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ORIGINAL ARTICLE

Development of molecular markers based on *CRa* gene sequencing of different clubroot disease-resistant cultivars of Chinese cabbage

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Abstract

Background *CRa* is a key gene in Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) that confers resistance to *Plasmodiophora brassicae*. In order to efficiently screen the clubroot resistance (CR) gene *CRa* in breeding, two functional codominant markers of the *CRa* gene were developed.

Methods and results In this study, through comparing the *CRa* allele sequences in resistant and susceptible cultivars of Chinese cabbage, we found two insertion and deletion of sequence variations in the fourth exon between resistant and susceptible cultivars. Two functional codominant markers for *CRa* gene were obtained based on the variations, namely, *CRa*EX04-1 and *CRa*EX04-3. The lengths of the extended fragment of *CRa*EX04-1 marker were 321 bp and 186 bp in resistant and susceptible cultivars, respectively. In contrast, those of *CRa*EX04-3 were 704 bp and 413 bp, respectively. We verified the genetic stability between the developed markers and *CRa* gene using 57 Chinese cabbage cultivars with known resistance and two genetic populations. The results showed that the marker identification was completely consistent with the known phenotypes in 57 cultivars. The marker identification results followed the 3:1 of Mendel's first law in the F_2 population, and the 1:1 of Mendel's first law in the BC_1.

Conclusions *CRa*EX04-1 and *CRa*EX04-3 can be used as a practical molecular marker for breeding and germplasm resource creation of clubroot disease-resistant Chinese cabbage.

Keywords Chinese cabbage · Clubroot · *CRa* gene · Molecular marker

Introduction

Clubroot is a cruciferous crop disease caused by *Plasmodiophora brassicae*, acting as a threat to Brassica crop productivity all over the world, including many provinces in China [1, 2]. The acceleration in the cross-regional circulation of crop seeds, development of the logistics industry, and mechanization of agricultural production in China have accelerated the speed of clubroot disease in the major producing areas for Chinese cabbage, leading to the more prominent damage by the disease to Chinese cabbage [3, 4]. Clubroot has become one of the most serious diseases of cruciferous crops worldwide, and it must be addressed urgently [5]. Agricultural and biochemical methods can not completely control the occurrence of clubroot, and excessive use of pesticides can cause environmental pollution. Breeding CR cultivars is a safe and effective method to manage clubroot [6], but the frequent variation and regional differences in the physiological race of *Plasmodiophora brassicae* can decrease the resistance in CR cultivars. Therefore, we need to mine and use more genetic resources for disease resistance. The rapid developments in molecular biology and the completion of Chinese cabbage whole-genome sequencing gave rise to a new breeding model combining conventional breeding and molecular marker-assisted selection. Using molecular markers closely linked to clubroot disease resistance genes, plant disease resistance can be accurately predicted. Hence, the use of effective molecular markers to screen and breed CR cultivars of Chinese cabbage has become one of the most effective approaches for the management of this disease [7, 8].

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So far, at least 22 CR genes have been mapped on six chromosomes of Chinese cabbage. Among them, *Crr2* is located on chromosome A01 [9], *CRc* and *Rcr8* are located on chromosome A02 [9–11], *CRa*, *CRb*, *CRbkato*, *CRk*, *Crr3*, *PbBa3.1*, *PbBa3.3*, *PbBa8.1*, *QS_B3.1*, *Rcr1*, *Rcr2*, *Rcr4* and *CRd* genes are located on chromosome A03 [10–21], *CrrA5* and *Crr4* are located on chromosome A05 and A06, respectively [22, 23], and *Crr1*, *CRs*, *Rcr3,* and *Rcr9* are located on chromosome A08 [9, 11], with the *Crr1* gene, including two sites, *Crr1a* and *Crr1b* [20, 24]. Among these genes, *CRa*, CRb^{kato} and *Crr1* have been cloned [20, 25, 26]. In 2014, Hui et al. found that genes *CRb*, *CRa*, and CRb^{kato} are closely linked on chromosome A03 [27]. Later, Hatakeyama et al. discovered that *CRa* and CRb^{kato} were the same gene by comparing their sequence information [25]. The identification of these CR loci and genes provides the basis for clubroot disease resistance breeding in Chinese cabbage. Scholars have developed many molecular markers based on these CR genes and utilized them for breeding. However, some markers had no polymorphism in material screening or had no polymorphism between disease-resistant and disease-sensitive materials. Therefore, new molecular markers for different disease resistance sites are still needed.

In Japan, the *CRa* gene of Chinese cabbage has been widely used in clubroot disease resistance breeding [26]. However, the distribution of *CRa* resistance genes is still unclear in many countries and regions, limiting the application of this significant gene in breeding practices. This problem urgently requires the development of practical molecular markers to help study the distribution of this gene in cultivars in different regions and breed clubroot-resistant cruciferous crops.

In the present study, we have focused on (1) The development of functional gene markers using the differences in *CRa* sequencing among different clubroot disease-resistant cultivars of Chinese cabbage, (2) Comparative analysis with known molecular markers detection results, and (3) Verification of the genetic stability of the developed markers using 64 Chinese cabbage cultivars, F_2 population and BC_1 population. The developed markers can then be used for breeding Chinese cabbage resistant to clubroot.

Materials and methods

Plant materials

The 64 Chinese cabbage cultivars used in the experiment are commercial varieties, and the detailed information is shown in Table S1. F_2 Population having a total of 290 plants, was obtained by selfing of CRLimin (F_1), and BC_1 population having a total of 155 plants, was obtained by crossing the susceptible parent 13s93 (P_1) with the CRLimin (F_1).

The *P. brassicae* (pathotype 4 of Williams' system) used in all experiments was provided by the vegetable genetics breeding laboratory of Shenyang Agricultural University.

Preparation of resting spore suspension

P. brassicae resting spores were prepared as described by Zheng et al. [1]. Root galls were homogenized in 10% sucrose (wt/vol) using a blender. The slurry was filtered through four layers of gauze, after which the suspension was clarified by centrifugation at 4000 rpm for 10 min. The pellet was suspended in sterile distilled water and transferred to a new tube. It was then centrifuged at 4000 rpm for 12 min to obtain resting spores. Resting spores were adjusted to a concentration of 10^8 spores/mL in sterile distilled water and stored at 4 °C.

Artificial inoculation identification

The seeds were germinated for five days at room temperature on moistened filter paper. The plants were transplanted into an autoclaved potting medium in 7 cm × 7 cm plastic pots, at a density of one seedling per pot. Of resting-spore suspension (1.0×10^8 spores/mL), 2 mL was injected into each pot. Twelve seedlings of each material were inoculated, with the treatments replicated three times. The substrate was kept moist, the humidity was kept at 80–90%, and the greenhouse temperature was kept between 20 and 28 °C. The plants were evaluated for clubroot disease severity 45 days after the inoculation.

Identification of disease resistance in the field

The test site is located in Taibai, Shaanxi, China (34° 5′ 27″ N, 107° 18′ 40″ E), a severely affected area of clubroot disease. The most common pathotype of *P. brassicae* in that area is pathotype 4 of Williams' system. The test field was turned deeply before winter, the 750 kg/ha of ammonium bicarbonate and 750 kg/ha chemical fertilizer (containing 18% N, 5% P_2O_5, 22% K_2O) were applied as base fertilizers prior to sowing. After the ground preparation was completed, it was covered by the mulch with a row spacing of 60 cm (mulch width 70 cm). The plants were planted on the covered ridge surface with a spacing of 50 cm. The planting density was 34,500 plants per ha. During the whole growth period, topdressing was performed twice: Once during the rosette stage and again during the heading period of Chinese cabbage. Urea was applied at 150 kg/ha and 300 kg/ha for the two stages, respectively. Chinese cabbage plants were grown in the test field with normal management. The experiment was repeated thrice

with a plot area of 11 m^2 and a random arrangement of plots. When the Chinese cabbage was harvested, the clubroot disease severity was evaluated in the field.

Disease investigation and resistance grading standards

Disease classification standards for artificial inoculation: 0 = No swelling; 1 = Root swelling accounted for less than 20% of the total roots; 2 = The main root is enlarged, and the proportion of fibrous and lateral root swelling accounts for 20 to 50 percent; 3 = The main root swelling is obvious, and the proportion of fibrous and lateral root swelling accounts for 50 to 75 percent; 4 = The main root is swollen abnormally, the fibrous root is close to disappearing, the lateral root is severely swollen, adhering to the main root to form a tumor, and the swelling ratio reaches 75–100%. Disease resistance classification standard: Test materials with disease index (DI) ≤ 20 are highly resistant (HR), 20 < DI ≤ 40 are resistant (R), 40 < DI ≤ 60 are moderately resistant (MR), and 60 < DI ≤ 100 are susceptible (S) to clubroot. The DI was calculated using the formula [5]:

$$\mathrm{DI} = \frac{\sum \left[(\text{Class no.})(\text{No. of plants in each class})\right]}{(\text{Total no. of plants per sample})(\text{Maximum classes no.})} \times 100\%$$

Field disease classification standards: 0 = No galling; 1 = Swelling only occurs on the lateral roots, with swollen roots accounting for 1–25% of the total root system; 3 = Swelling occurs on the tap root, with the number of swollen lateral roots accounting for more than 25% of the total root system and the number of swollen roots accounting for 26–50% of the root system; 5 = Swollen roots accounting for 5 l-75% of the root system, including the tap root; 7 = Swollen roots accounting for more than 75% of the root system, including the tap root; 9 = Severe galls with a rotten root system. Disease resistance classification standard: Test materials with DI ≤ 11.11 are highly resistant (HR), 11.11 < DI ≤ 33.33 are resistant (R), 33.33 < DI ≤ 55.55 are moderately resistant (MR), 55.55 < DI ≤ 77.77 are susceptible (S), and 77.77 < DI ≤ 100 are highly susceptible (HS) to clubroot.

DNA extraction and polymerase chain reaction (PCR) amplification system

The total genomic DNA was extracted from the fresh leaves of plants, according to the cetyl trimethyl ammonium bromide (CTAB) method [28], and the resulting DNA concentrations were adjusted to 50 ng μL^{-1} with TE buffer (pH 8.0) for subsequent analyses. The polymorphism of markers was validated by the PCR reaction. A 20 µL PCR mixture contained 2.0 µL template DNA (50 ng μL^{-1}), 10.0 µL 2 × PCR Master Mix (Tiangen Biotech), 10 µmol L^{-1} of each forward and reverse primer 1.0 µL, and ddH_2O 6.0 µL. Thermal Cycler (BIOER, Zhejiang, China) was used to perform PCR amplification with the following procedures: first denaturation step at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, annealing (temperature set was based on primers, usually 55 °C) for 30 s, 72 °C for 30 s, the final extension step at 72 °C for 10 min and storage at 8 °C. The PCR products were resolved on a 2% agarose gel.

Primer designing and synthesis

The *CRa* gene sequence was retrieved from GenBank (accession number is AB751516) [26]. We used DNAMAN software for multiple sequence alignment and developed molecular markers based on the results of sequence alignment. The primers for specific amplification were designed using Premier 5 software based on the differences in the sequences of disease-resistant and susceptible materials (Table 1). Gene sequencing and synthesis of all the used primers was done by Shanghai Shenggong Biological Co. Ltd.

Data analysis

Data were analyzed using SPSS and EXCEL software. The number of disease-resistant and susceptible plants in F_2 and BC_1 populations and clearly visible bands after gel electrophoresis were counted, the band patterns of individual plants in the population were recorded, and the statistical results were used for further analysis. The Chi-square (χ^2) test of the separation ratio of phenotype and genotype was completed using the EXCEL and SPSS software. We used

Table 1 Sequence information of marker primers

Name of primer	Primer sequence	Length of PCR product
*CRa*EX04-1		
*CRa*EXON4-FW1	5′ACTGCCACTAATCTGCAAACAT3′	321 bp (Resistance) & 186 bp (Susceptible)
*CRa*EXON4-RV1	5’TGAGCATCCAGTGAGAAGCAA3′	
*CRa*EX04-3		
*CRa*EXON4-FW3	5′TACTCTCGAGGAATTGCTTCTCAC3′	704 bp (Resistance) & 413 bp (Susceptible)
*CRa*EXON4-RV3	5′TGTTGATATTGATCGGAAGGGC3′	

the χ^2 test ($P \leq 0.05$) to determine the most suitable separation ratio and conduct a fitness test.

Results

Molecular marker development

The sequence of the *CRa* gene was obtained from GenBank (accession number AB751516) [26] and Premier 5 software was used to design the specific amplification primer *CRa*EXON4-2 (5'TCAACTGCCACTAATCTG C3'; 5'TGT TGATATTGATCGGAAGG3') on the richly diverse fourth exon sequence. It was used to perform PCR tests on 20 cultivars with different disease resistance levels (Table S1). The results showed that *CRa*EXON4-2 had polymorphic bands between the resistant and susceptible materials. Gene fragments of about 1000 bp were observed in resistant materials, whereas two types of fragments, either of 850 bp (numbers 11, 16, 19) or of 570 bp (numbers 9, 12, 16, 17, 19, and 20), were observed in susceptible cultivars (Fig. S1). Three different gene fragments were cloned and sequenced from materials 1, 17, and 19. Sequencing results revealed their sizes to be 994 bp, 569 bp and 859 bp, respectively. The sequence alignment of the three fragments showed that they were allelic fragments, with a 93% similarity to the *CRa* sequence. Two large insertion or deletion sequence variants between the disease-resistant and susceptible materials were found in the sequencing results (Fig. 1). Two pairs of primers named *CRa*EX04-1 and *CRa*EX04-3 were designed based on sequence variations (Table 1).

Specificity verification of markers in 57 Chinese cabbage cultivars

57 of the 64 materials showing consistent results after field and artificial disease resistance identification were selected for this part of the test. The PCR amplification results (Fig. S2) showed that the marker *CRa*EX04-1 produced 321 bp fragments in disease-resistant cultivars homozygous for genetic locus, 186 bp fragments in susceptible cultivars, and both these two fragments were shown in the heterozygous disease-resistant cultivars. *CRa*EX04-3 produced 704 bp fragments in disease-resistant cultivars homozygous for genetic locus, 413 bp fragments in sensitive materials, and both fragments also appeared in the heterozygous disease-resistant cultivars. The agreement between the detection results of the two molecular markers and the disease resistance identification resulted in the 57 materials was 100%. Of which 1/57 were homozygous genotypes for disease resistance, 3/57 were susceptible genotypes, and others were disease resistant heterozygous genotypes (Table 2). The above results indicated that the two molecular markers have good specificity.

Detection of coincidence between CRaEX04 markers and known marker results

Two previously reported markers, B0902 [29] and GC1250 [26], linked to *CRb* and *CRa* gene, were used to detect the genotype of 10 plants in the segregating population. Marker B0902 can amplify 250 bp (Susceptible, S) and 160 bp (Resistance, R) specific fragments in plants (Fig. 2a). GC1250 can amplify 1250 bp (S) and 660 bp (R) specific fragments in plants (Fig. 2b). The fragment size amplified by the two *CRa*EX04 markers differs from the previously reported markers (Fig. 2c, d). The results of our two markers in identifying the genotypes of individual plants were largely consistent with those of B0902 and GC1205 (Fig. 2), which further confirmed the feasibility of the two new developed markers in this study.

Stability verification of functional markers in F_2 and BC_1 populations

In order to further verify the accuracy of the markers, we constructed F_2 and BC_1 populations for testing. Firstly, we used the artificial inoculation method to identify disease resistance in F_2 and BC_1 populations. Out of a total of 290 plants in the F_2 population, 206 were resistant, and 84 were susceptible. A χ^2 test (Table S2) indicated that this distribution followed the theoretical 3:1 segregation ratio ($\chi^2 = 2.18 < \chi^2_{0.05,1} = 3.84$). Out of a total of 155 plants in the BC_1 population, 83 were resistant, and 72 were susceptible. The χ^2 test (Table S2) indicated that this distribution followed the theoretical 1:1 segregation ratio ($\chi^2 = 0.78 < \chi^2_{0.05,1} = 3.84$). Subsequently, we analyzed resistance to clubroot disease in the F_2 population using markers. As a result, three genotypes were found in this population (Fig. S3a, b). The χ^2 test indicated 1 (RR): 2 (Rr): 1 (rr) separation ratio ($\chi^2 = 1.55 < \chi^2_{0.05,2} = 5.99$) (Table 3). Two genotypes were obtained by analyzing the BC_1 population using the markers (Fig. S3c, d). The χ^2 test indicated 1 (Rr):1 (rr) separation ratio ($\chi^2 = 0.78 < \chi^2_{0.05,1} = 3.84$) (Table 3). The above results show that a single dominant gene controls resistance to clubroot and our markers can be used to judge the genotype.

Discussion

CRa was the first genetically identified clubroot resistant locus, originally derived from the ECD02 turnip line of the European Clubroot Differential (ECD) series and introduced into Chinese cabbage [12]. Previous studies on the molecular

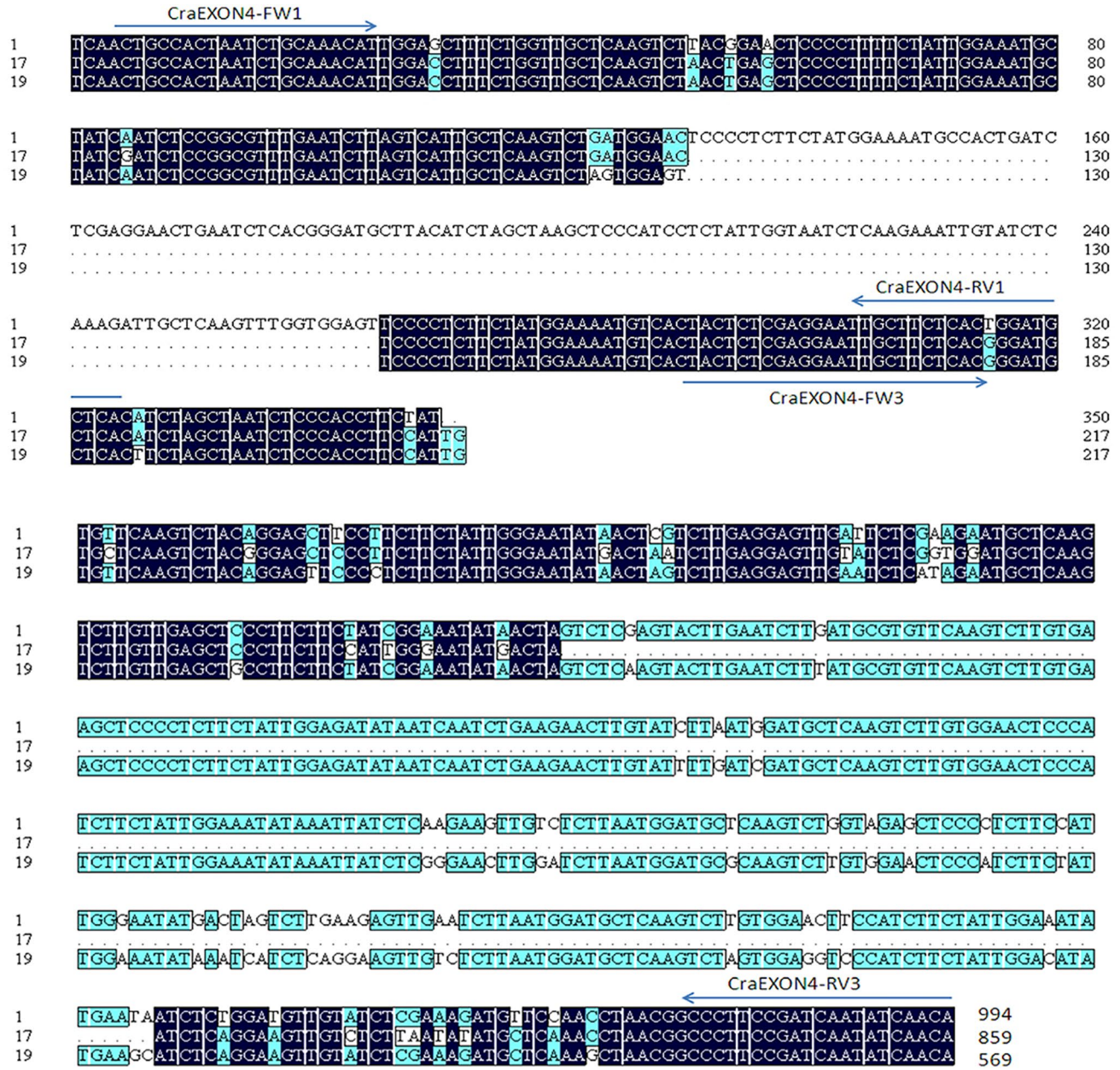

Fig. 1 The sequence comparison of the *CRa* exon4 in 3 Chinese cabbage cultivars and markers development

characteristics of the *CRa* gene have shown that it encodes a toll-interleukin-1-nucleotide binding site-leucine-rich repeat (TIR-NBS-LRR) protein and plant disease resistance can be triggered by specific recognition of microbial effectors by such NB-LRR receptors [26]. Therefore, it is of great significance to accurately detect the disease resistance gene in Chinese cabbage and other cruciferous crops. The utilization of molecular markers is a quick and easy way to detect the target gene. Developing molecular markers based on cloned disease resistance genes and using them to distinguish the target disease resistance can overcome some difficulties that faced in conventional breeding and improve breeding efficiency. Hence, we developed two markers of *CRa* gene by comparing the *CRa* allele sequences in resistant and susceptible materials of Chinese cabbage. They can detect the presence of *CRa* disease-resistant sites accurately. Ueno et al. [26] developed *CRaim* specific markers based on sequence differences, which could detect disease resistance genes and susceptibility genes, respectively. However, this labeling requires two simultaneous PCR reactions and high operating conditions. These problems could be avoided by using the two codominant markers developed by us. A single reaction is sufficient to detect the resistance gene and is easy to proceed with, making it more conducive to

Table 2 Evaluation of clubroot resistance and DNA markers analysis of Chinese cabbage materials

No	Resistance identification		EX04-1/bp		EX04-3/bp		No	Resistance identification		EX04-1/bp		EX04-3/bp	
	Field	Inoculation	321/R	186/S	704/R	413/S		Field	Inoculation	321/R	186/S	704/R	413/S
1	HR	HR	+	+	+	+	32	HR	R	+	+	+	+
2	HR	R	+	+	+	+	33	HR	MR	+	+	+	+
3	HR	HR	+	+	+	+	34	HR	HR	+	+	+	+
4	HR	HR	+	+	+	+	35	HR	HR	+	+	+	+
5	HR	HR	+	+	+	+	37	HR	R	+	+	+	+
6	HR	R	+	+	+	+	38	HR	R	+	+	+	+
7	HR	R	+	+	+	+	39	HR	R	+	+	+	+
8	HR	R	+	+	+	+	41	HR	R	+	+	+	+
9	HR	HR	+	+	+	+	42	HR	HR	+	+	+	+
10	HR	R	+	+	+	+	43	HR	HR	+	+	+	+
11	HR	HR	+	+	+	+	44	HR	R	+	+	+	+
12	HR	MR	+	+	+	+	45	HR	HR	+	+	+	+
13	HR	HR	+	+	+	+	46	HR	HR	+	+	+	+
14	HR	MR	+	+	+	+	47	HR	R	+	+	+	+
15	HR	R	+	+	+	+	48	HR	HR	+	+	+	+
16	HR	HR	+	−	+	−	49	HR	HR	+	+	+	+
19	HR	R	+	+	+	+	50	HR	HR	+	+	+	+
20	HR	HR	+	+	+	+	51	HR	HR	+	+	+	+
21	HR	HR	+	+	+	+	52	HR	R	+	+	+	+
22	S	S	−	+	−	+	53	HR	HR	+	+	+	+
23	HR	HR	+	+	+	+	54	HR	HR	+	+	+	+
24	HR	R	+	+	+	+	55	HR	HR	+	+	+	+
25	HR	R	+	+	+	+	56	HR	HR	+	+	+	+
26	HR	MR	+	+	+	+	58	HR	HR	+	+	+	+
27	HR	MR	+	+	+	+	59	HR	HR	+	+	+	+
28	HR	R	+	+	+	+	60	HR	HR	+	+	+	+
29	HR	R	+	+	+	+	63	HS	HS	−	+	−	+
30	HR	HR	+	+	+	+	64	S	S	−	+	−	+
31	HR	MR	+	+	+	+							

HR, R, MR, represent resistance to clubroot; S, HS represent no resistance to clubroot; +represents there are amplified

the accurate screening of materials containing the disease resistance gene. The functional marker for the *CRa* gene is derived from the gene itself. In comparison to the existing gene-linked marker [26, 29], it will not produce false-positive results due to gene recombination and has a higher selection efficiency for target genes.

The genetic mechanism of Chinese cabbage resistance to clubroot is complicated, with the inheritance of both qualitative and quantitative traits [6, 30, 31]. The disease resistance phenotype of clubroot is controlled not only by a single gene but also by the strong pathogenicity, diverse pathogenic types, and the complex population structure of *Plasmodiophora brassicae,* which is why the identification of disease resistance is greatly affected by the external environment [32–35]. This led to a certain deviation between the phenotype identification results of clubroot disease in the F_2 population and the molecular marker detection results. However, when 57 varieties with the same artificial and field clubroot resistance identification results were screened out from the 64 commercial varieties for molecular marker testing, the consistency of the results reached 100%. The results of markers identification in the F_2 population showed that among the 290 plants, 70 plants were homozygous for disease-resistant genotype (RR), 155 plants were heterozygous for disease-resistant genotype (Rr), and 65 plants were homozygous for disease-susceptible genotype (rr). The χ^2 test showed that the ratio of resistant to susceptible plants was 3:1. As expected, the disease-resistant site was found to be a dominant single-gene disease-resistant site. In addition, the marker detection results are in line with that of the known *CRa* and *CRb* gene-linked markers (*CRb* and *CRa* gene are closely linked on the chromosome [27]). This

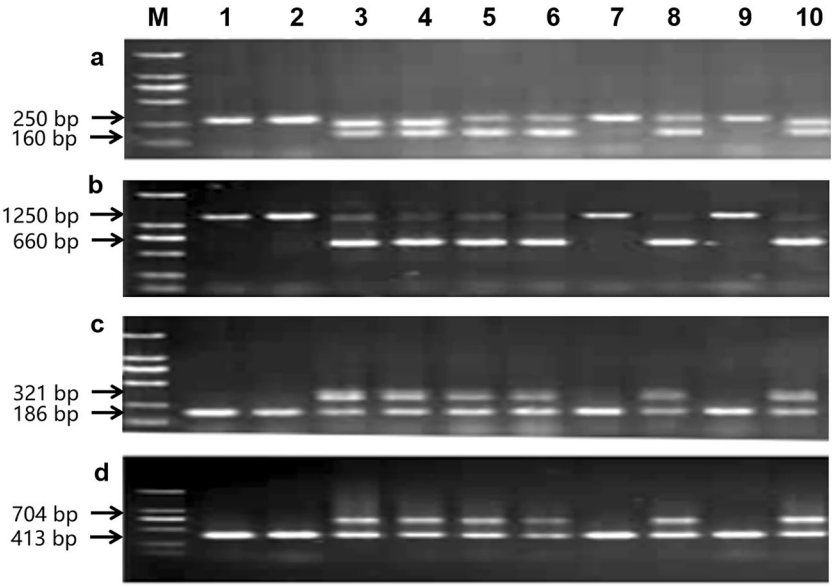

Fig. 2 Amplification results for development markers and known markers. a-d are the amplification results of B0902, GC1250, *CRa*EX04-1 and *CRa*EX04-3, respectively. 1–10 are the ten Chinese cabbage cultivars selected in Table S1

Table 3 Verification of resistance to clubroot disease in F_2 and BC_1 population by markers

Population	Total number of plants	Number of plants with different genotypes			Separation ratio	χ^2 value
		RR	Rr	rr		
F_2	290	70	155	65	1.07:2.39:1	1.55
BC_1	155	0	83	72	1.15:1	0.78

RR plants with homozygous disease resistance sites, *Rr* plants with heterozygous disease resistance sites, *rr* plants with homozygous susceptible sites

proves that the two markers developed by us can help in the screening of materials containing the *CRa* gene and eliminate the need for complicated artificial phenotyping. The deviation of phenotype and genotype observed during the test indicates that the resistance of a single CR gene to clubroot disease is insufficient. Hence, the integration of multiple resistance genes is an important direction for future clubroot disease resistance breeding.

Conclusion

In conclusion, two molecular markers CRaEX04-1 and CRaEX04-3 were developed in this work and both of them are functional molecular markers for the clubroot disease resistance gene, *CRa*. They have codominant characteristics that can distinguish the three genotypes of *CRa* gene in plants, namely, disease-resistant homozygotes, disease-resistant heterozygotes, and susceptible homozygotes at the same time. The test results showed that two molecular markers could effectively identify the presence of *CRa* gene in Chinese cabbage. They can be used as a practical molecular marker for breeding and germplasm resource creation of clubroot-resistant Chinese cabbage.

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Author contributions The first draft of the manuscript was written by TL. The main experiments were conducted and data collection and analysis were performed by NL. NL and TL contributed equally to this work. The artificial inoculation identification experiment was assisted by NL and JM. The experiments were designed by MH. The experimental materials were provided by LZ. All authors read, edited, and approved the manuscript.

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Declarations

Competing interests The authors have no competing interests to declare that are relevant to the content of this article.

Ethics approval Not applicable.

Data availability All data supporting the conclusions of this article are provided within the article (and its supplementary information files).

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