

Note

Distribution of *CRA* in clubroot resistance (CR) cultivars of Chinese cabbage

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Abstract Clubroot resistance (CR) is an important trait for Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*). Since no CR genes had been found throughout Chinese cabbage lines originally, they were introduced from CR European turnips. Distribution of CR genes in the commercial cultivar is important information for CR breeding, but it has been scarcely known at molecular level. In this work, CR of 47 Chinese cabbage cultivars were analyzed phenotypically and genotypically. More than 80% of them exhibited high resistance phenotype, and all of the cultivars with high resistance were found to have *CRA*, one of the genes controlling CR. Majority of these cultivars were heterozygote for resistance, which is reasonable because *CRA* controls the resistance single-dominantly. This is the first work reporting the distribution of CR gene in Chinese cabbage cultivars and provides important and practical information for breeders and farmers. Further applications of CR genes are also discussed.

Key words: Chinese cabbage, clubroot resistance, *CRA*, DNA marker.

Clubroot disease is a soilborne disease caused by *Plasmodiophora brassicae* Woronin. The disease seriously damages the production of *Brassica* crops and causes yield loss throughout the world (Kowata-Dresch and May-De Mio 2012). Several races/isolates of *P. brassicae* were reported (Kuginuki et al. 1999). To protect the crops against the disease, clubroot resistance (CR) cultivars have been bred actively. Originally, Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) were highly susceptible to clubroot disease. But breeding of clubroot resistance has been conducted since 1970s, and many CR cultivars have been bred, by employing European fodder turnips (*B. rapa* L. ssp. *rapifera*) as CR source materials (Diederichsen et al. 2009). The CR of European fodder turnips is suggested to be controlled by several genes, and until now 7 CR loci, *CRA*, *CRb*, *CRk*, *CRc*, *Crr1*, *Crr2* and *Crr3*, have been reported through the study of CR Chinese cabbage cultivars (Hirai et al. 2004; Matsumoto et al. 1998; Piao et al. 2004; Sakamoto et al. 2008; Suwabe et al. 2003). Many of the CR cultivars are considered to have only one of the 7 CR loci, because most CR cultivars show isolate-specific resistance to *P. brassicae*. This is

probably caused by selection pressure of specific clubroot isolates used in the individual breeding process.

CR cultivars vary in several characters: growth rate, cold tolerance, head formation and timing of bolting. They have been usefully cultivated in adaptable field conditions, and some of them were used as practical resistant materials equipped with economical characteristics. CR genes in these cultivars, however, are poorly understood because they have been bred without molecular knowledge of the CR genes, so further traceable applications are difficult when they are used as breeding materials. Molecular information of the resistance gene is required for certain selection.

CRA is a major CR locus exhibiting dominant resistance to *P. brassicae* M85 isolate (Matsumoto et al. 2005). The susceptible allele of *CRA* is considered to be originated from Chinese cabbage. HC352b was a RFLP marker closely linked to *CRA* (Matsumoto et al. 1998), and was later converted into a SCAR marker HC352b-SCAR, which located 2.9 cM from *CRA* (Hayashida et al. 2008). Recently, molecular characterization of *CRA* gene was achieved, indicating that the *CRA* gene encodes a

Abbreviations: CR, clubroot resistance; SCAR, sequence characterized amplified region.

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Table 1. Genotypes of *CRa* locus in commercial CR cultivars and their resistance to clubroot.

Cultivar	Source	CRaim genotype ^a	Averaged disease index	
			M85	K04
Chiyobuki 85	Sakata seed Co.	A	0.00	n.d. ^b
CR Akogare	Nippon Norin Seed Co.	H	0.23	2.96
CR Gekka	Kobayashi seed Co.	A	0.10	n.d.
CR Kanki	Nippon Norin Seed Co.	H	0.00	2.96
CR Kanko	Nippon Norin Seed Co.	H	0.00	2.59
CR Kikoma	Kobayashi seed Co.	H	0.00	n.d.
CR Kisaku 80	Marutane Co.	H	0.17	n.d.
CR Kyotakara 70	Marutane Co.	H	0.22	3.00
CR Ohken 75	Kyowa seed Co.	H	0.83	3.00
CR Satokaze	Sakata seed Co.	H	0.00	3.00
CR Seiga	Ishii seed Growers	H	0.00	3.00
CR Senmai 65	Tohoku seed Co.	B	3.00	n.d.
CR Sensyuu 65	Tohoku seed Co.	B	3.00	n.d.
CR Shinki	Takii seed Co.	H	0.00	3.00
CRR Emperor	Kobayashi seed Co.	A	0.00	n.d.
Daifuku 234	Tohoku seed Co.	B	3.00	3.00
Gokigen 65	Takayama seed Co.	B	3.00	3.00
Haregi 65	Takii seed Co.	A	0.00	n.d.
Fuyusae	Ishii seed Growers	H	0.00	n.d.
Kaname	Watanabe seed Co.	H	0.00	3.00
Kiai 65	Watanabe seed Co.	A	0.00	n.d.
Kien 75	Nippon Norin Seed Co.	H	0.00	n.d.
Kigokoro 65	Takii seed Co.	A	0.00	3.00
Kifuku 65	Nippon Norin Seed Co.	H	0.00	n.d.
Kinami 90	Ishii seed Growers Co.	A	0.00	3.00
Kiraboshi 65	Takii seed Co.	H	0.00	n.d.
Kiraku 60	Tohoku seed Co.	H	0.48	n.d.
Kiryoyoshi	Kaneko seed Co.	H	1.22	3.00
Kougetsu 77	Kaneko seed Co.	H	0.41	3.00
Koubou	Takii seed Co.	H	0.07	3.00
Kunki 70	Watanabe seed Co.	B	1.94	2.36
Kukai 65	Takii seed Co.	H	0.00	3.00
Masashige	Watanabe seed Co.	H	0.00	3.00
Minebuki 505	Sakata seed Co.	A	0.00	n.d.
Moegi	Kaneko seed Co.	H	0.13	3.00
Ryutoku	Watanabe seed Co.	H	0.07	3.00
Saegi 90	Ishii seed Growers Co.	A	0.00	n.d.
Satobuki 613	Sakata seed Co.	A	0.10	n.d.
Shingen	Watanabe seed Co.	H	0.00	3.00
Shinseiki	Tohoku seed Co.	B	3.00	3.00
Strong CR 75	Watanabe seed Co.	H	0.27	2.88
Super CR Hiroki	Kakinuma Breeding center	B	1.49	1.92
Super CR Kimi 85	Kakinuma Breeding center	B	2.21	2.68
Super CR Shinrisoh	Nippon Norin Seed Co.	H	0.00	2.58
Syouki	Kaneko seed Co.	A	0.38	n.d.
Yumebuki502	Sakata seed Co.	H	0.00	n.d.
Yuuki	Takii seed Co.	H	0.06	2.96
Muso (susceptible control)	Takii seed Co.	B	3.00	3.00

^a "A" indicates resistant homozygote. "H" indicates heterozygote. "B" indicates susceptible homozygote. ^b Not determined.

TIR-NBS-LRR protein, a typical product of plant disease resistance genes. Based on the specific sequence of *CRa*, a DNA marker CRaim was designed and it could be universally applicable to *CRa* detection (Ueno et al. 2012).

In this study, commercial CR varieties are tested to detect *CRa* allele using SCAR marker CRaim, to establish molecular information for the advanced CR breeding.

This is the first report for the distribution of CR gene in Chinese cabbage cultivars.

Forty-seven commercial varieties pronounced to be clubroot resistance were collected from Japanese breeding companies and used for inoculation tests and DNA analysis (Table 1). The S₁ populations (Tables 2, 3) were produced by self-pollination of the cultivars and subjected to the CR test and DNA analysis. Two doubled

Table 2. Segregation of resistance in selfed progenies of CR cultivars.

Cultivar	No. of resistant plants ^b	No. of susceptible plants ^c	χ^2 ^d (3:1)	
CR Akogare	24	7	0.0968	0.7 < p < 0.8
CR Kanki	39	16	0.4909	0.4 < p < 0.5
CR Kanko	34	9	0.2849	0.5 < p < 0.6
CR Kyotakara 70	46	11	0.9883	0.3 < p < 0.4
CR Ohken 75	27	5	1.5000	0.2 < p < 0.3
CR Satokaze	35	12	0.0071	0.9 < p < 0.95
CR Seiga	39	19	1.8621	0.1 < p < 0.2
CR Shinki	48	17	0.0462	0.8 < p < 0.9
Kaname	63	12	3.2400	0.05 < p < 0.1
Kien 75	35	9	0.4848	0.4 < p < 0.5
Kigokoro 65	42	11	0.5094	0.4 < p < 0.5
Kinami 90	44	3	8.6879	0.001 < p < 0.01
Kiryoyoshi ^a	38	15	0.3082	0.5 < p < 0.6
Kougetsu 77	35	20	3.7879	0.05 < p < 0.1
Koubou	49	15	0.0833	0.7 < p < 0.8
Kunki 70 ^a	10	19	25.3908	p < 0.001
Kukai 65	32	11	0.0077	0.9 < p < 0.95
Masashige	36	14	0.2400	0.6 < p < 0.7
Moegi	36	17	1.4151	0.2 < p < 0.3
Ryutoku	36	7	1.7441	0.1 < p < 0.2
Shingen	36	9	0.6000	0.4 < p < 0.5
Strong CR 75	45	13	0.2069	0.6 < p < 0.7
Super CR Hiroki ^a	10	19	25.3908	p < 0.001
Super CR Kimi 85 ^a	6	26	54.0000	p < 0.001
Super CR Shinrisoh	41	10	0.7908	0.3 < p < 0.4
Yuuki	31	12	0.1938	0.6 < p < 0.7

^a Cultivars that showed the values more than 1.00 as the disease indexes in Table 1. ^b Number of plants indicating 0 or 1 of disease index to M86 isolate of *P. brassicae*. ^c Number of plants indicating 2 or 3 of disease index to M86 isolate of *P. brassicae*. ^d χ^2 test was calculated with 5% level.

haploid Chinese cabbage lines, T136-8 and Q5, were used as positive and negative controls respectively.

Inoculation tests for resistance to clubroot were performed as previously reported (Matsumoto et al. 1998). As an inoculation source, M85 and K04 isolates of *P. brassicae* were used. They were identified as race 2 and 4 according to Williams's classification (Williams 1966), respectively. Twenty to thirty seeds of each cultivar and S₁ populations were sown in the pathogen-inoculated soil. Disease infection was rated 30 days after sowing. Evaluation of CR was based on the previous report (Matsumoto et al. 2005); disease index was scored 0 (no infection), 1 (a few small clubs on lateral roots), 2 (larger clubbing on lateral roots or slight swelling of the main root) and 3 (severe galling of the main roots). The averages of scored individuals were calculated for each cultivar. The inoculation test was replicated two or three times.

DNA was extracted from leaves as described (Ueno et al. 2012). A small piece of leaf, approximately 100 mg, was frozen at -80°C for 2 h in a tube containing HD ball (Nikkato, Osaka, Japan) and then crushed by shaking. Two-hundred microliters of extraction buffer, containing 200 mM Tris-HCl (pH 8.0), 250 mM NaCl, 25 mM EDTA and 0.5% SDS, were added to the crushed leaves, and the samples were vortexed. The vortexed samples were kept at room temperature for 10 min, and then centrifuged at

18,000 *g* for 10 min. The supernatants were transferred to new tubes, and purified with a PCI treatment, chloroform treatment and ethanol precipitation. The purified DNA was resuspended in 50 μl of TE buffer.

CRaim was analyzed by using two combinations of primers, which detect the *CRa* resistant allele, and the susceptible allele. The first combination, 5'-TAT ATT AAT GAT AAA GCA GAA GAA GAA A-3' and 5'-AAT GCG ACT GAG AAA GTT GTA G-3', was used to detect the resistant allele, and the other set, 5'-TGA AGA ATG CGG GCT ACG TCC TCT GAA ATC-3' and 5'-GAA GTA GAT GAA CGT GTT TAT TTT AGA AA-3', was used to detect the susceptible allele. The PCR reaction solution contained 1 μl of extracted DNA, 0.5 μM primers, 0.2 mM dNTPs, 1 μl of 10 \times reaction buffer, 4 mM MgCl₂, and 0.25 unit of Taq DNA polymerase (Bioline, London, UK) in a total volume of 10 μl .

For the resistant allele, thermal cycler (ThermoGen, Nagano, Japan) was programmed as follows: 94 $^{\circ}\text{C}$ for 1 min; 35 cycles of 94 $^{\circ}\text{C}$ for 30 s, 63 $^{\circ}\text{C}$ for 30 s, and 68 $^{\circ}\text{C}$ for 1 min; and 72 $^{\circ}\text{C}$ for 7 min. For the susceptible allele, the program was as follows: 94 $^{\circ}\text{C}$ for 1 min; 38 cycles of 94 $^{\circ}\text{C}$ for 30 s, 59 $^{\circ}\text{C}$ for 30 s, and 68 $^{\circ}\text{C}$ for 2 min; and 72 $^{\circ}\text{C}$ for 7 min depending on the amplification efficiency of the primers. PCR products were electrophoresed in 1% agarose gels and stained with ethidium bromide. Band patterns in the gels were observed using a Printgraph

Table 3. Genotypes of *CRa* locus in selfed progenies derived from commercial CR cultivars.

Cultivar	Total number of S_1 progeny	Resistance to M85 ^a	Number of plants ^b		
			A	H	B
CR Akogare	11	R	2	3	0
		S	0	0	6
CR Kanko	16	R	3	6	0
		S	0	0	7
CR Kyotakara 70	27	R	4	16	0
		S	0	0	7
CR Ohken 75	12	R	2	7	0
		S	0	0	3
CR Satokaze	20	R	7	11	0
		S	0	0	2
Kaname	27	R	5	13	0
		S	0	0	9
Kien 75	18	R	4	8	0
		S	0	0	6
Kiryoyoshi	27	R	1	15	0
		S	0	0	11
Kougetsu 77	26	R	5	9	0
		S	0	0	12
Koubou	19	R	5	8	0
		S	0	0	6
Kunki 70	12	R	0	0	4
		S	0	0	8
Kukai 65	16	R	5	5	0
		S	0	0	6
Masashige	15	R	2	8	0
		S	0	0	5
Ryutoku	28	R	10	14	0
		S	0	0	4
Strong CR 75	27	R	9	7	0
		S	0	0	11
Super CR Shinrisoh	24	R	8	6	0
		S	0	0	9
Yuuki	32	R	9	14	0
		S	0	0	9
T136-8	5	R	5	0	0
Q5	5	S	0	0	5

^a "R" and "S" indicate cultivars showing 0 or 1 of disease index and those showing 2 or 3 of disease index, respectively, to M85 isolate of *P. brassicae*. ^b The number of progenies showing A: resistant homozygote, H: heterozygote and B: susceptible homozygote of *CRa* genotype.

photo image instrument (ATTO, Tokyo, Japan). Fragment sizes of PCR products in all tested cultivars were same with controls.

Analyzed CR cultivars showed different responses against 2 isolates (Table 1). Many cultivars exhibited stable resistance to M85, but were susceptible to K04. 'CR Senmai 65', 'CR Sensyuu 65', 'Daifuku 234', 'Gokigen 65' and 'Shinseiki' were susceptible to M85. 'Kiryoyoshi', 'Kunki 70', 'Super CR Hiroki', and 'Super CR Kimi85' had moderate or low resistance to each isolate. PCR analysis indicated that 39 of the 47 cultivars (83%) showing high resistance against M85 had *CRa* (Table 1), while the other 8 cultivars only had the susceptible allele of *CRa*.

Twenty-six cultivars were additionally studied using self fertilized S_1 populations and M85. Most individuals derived from high resistance cultivars segregated at a ratio of 3 : 1 (resistant : susceptible, $p > 0.05$) as controlled

by a single dominant gene (Table 2). 'Kinami 90' was an exception in which the proportion of resistant was significantly high. Progenies of low resistance 'Kunki 70', 'Super CR Hiroki' and 'Super CR Kimi 85' segregated rather as recessive manner. On the other hand, 'Kiryoyoshi' segregated at a ratio of 3: 1 ($p > 0.05$) like the high resistance cultivars in spite of its moderate disease index. It could be caused by the incomplete uniformity of the seeds used in this study (data not shown).

Further analysis was done for the relationship between the resistance phenotype and the *CRa* genotype of S_1 individuals of 17 cultivars (Table 3). All of the resistant plants had *CRa*, except progenies of 'Kunki 70', whereas susceptible ones had a susceptible allele.

Many CR Chinese cabbage cultivars have been developed and prevail in Japan, employing several resistance genes. The genes differ in defending responses

to the varied isolates of *P. brassicae*, but the genetic background of their resistance is scarcely known, and each CR cultivar is thought to have only one or a few resistant gene(s).

From CR segregation ratio of selfed progenies in resistant cultivars to M85, it is presumed that these cultivars are heterozygote for resistance and a major resistance gene controls the resistance single-dominantly. Moreover, coincidence between the CR phenotype and the CRaim genotype in segregating populations supports that *CRA* is the only resistance gene in these cultivars.

Through our analysis, surprisingly more than 80% of the commercial varieties retained the *CRA* gene, indicating that the *CRA* is widely distributed in CR cultivars of Chinese cabbage. It also implies the possibility that *CRA* is a basic resistance gene against widespread pathotype of *P. brassicae* like M85. Information on *CRA* uncovered in this study will be helpful for breeders, because they will no longer need repetitive phenotype selection of CR candidates. *CRA* works dominantly and could be certainly introduced into cultivars by monitoring only CRaim.

In this study, it was shown that responses of the CR cultivars against 2 different isolates; many cultivars were resistant to the isolate M85, but were susceptible to the isolate K04. The CR cultivars widely used in major production areas do not have consistent results, i.e. the breakdown of resistance would corrupt against the outbreak of a new pathotype like K04. Pyramiding (A breeding technique that accumulates multiple genes of similar function into a line) of the resistance genes, or multiline (A method conducted by cultivating many isogenic lines in a field) technique are effective ways to avoid resistance breakdown. For both pyramiding and multiline, information on individual genes are necessary. Although *CRA* emerged as the representative CR gene in this work, it is not sufficient enough, and information on the other CR genes is required to achieve strong CR resistance, as in the fodder turnip. Accumulation of 3 genes are achieved (Matsumoto et al. 2012), but it is still an intermediate to the goal. It is expected that DNA markers, like CRaim, will be developed and applied, thus opening a new stage of CR breeding in Chinese cabbage.

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