Identification and Characterization of Crr1a, a Gene for Resistance to Clubroot Disease (Plasmodiophora brassicae Woronin) in Brassica rapa L.

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Abstract

Clubroot disease, caused by the obligate biotrophic protist Plasmodiophora brassicae Woronin, is one of the most economically important diseases of Brassica crops in the world. Although many clubroot resistance (CR) loci have been identified through genetic analysis and QTL mapping, the molecular mechanisms of defense responses against P. brassicae remain unknown. Fine mapping of the Crr1 locus, which was originally identified as a single locus, revealed that it comprises two gene loci, Crr1a and Crr1b. Here we report the map-based cloning and characterization of Crr1a, which confers resistance to clubroot in Brassica rapa. Crr1aG004, cloned from the resistant line G004, encodes a Toll-Interleukin-1 receptor/nucleotide-binding site/leucine-rich repeat (TIR-NB-LRR) protein expressed in the stele and cortex of hypocotyl and roots, where secondary infection of the pathogen occurs, but not in root hairs, where primary infection occurs. Gain-of-function analysis proved that Crr1aG004 alone conferred resistance to isolate Ano-01 in susceptible Arabidopsis and B. rapa. In comparison, the susceptible allele Crr1aG9750 encodes a truncated NB-LRR protein, which lacked more than half of the TIR domain on account of the insertion of a solo-long terminal repeat (LTR) in exon 1 and included several substitutions and insertion-deletions in the LRR domain. This study provides a basis for further molecular analysis of defense mechanisms against P. brassicae and will contribute to the breeding of resistant cultivars of Brassica vegetables by marker-assisted selection. Data deposition The sequence reported in this paper has been deposited in the GenBank database (accession no. AB605024).

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Introduction

Clubroot is one of the most serious diseases of cruciferous crops in the world. Plasmodiophora brassicae, the causal agent of this disease, is not a fungus but a member of the phylum Cercozoa in the kingdom Rhizaria [1,2,3]. The first record of clubroot in Japan dates from 1892 in cabbage and 1898 in Chinese cabbage [4], and the disease is now considered a major problem in cabbage and Chinese cabbage production in Japan and Korea [5]. The life cycle of P. brassicae is divided into two phases: a primary phase occurring in the root hairs and a secondary phase occurring in the stele and cortex of the hypocotyl and roots [4]. During the secondary phase, secondary plasmodial induced abnormal tissue proliferation of infected roots, leading to the formation of galls (clubs). These symptoms prevent the uptake of water and nutrients, stunting the infected plants and severely reducing crop yield and quality [6]. The primary phase has been observed in both susceptible and resistant plants. In the secondary phase, the development of the plasmodia is quantitatively reduced or delayed in resistant plants [7,8,9]. Because the resting spores released from decayed clubs can survive for many years in soil, agricultural practices such as liming and crop rotation are insufficient to keep crops healthy. In addition, reducing the use of agrochemicals is preferred for the production of vegetables. Therefore, the breeding of resistant cultivars is one of the most efficient ways to control clubroot.

European fodder turnips (Brassica rapa) were identified as sources of resistance and have been used to transfer their clubroot resistance (CR) genes into Chinese cabbage, oilseed rape, and Brassica oleracea [5,10]. The CR trait of European turnip cultivars such as Siloga, Gelria R, and Debra was considered at first to be controlled by a single dominant gene [11]. Yet after the release of more than 50 CR F1 cultivars of Chinese cabbage so far, the breakdown of resistance, caused by variation in the pathogenicity of P. brassicae, has been reported only recently [12,13]. Several differential tester sets have been used to study the interaction between P. brassicae and hosts [12,13,14,15,16]. Four pathotypes (groups 1 to 4) were identified in Japanese field isolates through the use of two commercial CR F1 cultivars of Chinese cabbage [12,13]. But since the number and identity of resistance genes in the tester sets are unknown [10], information on the performance or pathotype specificity of CR genes remains limited.
Genetic analysis and quantitative trait locus (QTL) mapping studies have identified at least 8 CR loci in B. rapa, 22 QTLs in B. oleracea, and 16 QTLs in Brassica napus [10,17]. In B. rapa, Crr1 and Crr2 were identified on chromosomes A08 and A01, respectively [10,19]. These two loci were detected by using two P. brassicae isolates, the mild Ano-01 and the more virulent Wakayama-01. Crr1 was necessary for the resistance to both isolates, but plants having Crr1 alone were susceptible to Wakayama-01. Crr2, which by itself does not show any effect against either isolate, was necessary for resistance to Wakayama-01 in a common pathway with Crr1. Therefore, Crr1 may play a role in a common pathway of resistance, and Crr2 may be a modifier locus for the resistance expressed by Crr1 [19]. Four CR loci–Crra, Crrb, Crrk, and Crrc–were identified. A BLAST search revealed that the predicted Crr1G004 protein showed similarity to Arabidopsis Ty1–copia LTRs bordered by 6 bp of target site duplication (TSD) (Fig. S3).

Alleles

Comparison of the genomic DNA and cDNA sequences revealed that Crr1G004 consists of 4 exons and 3 introns. Crr1G004 encodes a putative 1223-aa protein consisting of an N-terminal Toll-Interleukin-1 receptor (TIR) domain, a nucleotide-binding site (NB) domain, and leucine-rich repeats (LRRs) at the C-terminus of the NB domain (Fig. 3). Conserved motifs characteristic of the NB domain–the P-loop, kinase-2, RNBS-B, GLPL, and MHDV domains–were also identified [27]. In the LRR region, at least 11 imperfect repeats with lengths of 22 to 24 aa were identified. A BLAST search revealed that the predicted Crr1G004 protein showed similarity to A5g11250 (50% identity), which is a TIR-NB-LRR-type R gene in the TNL-G subgroup [27]. Among known R genes, the predicted Crr1G004 protein showed moderate similarity to Arabidopsis RPP1s (55% identity), which confer resistance to the biotrophic oomycete Peronospora parasitica (downy mildew) [28].

Structural Differences between Resistant and Susceptible Alleles

We determined the nucleotide sequence between markers B355H7 and B359C3 in the susceptible A9709 (Fig. 1) and compared it with that in the resistant G004. Crr1G004 had three large insertions: a 357-bp insertion 37 bp downstream of the start codon, and 333- and 4982-bp insertions in exon 4 (Fig. 4). The 4982-bp insertion, 157 bp upstream of the termination codon of Crr1G004, showed high similarity to a Ty1-copia long terminal repeat (LTR) retrotransposon comprising two identical 171-bp LTRs bordered by 6 bp of target site duplication (TSD) (Fig. S3).
Interestingly, 5’- and 3’-RACE analysis placed the 5’ end of Crr1aA9709 25 bp downstream of the 3’ end of the 357-bp insertion (Fig. 4) and the 3’ end in the first LTR region of the retrotransposon (Fig. S3). Translation from the first available in-frame start codon would yield an NB-LRR protein lacking 80 residues of the TIR domain and 49 residues of the C-terminal domain (Fig. 3). Comparison of the predicted proteins of both alleles showed that the remaining TIR and NB domains were well conserved, but the LRR domain was highly variable (56% identity; Fig. 3). The LRR domain of the predicted Crr1aA9709 protein included 50 substitutions, 2 deletions, and insertions of 2 and 111 residues relative to the predicted Crr1aG004 protein.

We found a highly homologous sequence (Bra020861) in the reference B. rapa genome sequence derived from a clubroot-susceptible line, Chiifu-401 [29], and considered that it is an allele of Crr1a. Comparison of the nucleotide sequences between Crr1aA9709 and Crr1aChiifu-401 revealed that the sequences between markers B355H7 and B359C3 were almost identical except in the insertion in exon 1. Interestingly, large insertions in exons 1 and 4 were also found in Crr1aChiifu-401, both with high similarity to the LTR retrotransposon (Fig. 4, Fig. S3). Although the sequence similarity and domain order suggest that the insertion in exon 1 of Crr1aChiifu-401 is a Ty1-copia LTR retrotransposon, it is not probably an intact retrotransposon, because it has no start codon and it has an inverted repeat of LTRs at the 5’-end. Because the 357-bp insertion in Crr1aA9709 showed high similarity to the LTR region at the 5’-end of the insertion in Crr1aChiifu-401 and TSDs were well conserved between both alleles (Fig. 4), it is likely that the 357-bp insertion is a solo-LTR.

**Expression Analysis of Crr1G004**

Semi-quantitative RT-PCR revealed Crr1aG004 transcripts in roots and leaves of the resistant R4-8-1 (see “Experimental procedures”) and the susceptible A9709, more so in the former (Fig. 5A). We examined the expression of Crr1aG004 in transgenic Arabidopsis carrying a Crr1aG004 promoter::GUS reporter gene.
construct. In 12-day-old seedlings, the construct was expressed in the stele and cortex of the primary root and hypocotyl, the vascular bundles of the cotyledons and leaves, and at the center of rosettes in a region corresponding to the shoot apical meristem (Fig. 5B–D). No expression was detected in root hairs, the root cap, or the adjacent elongation zone (Fig. 5B–D).

In the course of cloning the full-length cDNAs by RT-PCR, we obtained two clones, each containing a cryptic intron, one in exon 2 and the other in exon 4. This result indicates that *Crr1aG004* is alternatively spliced like other TIR-NB-LRR–type R genes [30,31]. RT-PCR with primers flanking introns 2 and 3 produced faint bands in addition to the major band. We cloned and sequenced the PCR products and, in addition to the regular transcript with introns spliced out (RT-a and RT-d in Fig. 5E), obtained a longer transcript with a retained intron 2 (RT-b). In addition, the shorter PCR product obtained with primers flanking introns 2 and 3 represented transcripts in which a cryptic intron within exon 2 or exon 4 was spliced out (RT-c and RT-e, respectively). No PCR products with retained introns 1 and 3 were obtained. Because the retained and cryptic introns gave rise to in-frame stop codons or a frame shift, all three alternative transcripts encode truncated TIR-NB proteins.

**Characterization of Transgenic B. rapa Plants Expressing Crr1a cDNA**

To assess the ability of the *Crr1aG004* allele to confer resistance in susceptible *B. rapa*, we inserted the *Crr1aG004* promoter::*Crr1aG004* cDNA construct into a susceptible cultivar of *B. rapa*. T1 seeds of 1.5 independent transgenic plants (T0) were tested for resistance to Ano-01. Five of the T0 lines produced resistant T1 plants (DI = 0 or 1; Table 1, Fig. S4). Other T1 plants with the transgene showed severe (DI = 3.0) or moderate swelling (DI = 2.5) on the main roots. Because *Crr1aG004* was expressed in both roots and leaves, we analyzed the expression of the transgene in leaves of resistant and susceptible T1 plants derived from two resistant T0 lines. Expression of the transgene in the resistant plants was 2 to 3 times that in the susceptible plants (Fig. 6). Similar results were observed in 2 independent tests. These results indicate that *Crr1aG004* functions in susceptible *B. rapa* and dose-dependently confers resistance to Ano-01.

**Discussion**

The *Crl* locus was originally identified as a single locus for resistance to clubroot isolate Ano-01 [19]. Fine mapping of this
locus revealed two gene loci [3], Crr1, with a major effect, and Crr1b, with a minor effect. We have now cloned and characterized Crr1aG004. Recent findings show that a pair of NB-LRR genes function together in disease resistance [32]. In contrast, our finding that Crr1aG004 alone could confer resistance to Ano-01 in transgenic Arabidopsis and B. rapa suggests that Crr1b is not required for Crr1aG004-mediated resistance.

Crr1aG004 encodes the TIR-NB-LRR class of R protein (Fig. 3), which confers resistance to viral, fungal, and oomycete pathogens. Resistance to the obligate biotroph protist P. brassicae also might be mediated via a gene-for-gene interaction, called effector-triggered immunity [33]. Expression of Crr1aG004 was detected in the stele and cortex of hypocotyl and root, where secondary infection occurs, but not in root hairs, where primary infection occurs (Fig. 5B–D). These results are consistent with previous histological findings of the incompatible interaction [7,8,9,10]. Because primary infection occurs in both resistant and susceptible plants [10], this phase is not likely to be associated with resistance.

Figure 3. Alignment of deduced amino acid sequences of Crr1a between resistant G004 and susceptible A9709 alleles. Asterisks, identical amino acid residues; dashed lines, gaps for alignment. Blue, TIR domain; green, NB domain; red, LRR domain.
Tanaka et al. [9] reported that the plasmodia remained immature with a small number of nuclei and did not form resting spores even by 40 days after inoculation of Kukai 70, a CR cultivar of Chinese cabbage. Similar findings were also reported in resistant radish cultivars and accesses of Arabidopsis [7,8,9]. Therefore, Crr1aG004 may inhibit plasmodial development during the secondary infection phase.

Gain-of-function analysis proved that Crr1aG004 alone confers resistance to Ano-01 in susceptible Arabidopsis and B. rapa. Resistant and susceptible T1 plants of B. rapa segregated, with the level of resistance dependent on the expression level of the transgene (Fig. 6). It is likely that T1 plants homozygous for the transgene were resistant and plants heterozygous were susceptible. Our unpublished data suggest that Crr1 is incompletely dominant, because genetic analysis of an F2 population derived from a cross between G004 and A9709 showed that a heterozygous Crr1 locus was insufficient for complete resistance to Ano-01. Our results here suggest that a threshold level of Crr1a expression is required for complete resistance, and the level of expression may explain the incomplete dominance of Crr1. However, we cannot rule out the possibility that a low level of susceptible Crr1a protein acts as a dominant-negative regulator of Crr1aG004 in heterozygous plants. In the case of the tobacco N gene, a member of the TIR-NB-LRR class of R genes, loss-of-function alleles caused by TIR deletion or point mutations in TIR interfered with the wild-type N function in heterozygous plants [34]. In Arabidopsis, all T2 plants with the transgene showed resistance, and Crr1aG004 behaved as a dominant resistance gene (Fig. 2). This different behavior may be due to the difference in inoculation methods between Arabidopsis and B. rapa. The level of expression of the transgene in heterozygous plants might be high enough to confer resistance in to lower concentration of resting spores.

The genomic regions of B. rapa adjacent to Crr1, Crr2, and Crr6 are syntenic with Arabidopsis chromosome 4 [19,23,25]. Therefore, these CR loci may have been derived from the same region of the ancestral genome, and triplicated and dispersed among 3 chromosomes in B. rapa during the evolution of the Brassica genome [5]. The genomic region around Crr3 is syntenic with Arabidopsis chromosome 3, and this locus is thought to have a different origin from Crr1, Crr2, and Crr6 [25]. Here, however, we found that Crr1aG004 showed the highest similarity to At5g11250 on Arabidopsis chromosome 5. Because two additive QTLs controlling partial resistance to clubroot in Arabidopsis accession Bur-0 have been identified on chromosome 5 and one of them co-localized with several clusters of resistance genes [35], either locus might be a functional ortholog of Crr1a and the ancestor of CR genes. Sequence information on Crr1aG004 will accelerate the cloning of other CR genes identified in Brassica and Arabidopsis, and may clarify the evolution of CR genes. In fact, a BLASTP search of the B. rapa genome sequence revealed a large number of predicted proteins with partial similarity to Crr1aG004, some near markers linked to other, previously reported CR loci (data not shown).

The susceptible Crr1aA9709 allele had the solo-LTR in exon 1 and appeared to generate a truncated NB-LRR protein lacking more than half of the TIR domain (Fig. 3). The TIR domain of the NB-LRR protein plays an important role in the induction of defense responses or in recognition specificity for the pathogen effector [36,37,38]. Therefore, it is likely that deletion of the TIR domain abolishes the Crr1aA9709 function and results in susceptibility. The large LTR retrotransposon-like insertion in exon 1 was also found in the susceptible Crr1aA9709 allele, and the insertion site was conserved between Crr1aA9709 and Crr1aChiifu-401. These results suggest that both susceptible alleles are derived from the same retrotransposon insertion event, which causes loss of resistance, and that this retrotransposon has a role in the differentiation of CR genes. However, this insertion is not the sole cause of susceptibility alleles in B. rapa, because PCR analysis using primers flanking this insertion revealed that 18 of 24 non-CR cultivars of Chinese cabbage and turnips tested did not have such an insertion (data not shown). Furthermore, since the LRR domain plays an important role in recognition specificity [37], we cannot exclude the possibility that indels or substitutions in the LRR domain result in susceptibility.

Crr1aChiifu-401 was alternatively spliced (Fig. 5E). Alternative splicing is a common feature of NB-LRR-type R genes. Although its
The biological role in the function of R genes is still unknown, transcript variants of tobacco N and Arabidopsis RPS4 are required for complete resistance, and the expression of the alternative transcripts is induced during defense responses [30,31]. Our transgenic plants with the full-length Crr1 cDNA, which did not produce truncated transcripts by alternative splicing, were resistant to Ano-01 (Fig. 2, 6); this result suggests that transcript variants are not necessary for Crr1G004 function. A similar finding was also reported in relation to the flax rust resistance gene L5 [39].

An effective measure to increase the durability of resistance is the pyramiding of 3 or more CR loci in a single cultivar by marker-assisted selection [5]. Our results will enable the development of Crr1a-specific or closely linked markers that will improve the efficiency of marker-assisted selection and avoid the introduction of undesirable traits into improved cultivars by linkage drag [40]. Furthermore, knowledge of the pathotype specificity of CR genes is important. Crr1aG004 was effective against pathotypes of groups 2 and 4, but not groups 1 and 3 (Fig. S2). The CR line PL9, with both Crr1 and Crr2, is effective against isolate No. 5 of group 1 [41], and Wakayama-01 distinguished groups 1 and 2 [18], indicating that Crr2 is useful for resistance to the more virulent isolates. Because Crr1 comprises Crr1a and Crr1b, further analysis of the relationships among Crr1a, Crr1b, and Crr2 in resistance to different pathotypes is necessary. Recently, Kato et al. [41] showed that CRb was effective against isolates of group 3. The pyramiding of Crr1aG004 and/or Crr1b, Crr2, and CRb could confer resistance to most isolates currently present in Japan. In this study, we used field isolates, which are regarded as heterogenic; and multi-pathogenic races have been found in a single field [42,43]. Single-spore-derived isolates (SSIs) are considered to be most valuable for the genetic study of resistance and virulence surveys of pathogens [43]. The availability of single CR genes and SSIs of P. brassicae will allow us to differentiate pathogenicity and pathogen-host interactions more precisely.

**Experimental Procedures**

**Plant Materials**

A clubroot-resistant (CR) doubled-haploid (DH) line, G004, with Crr1 and Crr2, and a susceptible DH line, A9709, were used as parents for the F2 population [19]. To develop a CR inbred line with Crr1 and Crr2 in the A9709 background, we selfed one of these CR F2 plants to generate a CR F3 plant, and backcrossed that line three times to A9709 to generate BC3F3 plants. During this process, SSR markers linked to Crr1 and Crr2, and CRb could confer resistance to most isolates currently present in Japan. In this study, we used field isolates, which are regarded as heterogenic; and multi-pathogenic races have been found in a single field [42,43]. Single-spore-derived isolates (SSIs) are considered to be most valuable for the genetic study of resistance and virulence surveys of pathogens [43]. The availability of single CR genes and SSIs of P. brassicae will allow us to differentiate pathogenicity and pathogen-host interactions more precisely.

**Test for Clubroot Resistance**

The P. brassicae field isolates Ano-01, Wakayama-01, and Nos. 5, 7, 9, and 14 were used [12,13,18] to test clubroot resistance in A. thaliana according to Jubault et al. [35] with modifications; T2 seeds were plated on MS medium containing kanamycin to select plants carrying the transgene. Ten-day-old kanamycin-resistant T2 plants were transplanted into soil and inoculated by the injection of 2–4 mL of a resting-spore suspension (1.0–1.5×107 spores/mL) into the soil near the roots. Inoculated plants were grown in a controlled environment under 14-h light/10-h dark at 23/18°C. Resistance responses were evaluated 3 to 4 weeks after inoculation, and the disease index (DI) was scored on a scale of 0 to 3 (Fig. S5). The mean DI of each T1 line was expressed as the mean of two or
three clubroot tests (9 T2 seedlings per test) on different dates. Clubroot tests of B. rapa were carried out by the insertion method [11,12]. Each of 2 or 3 tests, performed on different dates, used 8 T1 seedlings derived from an independent transgenic B. rapa (T0).

We determined whether the T1 plants had the transgene or not by PCR analysis before evaluating resistance. Root symptoms were graded as: 0, no symptoms; 1, very slight swelling on main roots; 2, a small gall on main roots; 2.5, moderate swelling on main roots; 3, severe swelling on main roots (Fig. S4).

Map-based Cloning of Crr1

A BAC library constructed from the resistant G004 was screened with markers BSA2, AT27, and BSA7 as anchors, and a BAC contig covering 0.6 cM between BSA2 and BSA7 was assembled [3]. We selected F2 plants in which recombination occurred in the interval between markers developed from BAC-end sequences. F3 plants derived by selfing of the selected F2 plants were tested for resistance to Ano-01. To sequence the candidate region for Crr1a, we shotgun-sequenced BAC clones, 355H7 and 208F8. Sequences were assembled with Sequencher v. 2 software (Hitachi). ORFs were predicted with Genetyx v. 10 software (Genetyx).

Vector Construction and Transformation

A 3.7-kb coding sequence of Crr1aG004 was amplified from first-strand cDNA synthesized from root poly(A) + RNA. The Lactuca sativa Ubiquitin (LsUbi) promoter::Crr1aG004 and Crr1aG004 promoter::Crr1aG004 constructs were generated from the pUC198AAUGU plasmid [44], in which the 1.9-kb LsUbi promoter, the GUS gene, and the 0.6-kb LsUbi terminator were inserted in a modified pUC19 vector, pUC198AA [44]. The amplified Crr1aG004 cDNA replaced GUS between the LsUbi promoter and terminator in pUC198AAUGU to generate the LsUbi promoter::Crr1aG004 construct. A 2.5-kb 5′-upstream sequence of Crr1aG004 replaced the LsUbi promoter to generate the Crr1aG004 promoter::Crr1aG004 construct. The gene cassettes were ligated into the plant binary vector pZK3B [45]. These constructs were transformed into Arabidopsis Col-0 using Agrobacterium tumefaciens strain GV3101 by the floral dip method [46]. For B. rapa transformation, a commercial F1 cultivar, Osome (Takii Seed Co.), was used as the recipient, and hypocotyl explants were transformed with A. tumefaciens strain GV3101 as described [47]. Primer sequences used for vector construction are listed in Table S1.

GUS Assay Construct and Histochemical Staining

The 2.5-kb 5′-upstream sequence of Crr1aG004 replaced the LsUbi promoter of pUC198AAUGU to generate the Crr1aG004 promoter::GUS construct. The resulting construct was introduced into Arabidopsis Col-0, and T2 plants were used in the reporter gene assay. The GUS assay was carried out according to Ariizumi et al. [48].

RT-PCR and RACE Analysis

R4-8-1, A9709, and Col-0 plants were inoculated with Ano-01. Total RNA was extracted with a TRIzol Plus RNA Purification Kit (Invitrogen) or an RNaseasy Plant Mini Kit (Qiagen) by on-

![Figure 6. Expression of the Crr1aG004 transgene in T1 lines inoculated with Ano-01.](image)

Transcript levels of 5 representative T1 plants derived from each of 2 resistant T0 lines and 1 plant derived from each of 2 susceptible T0 lines are shown. Osome is the recipient. The disease index of each plant is indicated in parentheses above each bar. Similar results were obtained in 2 independent tests.

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| Cultivar/Line (T0) | disease index (DI) | No. plants tested | mean DI |
|--------------------|-------------------|------------------|---------|
| Osome              | 0 0 0 0 16 16     | 3.0              |
| OsCpCc_03          | 0 1 2 2.5 3       | 2.2              |
| OsCpCc_04          | 0 0 0 0 30 32     | 3.0              |
| OsCpCc_05          | 0 0 0 1 41 42     | 3.0              |
| OsCpCc_07          | 0 1 1 9 13 23     | 2.8              |
| OsCpCc_08          | 0 5 6 10 12 33    | 2.4              |
| OsCpCc_09          | 0 0 5 1 26 21     | 2.9              |
| OsCpCc_10          | 2 1 1 5 4 8 30    | 1.8              |
| OsCpCc_12          | 0 2 0 4 9 15      | 2.6              |
| OsCpCc_15          | 0 7 8 4 9 28      | 2.1              |
| OsCpCc_16          | 0 0 0 0 20 20     | 3.0              |
| OsCpCc_18          | 0 0 0 0 10 10     | 3.0              |
| OsCpCc_19          | 0 0 0 3 12 15     | 2.9              |
| OsCpCc_20          | 0 0 0 1 10 12     | 2.9              |
| OsCpCc_21          | 0 0 0 0 12 12     | 3.0              |
| OsCpCc_22          | 0 0 0 0 4 4       | 3.0              |

Root symptoms of each disease index are shown in Fig. S1.
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column DNase 1 treatment, and then converted into first-strand cDNA with a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Semi-quantitative RT-PCR for Crr1aG004 was performed using cDNA synthesized from RNA isolated from plants at 14 days after inoculation with Ano-01. To test splicing variation, we performed PCR using primer pairs flanking intron 2 and 3 and cloned the PCR products into the pGEM-T Easy vector (Promega) for sequencing. RACE was performed with a BD SMART RACE cDNA Amplification Kit (TaKaRa Bio); 2 independent RACE experiments were carried out. Primer sequences used for these amplifications are listed in Table S1.

Real-time PCR Analysis
‘Osome’ were inoculated with Ano-01, and total RNA was isolated from leaves 4 weeks after inoculation. Transcript levels of the Crr1aG004 transgene were analyzed in 2 independent clubroot tests by real-time PCR using SYBR Premix Ex Taq II (TaKaRa Bio). BiACT2 was used as an internal control for normalization [19]. Primer sequences used for these amplifications are listed in Table S1.

Supporting Information
Figure S1 Resistance responses of transgenic Col-0 lines carrying Crr1 promoter: Crr1G004 cDNA construct (Cp_01–19) or wild-type Col-0 plants, to Ano-01. The mean (±SD) is based on the average of 3 clubroot tests. (TIF)

Figure S2 Resistance of transgenic Col-0 plants with Crr1G004 to different pathotypes. Col-0 and 2 T1 lines were inoculated with a representative isolate of each pathotype: No. 5 (group 1), No. 7 (group 2), No. 14 (group 3), and No. 9 (group 4). The mean (±SD) is based on the average of 3 tests. (TIF)

Figure S3 Schematic representation of Crr1a allelic structure in resistant G004 and susceptible A9709. Black boxes, exons; black lines, introns; arrowheads, large insertions. White box, Ty1-copia-type retrotransposon sequences inserted at 3’-end of exon 4. Sequences of 3’- and 3’-ends of the retrotransposon are shown. Long terminal repeat (LTR) elements (arrows in white box) are underlined in sequences. Putative target site duplication is boxed. Deduced amino acid sequence of Crr1aG004 is indicated. Asterisk, putative stop codon. (TIF)

Figure S4 Typical root symptoms of transgenic Brassica rapa inoculated with Ano-01. Resistance responses were evaluated 5 weeks after inoculation. Root symptoms were graded as: 0, no symptoms; 1, very slight swelling on main roots; 2, a small gall on main roots; 2.5, moderate swelling on main roots; 3, severe swelling on main roots. Scale bar indicates 5.0 cm. (TIF)

Figure S5 Typical root symptoms of Arabidopsis (Col-0) inoculated with P. brassicae. Resistance responses were evaluated 3 weeks after inoculation. Root symptoms were graded as: 0, no symptoms; 1, very slight swelling on lateral roots; 2, moderate swelling on lateral roots and taproot; 2.5, severe swelling on all roots but no swelling on hypocotyl; 3, severe swelling on all roots and hypocotyl. Scale bar indicates 1.0 cm. (TIF)

Table S1 Sequences of primers used in this study. (XLS)

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Author Contributions
Conceived and designed the experiments: KH SM. Performed the experiments: KH KS RNT TN HF SM TK. Wrote the paper: KH SM.

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