F1          | CAAS-IVF, Beijing, China | HR      | 2021 |
| B581       | Turnip (Brassica rapa L. ssp. rapa) | F11         | CAAS-IVF, Beijing, China | HR      | 2021 |
| B582       | Yellow rocket (Barbarea vulgaris R. Br.) | F11         | CAAS-IVF, Beijing, China | I       | 2021 |
| B606       | Red cabbage (Brassica oleracea L. var. capitata) Turnip (Brassica rapa L. ssp. rapa) | BCIF3      | CAAS-IVF, Beijing, China | S, R, MR | 2021 |
Table 1. Cont.

| Accessions | Species | Generations | Source Origin | Disease Resistant | Year |
|------------|---------|-------------|---------------|-------------------|------|
| B607       | Red cabbage (Brassica oleracea L. var. capitata) Turnip (Brassica rapa L. ssp. rapa) | BC1F3 | CAAS-IVF, Beijing, China | S, R, MR | 2021 |
| B608       | Black mustard (Brassica nigra) | BC1F3 | CAAS-IVF, Beijing, China | S | 2021 |
| B611       | Red cabbage (Brassica oleracea L. var. capitata) Turnip (Brassica rapa L. ssp. rapa) | BC1F3 | CAAS-IVF, Beijing, China | S, R | 2021 |
| B612       | Red cabbage (Brassica oleracea L. var. capitata) Turnip (Brassica rapa L. ssp. rapa) | BC1F3 | CAAS-IVF, Beijing, China | S, R | 2021 |
| B613       | Red cabbage (Brassica oleracea L. var. capitata) Turnip (Brassica rapa L. ssp. rapa) | BC1F3 | CAAS-IVF, Beijing, China | S, R | 2021 |
| B614       | Red cabbage (Brassica oleracea L. var. capitata) Turnip (Brassica rapa L. ssp. rapa) | BC1F3 | CAAS-IVF, Beijing, China | S, R | 2021 |
| B621       | Red cabbage (Brassica oleracea L. var. capitata) Kohlrabi (Brassica oleracea L. var. caulorapa) | BC2 | CAAS-IVF, Beijing, China | S, R | 2021 |
| B831       | Broccoli (Brassica oleracea L. var. italica) Wild cabbage (Brassica macrocarpa Guss.) Rape (Brassica napus L.) | BC1 | CAAS-IVF, Beijing, China | S, R | 2021 |
| B832       | Broccoli (Brassica oleracea L. var. italica) Wild cabbage (Brassica macrocarpa Guss.) Rape (Brassica napus L.) | BC1 | CAAS-IVF, Beijing, China | S, R | 2021 |
| B908       | Broccoli (Brassica oleracea L. var. italica) Turnip (Brassica rapa L. ssp. rapa) Rape (Brassica napus L.) | F1 | CAAS-IVF, Beijing, China | S, R, HR | 2021 |
| B909       | Broccoli (Brassica oleracea L. var. italica) Turnip (Brassica rapa L. ssp. rapa) | F1 | CAAS-IVF, Beijing, China | S, R, HR | 2021 |
| B910       | Broccoli (Brassica oleracea L. var. italica) Turnip (Brassica rapa L. ssp. rapa) | F1 | CAAS-IVF, Beijing, China | S, R, HR | 2021 |
| B932       | Broccoli (Brassica oleracea L. var. italica) Turnip (Brassica rapa L. ssp. rapa) | F1 | CAAS-IVF, Beijing, China | S, R, HR | 2021 |
| B933       | Broccoli (Brassica oleracea L. var. italica) Turnip (Brassica rapa L. ssp. rapa) | F1 | CAAS-IVF, Beijing, China | S, R, HR | 2021 |
| B934       | Broccoli (Brassica oleracea L. var. italica) Turnip (Brassica rapa L. ssp. rapa) | F1 | CAAS-IVF, Beijing, China | S, R, HR | 2021 |
| B935       | Broccoli (Brassica oleracea L. var. italica) Turnip (Brassica rapa L. ssp. rapa) | F1 | CAAS-IVF, Beijing, China | S, R, HR | 2021 |
| B936       | Broccoli (Brassica oleracea L. var. italica) Turnip (Brassica rapa L. ssp. rapa) | BC1 | CAAS-IVF, Beijing, China | S, R, HR | 2021 |
| B1018      | Cabbage (Brassica oleracea L. var. capitata) Turnip (Brassica rapa L. ssp. rapa) | F1 | CAAS-IVF, Beijing, China | S, R, HR | 2021 |
| B1019      | Cabbage (Brassica oleracea L. var. capitata) Turnip (Brassica rapa L. ssp. rapa) | F1 | CAAS-IVF, Beijing, China | S, R, HR | 2021 |
| B1024      | Cabbage (Brassica oleracea L. var. capitata) Turnip (Brassica rapa L. ssp. rapa) | F1 | CAAS-IVF, Beijing, China | S, R, HR | 2021 |
| B1025      | Cabbage (Brassica oleracea L. var. capitata) Turnip (Brassica rapa L. ssp. rapa) | F1 | CAAS-IVF, Beijing, China | S, R, HR | 2021 |
| B1026      | Broccoli (Brassica oleracea L. var. italica) Rape (Brassica napus L.) | F1 | CAAS-IVF, Beijing, China | S, R, HR | 2021 |
| B1027      | Broccoli (Brassica oleracea L. var. italica) Rape (Brassica napus L.) | BC1 | CAAS-IVF, Beijing, China | S, R, HR | 2021 |

2.2. Molecular Markers and Identification of Clubroot Resistance Genes

Ten pairs of primers for clubroot resistance markers described in previous studies were used to identify CR genes in the experimental materials (Table 2). Among these primers, SC2930-T-FW/SC2930-RV and SC2930-Q-FW/SC2930-RV were linked to the clubroot resistance gene Crr3, KBrH129J18R was linked to Crr1, B50-C9-FW/-RV and B50-6R-FW/-RV were linked to Crr2, HC688-4-FW/-7-RV was linked to Crrk [18], OPC11-2S was linked to Crr3 and BRMS-088 and BRMS-096 were linked to the genes of Crr1 and Crr2, respectively [12,13,19].
Table 2. Information of selected specific CR markers.

| Primer Names | Loci   | Primer Sequences (5′-3′)                  | Product Size (bp) |
|--------------|--------|------------------------------------------|-------------------|
| C2930-T-FW   | CrA    | TAGACCTTTTTTCTCCTTTTCTACCT               | 800               |
| SC2930-R-FW  | CrA    | CAGACTAGCTTTTGTCTTTTCTGACG              | 800               |
| SC2930-RV    | CrA    | AAGGCAATGAAATACGGTC                   | 800               |
| KBrH129J18R-F| CrB    | AGAGCAGAGTGAAACAGAAGCT               | 254               |
| KBrH129J18R-R| CrB    | GTTCTCAGTTCAGTTTCTTGTGAG            | 194               |
| B50-C9-FW    | CrC    | GATTCATACCTTATCTCTTCAG                | 800               |
| B50-6R-FW    | CrC    | ATGCATTTTCGCTCAAC                      | 800               |
| B50-RV       | CrC    | CTCTATACATTTCTCTTCTGAGGTGGA           | 1000              |
| HC688-4-FW   | CrK    | ATATGCATTTTCGCTCAAC                    | 1000              |
| HC688-7-RV   | CrK    | ATATGCATTTTCGCTCAAC                    | 1000              |
| BRMS-088T    | CrR1   | TATCCGTTACTCTTTGCTTCTCTCAAC           | 263               |
| BRMS-088R    | CrR2   | ATCGCTTACTCTTTGCTTCTCTCAAC           | 220               |
| BRMS-096R    | CrR3   | TGAAGAAGGATGAAACGTGGTGTTGG           | 189               |
| OPC11-2ST    | CrR3   | GTAACTTGTGTCGACAGACGACAT             | 1300              |
| OPC11-2SR    | CrR3   | ATCTGCTCTAACTGAAACATGATG             | 1000              |

Genomic DNA was extracted from young leaves using the improved cetyltrimethylammonium bromide (CTAB) method [7,9], and the reagents for PCR were purchased from Vazyme Biotech Co., Ltd. (Virginia, USA). The PCR amplification was carried out in a 10.0 µL reaction mixture containing 2.0 µL of DNA template, 2.0 µL of buffer, 1.0 µL of primers and 5.0 µL of Taq Plus Master Mix. PCR products larger than 300 bp were identified using 1% agarose gel electrophoresis followed by visualization of the gel in an automatic gel imaging system. PCR products shorter than 300 bp were developed using 8.0% polyacrylamide gel electrophoresis and silver staining.

2.3. Pathogen Inoculation and Molecular Verification

Root gall samples from Chinese cabbage plants were collected from Xinye, Henan province, and the pathogen was identified and reported as race 4 [20–22]. Inoculation and resistance testing were performed using our previously described method [23]. DNA was extracted from the gall samples using the CTAB method, and PCR amplification and identification were carried out with the previously reported clubroot strain-specific primers (Table 3). The PCR system was the same as that used for the identification of clubroot resistance loci. The amplification protocol was as follows: 94 °C for 3.0 min (predenaturation); denaturation at 94 °C for 30.0 s, annealing at 55 °C for 30.0 s and extension at 72 °C for 45.0 s; a final extension at 72 °C for 7.0 min; and storage at 10 °C.

Table 3. The information of clubroot pathogen identification markers.

| Primer Names | Sequences (5′-3′) | Tm (°C) | Products (bp) |
|--------------|-------------------|---------|--------------|
| Actin1       | F: GGGACATCACCGCATACATCG  |
|              | R: ACTGCTCCGAATGGACATC    | 57      | 160          |
| Novel342-2   | F: CACCGGTATACCCCGGAAGAG  |
|              | R: CAACAGGACGGCGTGGAAGAG  | 58      | 666          |
| Novel407-2   | F: GTCTGTGTGTGCGGGGAAGAT  |
|              | R: GTCATAGGGTGTCGGAACGG   | 58      | 683          |
| PBRA_007750-2| F: ATCTGCTTGCAGTGCAGCTCT  |
|              | R: GAGTGTACAGGGTCTCGCAT   | 58      | 1034         |
| PBRA_008439-1| F: TCAGCGACCTAGCGACGAA    |
|              | R: TCAACATCCGAGATATGAC    | 58      | 651          |
| PBRA_009348-1| F: CACTGCTATCGTCTCCCTG    |
|              | R: CTCGCAATCTTTCGCTACGA   | 57      | 509          |
2.4. Staining and Observation

The susceptible broccoli cultivar B991 and the resistant cultivar “Yacui91” were selected for the hydroponic experiments. After germination, 24 seedlings of each variety were retained for observation. The infection process was carried out in a hydroponic solution system. When the broccoli seedlings produced one true leaf, they were moved to a centrifuge tube with the standard bacterial solution of $4 \times 10^7$ spores/mL. The system was put in an artificial climate incubator under the following conditions: 16.0 h of light at 25 °C, 8.0 h of dark at 20 °C with the relative humidity of 75.0%. At 0th, 7th and 14th days, the samples of broccoli root hairs and cortex were gathered and treated for infection observation, 3 individual plant distribution observations were selected at each time point. Firstly, broccoli root was placed in formal-acetic-alcohol (FAA) fixative for 24.0 h, and then FAA solution fixative was washed off with distilled water, stained in 0.5% Phloxine for 20.0 min and rinsed well with water. Finally, we conducted microscopic observations. Three replicates were selected for observation and imaging (n = 3).

2.5. Ploidy Detection by Flow Cytometry

Leaves were sampled from plants with five or more disease resistance genes, and broccoli (CC) and oilseed rape (AACC) were used as controls to establish the population for measuring the DNA levels. The corresponding peak value of the $G_0/G_1$ phase was adjusted to 200. We treated a 200-mg sample with 2.0 mL of Galbraith’s buffer, and a disposable blade was used to chop the tissue. After filtering through a 400-mesh filter, the liquid was added to a 2 mL centrifuge tube and centrifuged at 500 r/min for 3.0 min, then, the supernatant was discarded. Finally, PI dye solution was added to stain the nuclei, and the nuclei were visualized by a microscope [24–26].

3. Results

3.1. Diversity of Clubroot Resistance and Plant Ploidy

The results showed that seven clubroot resistance genes, namely, $CR_a$, $CR_b$, $CR_c$, $CR_k$, $Crr_1$, $Crr_2$ and $Crr_3$, were all well amplified in the test materials (Table 4) (Figure 1). Among these CR genes, four materials were detected in 2020, and $CR_a$ was found only in black mustard B1086. The CR gene $Crr_3$ was detected in all the materials except Porphyra yezoensis and black mustard. The other materials contained homozygous $Crr_2$ genes. In 2021, some varieties were detected with seven CR genes. Only the homozygous and heterozygous CR gene of $CR_k$ were detected in oilseed rape and the hybrids cross between cabbage and Chinese cabbage. In this study, $CR_a$ and $CR_b$ genes were widely detected in all the materials, but $CR_a$ was absent in two varieties of B571 and B582. Homozygous and heterozygous CR genes were all detected in the oilseed rape varieties “Huayouza62R” (B578), B1024, B1025 and B1026. The CR gene was found in Chinese cabbage, oilseed rape and turnip. The heterozygous $Crr_1$ gene was detected in “Huayouza62R”, turnip, “Yacui91” and broccoli hybrids. It is noteworthy that “Yacui91” and European mustard contained only $CR_b$ gene. The CR gene of $Crr_2$ was present in all the test materials except European mustard, “Yacui91” and broccoli. B613 and B614 were hybrids cross between cabbage and turnip without $CR_k$ gene. Homozygous and heterozygous CR genes could be detected in the other six varieties. Chinese cabbage named B608 was obtained from the National Germplasm Bank and harbored four CR genes. In our study, most of the materials contained two or more CR genes. The hybrids crossed between Chinese cabbage and “Huayouza62R” contained seven CR genes, the hybrids crossed between cabbage and turnip contained six CR genes, and the other materials contained five CR genes. The growth diagram of materials and their aggregated resistance genes were shown in Figure 2.
Table 4. Identification of 41 cruciferous genotypes by CR markers.

| Number | CRa | CRb | CRc | CRk | Crr1 | Crr2 | Crr3 |
|--------|-----|-----|-----|-----|------|------|------|
| B891   | /   | /   | /   | /   | R    | R    | R    |
| B991   | /   | /   | /   | /   | R    | R    | R    |
| B1007  | /   | /   | /   | /   | R    | /    | R    |
| B1081  | /   | /   | /   | /   | R    | R    | R    |
| B1082  | /   | /   | /   | /   | R    | R    | R    |
| B1083  | /   | /   | /   | /   | /    | /    | R    |
| B1084  | /   | /   | /   | /   | /    | /    | R    |
| B1086  | R   | /   | /   | /   | R    | /    | R    |
| B359   | S   | S   | /   | /   | /    | /    | /    |
| B366   | S   | S   | /   | R   | /    | S    | S    |
| B368   | S   | S   | R   | /   | /    | S    | S    |
| B369   | S   | S   | R   | /   | /    | S    | S    |
| B371   | /   | H   | /   | /   | /    | /    | /    |
| B578   | H/S | H/R | H/S | R   | H    | H/R  | S    |
| B581   | S   | S   | /   | /   | H    | H    | /    |
| B582   | /   | R   | /   | /   | /    | /    | /    |
| B606   | H/S | H   | S   | /   | /    | H/S  | R    |
| B607   | H/S | H   | S   | /   | /    | H    | S    |
| B608   | S   | H   | R   | /   | /    | S    | /    |
| B611   | H/S | H   | S   | /   | /    | S    | S    |
| B612   | H/R/S | H   | S   | /   | /    | H/R/S | /   |
| B613   | H/S | H   | R   | /   | S    | H/S  | S    |
| B614   | H/S | H/R/S | R   | /   | S    | H/R  | S    |
| B621   | S   | S   | /   | /   | S    | S    | S    |
| B831   | S   | S   | /   | /   | S    | S    | S    |
| B832   | S   | S   | /   | /   | S    | S    | S    |
| B908   | H/S | H/R/S | S   | /   | S    | /    | S    |
| B909   | H/R/S | H/R/S | S   | /   | S    | /    | S    |
| B910   | H/S | H/R/S | S   | /   | S    | R    | |
| B932   | H/S | S   | /   | /   | /    | /    | /    |
| B933   | S   | S   | /   | /   | /    | S    | R    |
| B934   | H/S | H/S  | /   | /   | /    | H/S  | R/S  | R    |
| B935   | H/R/S | H/S  | /   | /   | H/S  | H/R/S | R    |
| B936   | H/S | H/S  | /   | /   | S    | S    | R    |
| B1018  | H/S | H/S  | /   | /   | S    | /    | R    |
| B1019  | H/S | H/S  | /   | /   | S    | H    | R    |
| B1024  | R/S | H/S  | S   | S   | S    | H    | S    |
| B1025  | H/R/S | H/S  | S   | R/S  | S    | S    | S    |
| B1026  | S   | S   | S   | R   | S    | S    | R/S  |
| B1027  | S   | S   | /   | /   | S    | S    | R    |

Note: R indicates a homozygous disease resistance site, S indicates a homozygous susceptibility site and H indicates a heterozygous disease resistance site and/or an undetected disease resistance site.

As shown in Table 4 and Figure 2, morphological diversity and CR genes were found in these improved cruciferous germplasms. At the same time, the detection of various ploidy levels via DNA content measurement also verified the genetic information (Figure 3). As shown in Figure 3, there were six diploid materials in total, which were B606, B611, B831, B1019, B1024 and B1027. B606 and B611 were BC1F3 generations of red cabbage (B. oleracea L. var. capitata) × turnip (B. rapa L. ssp. rapa), B381 was the BC1 generation of broccoli (B. oleracea L. var. italica) × wild cabbage (B. macrocarpa Guss.) × rape (B. napus L.), B1024 was the F1 generation of choi sum (B. campestris L. ssp. chinensis var. utilis Tsen et Lee) × Chinese cabbage (B. pekinensis Rupr.) and B1027 was the BC1 generation of broccoli (B. oleracea L. var. italica) × rape (B. napus L.). B578 was a tetraploid oil rape variety. In addition, B368, B910 and B936 were identified as triploid, their fluorescence peaks were between 200 and 400, B910 and B936 belonged to F1 and BC1 was the generation of broccoli (B. oleracea L. var. italica) × turnip (B. rapa L. ssp. rapa).
**Figure 1.** Amplification results of different disease resistance genes in the experimental materials.

**Figure 2.** Species-aggregated multiple resistance sites. (a–l) letters represented experimental materials of B571, B578, B581, B582, B607, B608, B612, B910, B934, B1018, B1024, and B1026, respectively.
3.2. Disease Identification and Phylogenetic Analysis of CR Genes

In this study, six pairs of specific primers, namely, novel342-2, novel407-2, PBRA_007750-2, PBRA_008439-1, PBRA_009348-1 and actin1, amplified the target bands (Figure 4a), which might infer that the main strain collected in Xinye was a pathogen \[3,23\]. This result was consistent with previous reports and our identification. Moreover, “Huayouza62R” (B578), turnip (B581) and broccoli “Yacui91” exhibited high pathogen resistance (Figure 4c) (Table 1). The results of individual detection in hybrids of cabbage and turnip showed that 4 of 31 individual B612 plants were diseased, and 3 of 29 individual B613 plants were diseased, while only 1 of 58 individual B614 plants was diseased. The ratio of resistance to susceptibility was 59 to 4, and some traits were separated in the tested offspring, which was consistent with the results of molecular identification.

![Figure 3. Detection of ploidy by flow cytometry. The letters (a–h) represented experimental materials of B368, B130, B578, B831, B910, B936, B1024, and B1027, respectively.](image-url)
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In addition, we evaluated the evolutionary relationships of CR genes using the maximum likelihood method in MEGA7 software (Auckland, New Zealand). As shown in Figure 4b, the CR genes could be divided into three branches with a genetic similarity coefficient of 1.1; there were three genes in subgroup I, namely, CRa, CRb and Crr1 and CRa was the same as CRb reported in previous studies [1,3,18]. Subgroup II included only one CR gene, Crr3, suggesting that the relationship was different from the others. Crr3 and CRc were clustered together and belonged to subgroup III.

3.3. Comparisons of the Infection Processes of P. brassicae in Resistant and Susceptible Broccoli Cultivars

The root hairs of broccoli infected by P. brassicae were red, and the uninfected root hairs were colorless. We found that the root sections were initially not infected as shown in Figure 5a. Moreover, a large number of zoospores were observed in the susceptible broccoli roots, indicating that P. brassicae could easily infect the root hairs. As shown in Figure 5, some resting spores entered the cortical cells from the root hairs, producing many zoospores (Figure 5b). P. brassicae obviously infected the root cortical cells, as shown in Figure 5c, and a large number of sporangia were formed in the susceptible broccoli variety B991, inducing cortical infection. With the extension of infection time, the root hair infection rate on the 14th day was significantly higher than that on the 7th day. P. brassicae was found to rapidly infect susceptible broccoli on the 7th and 14th days.

As shown in Figure 5e,f, a different result was obtained. P. brassicae began to infect the root hairs of the resistant broccoli variety “Yacui91” on the 7th day, but fewer root hairs were infected. Although P. brassicae could invade the root cells of broccoli “Yacui91” on the 14th day, fewer zoospores and secondary zoospores were found in the root cells, and no sporangium formation was observed. At the same time, the root hair infection rate on the 14th day was significantly lower than that on the 7th day in resistant broccoli than in susceptible broccoli.
CRb which was also located upstream of CRk. It has been proposed that CRd is located in a 60-kb region between chromosome markers yau389 and yau376 on chromosome A03 [3]. Based on the physical location of the Crr3 linkage marker, CRd was also located upstream of Crr3; meanwhile CRa, CRb and CRbKato were located in the region between 23.6 Mb and 25.6 Mb of the Chinese cabbage A03 linkage group [30] in which CRb was identified in the area of 23.6–23.7 Mb and CRa was located between 24.2 Mb and 24.5 Mb (Figure 6). Therefore, this study provides necessary CR materials to help us understand the efficiency of these reported genes in cruciferous plants.

4. Discussion

According to previous studies, these CR genes are initially absent in B. oleracea plants and are usually different from those in Brassica pekinensis. After extensive screening of B. oleracea species, we found that most varieties of broccoli, cabbage, cauliflower, Chinese kale and kohlrabi were susceptible, and only a few materials were resistant and potentially useful in breeding. We also clarified the relationship between the disease resistance loci and the characteristics of different materials in each cruciferous family [10,27]. In this study, a number of cruciferous species were created and identified by major possible CR genes. To date, it is reported that both CRa and CRb are located on chromosome A03, and CRa is located on chromosome A03. Moreover, two RFLP molecular markers linked to the anti-root-tumor gene were obtained [19]. CRb and its linked markers TCR05 and TCR09 in disease-resistant Chinese cabbage and turnip ECD01 have been identified and it is predicted that they may be alleles or two closely linked disease resistance loci [5,28,29]. It was reported that CRb and CRa were two closely linked disease resistance loci [30]. In our study, only CRa, not CRb, was detected in broccoli, cabbage, rape, black mustard and some turnip and Chinese cabbage hybrids, and the results for these two loci were not completely consistent among the different materials, which might indicate gene preferences of CR genes occurred in the Brassica species [31]. The Crr3 gene has been fine-mapped through comparison with Arabidopsis thaliana [32], and the CRk gene was also found to be located on the Chinese cabbage A03 chromosome in 2008 [33]. It has been proposed that Crr3 and CRk may also be alleles or two closely linked disease resistance loci [3]. However, in this experiment, CRk was the only detected homozygous-susceptible locus in turnip and black mustard, while Crr3 was detected in all of these clubroot resistant materials. From this result, we inferred that CRk might be a more efficient major gene. It has been reported that CRd is located in a 60-kb region between chromosome markers yau389 and yau376 on chromosome A03 [3]. Based on the physical location of the Crr3 linkage marker, CRd was also located upstream of Crr3; meanwhile CRa, CRb and CRbKato were located in the region between 23.6 Mb and 25.6 Mb of the Chinese cabbage A03 linkage group [30] in which CRb was identified in the area of 23.6–23.7 Mb and CRa was located between 24.2 Mb and 24.5 Mb (Figure 6). Therefore, this study provides necessary CR materials to help us understand the efficiency of these reported genes in cruciferous plants.
European turnip and some oilseed rape varieties contained several important resistance genes that conferred vertical resistance to different physiological strains of \textit{P. brassicae}. By constructing a genetic map of a disease-resistant segregated Chinese cabbage population derived from the Siloga turnip, three genes, namely, \textit{Crr1}, \textit{Crr2} and \textit{Crr4}, were identified, and \textit{Crr1} was found to be located on chromosome A08 [12]. The homologous sequence information of \textit{Arabidopsis thaliana} was used for cloning, and \textit{Crr2} was located on chromosome A01, while \textit{Crr4} was located on chromosome A06. In our study, the experimental materials were all identified using published clubroot resistance markers, and it was found that most of the materials contained \textit{Crr1}, \textit{Crr2} and \textit{Crr3} resistance sites. The \textit{CrC} gene was detected in only four crops: broccoli and rape hybrids, rape, black mustard and cabbage and turnip hybrids. Ninety percent of the materials contained two or more disease resistance genes. The results of the molecular marker detection theoretically revealed the reasons for disease resistance.

In this a pioneering study, the infection process of \textit{P. brassicae} in resistant and susceptible broccoli under hydroponic conditions was elucidated, and the result might provide a rapid and reliable detection method for clubroot compared to traditional methods usually requiring more than 30 days. Moreover, the differences in \textit{P. brassicae} infection in resistant and susceptible broccoli suggested that, although the resting spores could be transformed to zoospores in root hairs of resistant broccoli plants, the sporangium could be formed, and the formation of more zoospores and its invasion were prevented after the 10th day in the hydroponic environment. However, in susceptible broccoli plants, \textit{P. brassicae} could achieve rapid infection and produce a large number of conidia and sporangia. The reason for this large difference might be closely related to the composition of the root exudates, which needs to be further studied. Therefore, this study also provided new evidence and approaches for studying the molecular mechanisms of clubroot resistance in \textit{Brassica} plants [34,35]. Finally, germplasm enhancement and identification of loci conferring resistance against domain pathotypes of \textit{P. brassicae} in broccoli and other cruciferous species would be beneficial for breeding disease-resistant varieties [10,27,36].

5. Conclusions

Based on 41 Brassicaceae plants, we have firstly created and then obtained six essential materials together with six or more CR genes currently lacking in \textit{Brassica} crops. Meanwhile, a total of 32% of all materials carried five CR genes, which is helpful for breeding disease-resistant varieties of broccoli and the other \textit{Brassica} crops. Here we provide these essential materials which will be beneficial to more in-depth research in \textit{Brassica} crops and international co-operation in the exchange of clubroot resistant materials for better global
breeding of cruciferous crops. Furthermore, in this study, we also explored and discussed the profiles of *P. brassicae* infecting the resistant and susceptible materials of broccoli, which provide new insight into the infection mechanism of clubroot in *Brassica* crops.

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