Doctoral Dissertation (Shinshu University)

The study on DNA markers for disease resistance breeding in lettuce (*Lactuca sativa* L.) and Chinese cabbage (*Brassica rapa* L. ssp *pekinensis*)

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Abbreviation

AFLP (amplified fragment length polymorphism)

CR (clubroot resistance)

FOL (*Fusarium oxysporum* f. sp. *lactucae*)

MAS (marker-assisted selection)

QTL (quantitative trait loci)

RAPD (Random amplified polymorphic DNA)

RFLP (restriction fragment length polymorphism)

RRD2 (resistance for root rot disease race 2)

SCAR (sequence characterized amplified region)

Summary

The purpose of breeding is improvement of crops reflecting the request from farmers and consumers on the features such as high yield, easy cultivation, high quality and easy processing. For the purpose, many years and laborious work are spent repeating cross of the material lines and phenotype evaluation of thousands of the progeny. Development of new technique is needed for efficient evaluation and selection. MAS (Marker-assisted selection) is an efficient technique to accelerate breeding programs. Application of MAS requires a DNA marker that is linked to interested gene.

Disease resistance is one of the important agronomic traits. It conserves the cultivation efficiency, yield and quality. Root rot of lettuce and clubroot disease of Chinese cabbage have become serious problems in production field of these crops. Rapid supply of disease resistance cultivars is required, but breeding programs for new cultivar take more than 10 years without MAS. In this thesis, DNA markers in lettuce and Chinese cabbage were studied to enable MAS breeding.

In chapter 1, DNA marker for root rot resistance in lettuce was studied. Root rot of lettuce is caused by *Fusarium oxysporum* f. sp. *lactucae* (FOL). The *RRD2* (resistance for root rot disease race 2) locus, which confers resistance to FOL race 2 was analyzed using two cultivars of crisphead lettuce: VP1013 (resistant) and Patriot (susceptible). The segregation patterns of resistant phenotypes in F_2 and F_3 indicated that the resistance was regulated by a single major locus and several minor loci. To define the positions of the resistance loci, a linkage map was newly constructed using amplified fragment length polymorphism (AFLP) and

random amplified polymorphic DNA (RAPD) markers. Quantitative trait loci (QTL) analyses revealed that resistance loci were located on linkage group 2. Detection pattern of RAPD marker WF25-42 was demonstrated to represent the phenotype of *RRD2* locus perfectly, throughout the various cultivars and lines studied. In addition, WF25-42 was converted into sequence characterized amplified region (SCAR) marker.

In chapter 2, DNA marker for clubroot resistance in Chinese cabbage was studied. A restriction fragment length polymorphism (RFLP) marker, HC352b, had been identified to be closely linked to the *CRa* locus, which is responsible for clubroot resistance (CR). Clubroot is soilborne disease caused by *Plasmodiophora brassicae*. The HC352b is complicated to detect, because it represented multiple-copy loci. Then, HC352b was analyzed and converted into a PCR based SCAR marker. The developed HC352b-SCAR detected the *CRa* locus easily and accurately more than the original HC352b.

In chapter 3, distribution of *CRa* gene was analyzed. In the commercial CR Chinese cabbage cultivars, retention of the *CRa* locus was identified by CR test. And the genotype of *CRa* gene was judged by DNA marker designed on the *CRa* gene. It was turned out that *CRa* was found in more than 78 % of cultivars tested, indicating it is the major CR gene in the commercial cultivars.

The DNA markers developed in this study will enable efficient MAS for breeding programs. WF25-42-SCAR marker in lettuce is planned by breeders to be used in breeding programs to develop new resistant cultivars. HC352b-SCAR in Chinese cabbage established identification of *CRa* gene and development of novel line possessing 3 individual CR loci.

The information of *CRa* distribution in the CR cultivars is expected to enhance the breeding programs to develop novel CR characteristic cultivars utilizing commercial cultivars.

Preface

Plant breeding is a development of new plant varieties that is valuable for agricultural production, and has been carried out since the beginning of farming. Domesticated plant species have features such as faster growth, larger fruits and higher yield, compared with their wild species. These characteristics have been acquired through the selection without the understanding of scientific basis of plant genetics. Since the early 1900s breeding science has begun as field academically and systematically studied referring the fundamental discoveries by Darwin, Mendel and following classical genetics. Then the findings in various modern sciences including genetics, physiology, molecular biology and pathology were further introduced, and formed current style of breeding programs.

The purpose of breeding is improvement of crops reflecting the request from farmers and consumers on the features such as high yield, easy cultivation, high quality and easy processing. For the purpose, many years and laborious work are spent repeating cross of the material lines, phenotype evaluation of the progeny populations, selection of promising lines from thousands of candidates, and finally reduction of dispersion of the features to fix the line as a new cultivar. Due to these complexed procedures, rapid implementation of breeding process is difficult, and usually it takes more than ten years even in annual crops. In particular, the evaluation and selection step need a lot of time and effort. Development of new technique is needed for efficient evaluation and selection.

The linkage analysis is the common strategy to reveal the positions (loci) of the interested genes, and provide the DNA markers that is linked

to the features. These DNA markers could be employed in breeding programs that are called Marker-assisted selection (MAS). MAS could select the ideal progeny at seedling time without evaluation of phenotype, making MAS a powerful technique that could efficiently and rapidly select through great numbers of plants. Thus, it could reduce the time and expense of fieldwork. DNA markers that are tightly linked to objective phenotype are required for MAS; less than 5 cM of genetic distance is preferable (Mohan et al., 1997: Collard and Mackill, 2008).

Many important agricultural features, such as morphology, amount of edible part, timing of bolting and some disease resistance, show continual changes. These features are controlled by polygene and called QTL (quantitative trait loci). To dissect responsible genes and to make DNA markers for the individual responsible genes, QTL analysis is employed. QTL analysis is computer assisted linkage analysis assuming responsible genes among the linked markers, and provides probability of contribution ratio.

Development of disease resistant cultivar is important to ensure stable crop production. Information of disease resistant genes and linked markers should enable breeding programs for disease resistant cultivars with MAS. However, identification of disease resistant genes are difficult and complicated depending its pathogens. Plant pathogens could be classified into several races that differ in pathogenicity. Interaction between pathogen races and host-plants could mostly be explained by the gene-for-gene theory (Van Der Blezen and Jones, 1998; Stukenbrock and McDonald, 2009). In this theory, a single dominant gene commonly controls disease resistance to one of the pathogen. On the other hand, some

disease resistances that are controlled by polygene have been reported (Zhang et al., 2008; Grimmer et al., 2008; Talukder et al., 2004). In major crops, resistance genes or loci have been identified by linkage analysis (Jeon et al., 2003; Bryan et al., 2002; Robert et al., 2001; Dussle et al., 2002; Donald et al., 2002). However, many pathogens and host-plants that are important for crop production remains unstudied.

Head vegetables, lettuce (*Lactuca sativa* L.) and Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) are largely produced in Japan, and Nagano prefecture is a major production area of these vegetables. Recently, root rot of lettuce and clubroot disease of Chinese cabbage have been occurring in the production field. For the acceleration of breeding programs using MAS for resistant cultivars to those diseases, it is needed to find and understand resistant genes together with its linked DNA markers. Then, in this thesis, in chapter 1, the DNA markers linked to FOL resistance is reported. This is the first report on the development of the DNA marker linked to FOL resistance. In chapter 2, improvement of a DNA marker linked to clubroot resistance (CR) is reported in Chinese cabbage. In chapter 3, distribution of the *CRa,* one of the major CR genes, in the commercial CR cultivars is reported based on newly developed DNA marker.

Chapter 1

Analysis of RAPD and AFLP markers linked to resistance to *Fusarium oxysporum* f. sp. *lactucae* race 2 in lettuce (*Lactuca sativa* L.)

Chapter 1. Analysis of RAPD and AFLP markers linked to resistance to *Fusarium oxysporum* f. sp. *lactucae* race 2 in lettuce (*Lactuca sativa* L.)

1-1. Introduction

Root rot of lettuce, a soilborne disease that occurs in many countries, has become a serious problem for lettuce production (Scott et al., 2010). In Japan, especially its infestation has become significant in Nagano prefecture. This disease is caused by FOL. Lettuce plants infected by FOL initially show yellowing in the leaf marginal region, then the root vascular bundles turn brown and growth of the aerial parts is inhibited. Mild symptoms lead to failure of head formation but the plant remains alive, whereas severe symptoms kill infected plants. FOL is classified into three races, 1, 2, and 3 (Fujinaga et al., 2001, 2003). Genetic resources of resistant lettuce were evaluated and selected using these races by Tsuchiya et al. (2004a). The cultivar 'Shinano Hope' was bred as resistant to FOL race 1 (Tsuchiya et al., 2004b), and 'Chouya No. 37' was bred as resistant to FOL race 2 (Tsuchiya, 2009). These breeding programs were carried out using conventional selection method with pathogen inoculating resistance tests in the field.

Several genetic maps of lettuce have been reported using populations raised from intra- or inter-specific crosses (Kesseli et al., 1994; Witsenboer et al., 1997; Waycott et al., 1999; Johnson et al., 2000; Jeuken et al., 2001; Syed et al., 2006). The current reference map contains 36 loci associated with resistant phenotypes for six diseases and one insect pest: downy mildew, root downy mildew, corky root rot, lettuce mosaic virus, turnip mosaic virus, lettuce dieback, and root aphids (McHale et al., 2009).

To date, however, no gene loci have been identified for resistance to FOL. To improve breeding efficiency with MAS, identification of resistant loci for FOL with DNA maker is necessary.

Then, the aim of this chapter is identification of genetic loci for resistance to FOL race 2, together with DNA markers linked to the loci. For the purpose, construction of a linkage map using AFLP and RAPD markers and QTL was performed to identify FOL resistance loci.

1-2. Materials and Methods

Plant materials

The crisphead lettuce cultivars VP1013 (resistant to FOL race 2) and Patriot (susceptible) were used as parental lines. Fifty-eight individuals of the F_2 progeny from a cross between VP1013 and Patriot were used for linkage analysis. To estimate the genotypes of F_2 individuals, 14–22 plants of the selfed F_3 population were used for disease resistance test. DNA markers were evaluated using an additional 18 fixed lines and 17 commercial cultivars (see Table 1-1).

Disease resistance test

The resistance test for FOL race 2 was performed as described by Tsuchiya et al. (2004a). Seeds (18 of both parental lines, and 14–22 of each F_3 population) were sown directly into artificially inoculated soil. Approximately 1 month after germination, seedlings were examined and scored using the disease index described below, and the disease incidence and disease severity were calculated.

The disease index for leaves (including all shoots) was as follows: 0,

no symptoms; 1, partial necrosis of leaf only; 2, leaf necrosis, stunting, and wilting; 3, severe wilting and death.

The disease index for roots was as follows: 0, no symptoms; 1, some brown roots; 2, many brown roots; 3, inside of crown rotten and brown.

Disease incidence and disease severity per F_3-line were calculated as follows:

Disease incidence (%) = number of diseased plants × 100/number of examined plants.

Disease severity = (3A+2B+C) × 100/(3N), where A is the number of category '3' plants, B is the number of '2' plants, C is the number of '1' plants, and 'N' is the total number of plants examined.

DNA extraction

DNA was extracted from the leaves of parental lines and F_2 plants according to the cetyl trimethyl ammonium bromide (CTAB) method (Murray and Thompson, 1980).

AFLP analysis

AFLP analysis (Vos et al., 1995) was carried out using the AFLP™ Plant Mapping Kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions. Selective PCRs were performed using 20 selective primer combinations, 2 *Eco*RI+AGS (S is G or C) × 8 *Mse*I+CWN (W is A or T and N is A, C, G, or T), *Eco*RI+ACA × *Mse*I+CTG, *Eco*RI+ACC × *Mse*I+CTA, *Eco*RI+AAG × *Mse*I+CTT, and *Eco*RI+ACT × *Mse*I+CAT with a GeneAmp® PCR System 2700 (Applied Biosystems). Fluorescence detection was performed using an ABI Prism

3100 Genetic Analyzer (Applied Biosystems).

RAPD analysis

RAPD analysis (Williams et al., 1990) was carried out with 435 combinations of random 12-mer oligonucleotides (Wako Pure Chemicals, Osaka, Japan). PCR was performed in a 10 μL reaction solution containing 3 ng template DNA, 0.5 μM primers, 0.2 mM dNTPs, 1 μL 10× manufacturer's reaction buffer, 4 mM $MgCl_2$, and 0.25 U Taq DNA polymerase (Bioline, London, UK). Amplification was performed in a thermal cycler (ThermoGen, Nagano, Japan) programmed as follows: 94°C for 1 min; 20 cycles of 94°C for 15 s, annealing temperatures decreasing by 0.5°C each cycle starting with 45°C for 2 min, 72°C for 2 min; 25 cycles of 94°C for 15 s, 35°C for 2 min, and 72°C for 2 min; and 72°C for 7 min. PCR products were electrophoresed in 2% agarose gels and stained with ethidium bromide. Band patterns in the gels were observed using a Typhoon trio imager (GE Healthcare, UK).

Bulked segregant analysis

Based on the disease severity, resistant and susceptible bulks were constructed with eight F_2 plants, respectively. The resistant bulk consisted of plants with disease severities ranging from 0 to 5; the susceptible bulk consisted of plants with disease severities ranging from 98 to 100.

Linkage analysis and QTL analysis

For individual markers, goodness-of-fit to expected segregation ratios was tested by chi-squared tests ($p > 0.05$). Linkage data was analyzed

using JoinMap 4.0 (van Ooijen, 2006). Mapping was carried out with the following thresholds: recombination frequency < 0.4, LOD > 1, and jump = 5. Recombination frequencies were converted to map distances in centimorgans (cM) using the Kosambi function (Kosambi, 1944).

Detection of QTLs was carried out using QGene 4.0 (Joehanes & Nelson, 2008) using interval mapping (IM) and composite interval mapping (CIM). Cofactor of CIM was "forward cofactor selection". IM and CIM were performed at LOD threshold values that were estimated by means of a permutation test with 1,000 permutations. The interval was 1 cM.

Development of SCAR marker

A sequence characterized amplified region (SCAR) marker WF25-42-SCAR was developed based on the RAPD marker WF25-42. The DNA sequence of the WF25-42 fragment was analyzed and a primer set was designed (5′- GAGCAATTGATACATACCTGCAACATTGTT-3′ and 5′- GGAGGGGCAATAAGAACATTTGCTCTT-3′). For detection of WF25-42-SCAR, PCR was performed in a 10 μL reaction solution containing 3 ng template DNA, 0.5 μM primers, 0.2 mM dNTPs, 1 μL 10× manufacturer's reaction buffer, 4 mM $MgCl_2$, and 0.25 U Taq DNA polymerase (Bioline). Amplification was performed in a thermal cycler (ThermoGen) programmed as follows: 94°C for 1 min; 35 cycles of 94°C for 15 s, 56°C for 5 s, and 72°C for 30 s; and 72°C for 5 min. PCR products were electrophoresed in 2% agarose gels and stained with ethidium bromide. Band patterns in the gels were observed using a Printgraph photo image instrument (ATTO, Tokyo, Japan).

1-3. Results

Segregation of resistance to FOL race 2

The disease severity of the F_2 progenies derived from the cross between resistant and susceptible cultivars was used to understand the pattern of inheritance of resistance to FOL race 2. The disease severities of VP1013 and Patriot were reported previously by Fujinaga et al. (2001; 2003). The disease severities of F_2 populations were evaluated according to the results of disease resistance tests of F_3 populations raised from selfed F_2 individuals. Of the 58 F_2 individuals, the disease severities of leaves of their progenies were distributed as follows: 12 plants, 0–20; 32 plants, 20–80; 14 plants, 80–100. The disease severities of roots were distributed as follows: 11 plants, 0–20; 32 plants, 20–80; 15 plants, 80–100. Regardless of tissue, resistance to FOL race 2 as scored by the disease severity segregated at the expected ratio of 1:2:1 in the F_2 population (Fig. 1-1). However, segregation patterns of F_3 progenies of several F_2 plants categorized an intermediate did not show the theoretical ratio of 3:1, particularly in the roots (Fig. 1-2). Additionally, the mean disease severity of 'intermediate' F_2 plants did not correspond to an expected value of 25 if single gene regulation was involved. These results suggested resistance to FOL race 2 might be determined by a single major dominant locus, but with minor loci that contribute some modifications. The major locus as *RRD2* (resistance for root rot disease race 2) was designated.

Establishment of molecular markers

AFLP analysis was carried out to estimate the position of *RRD2*. The marker candidates that showed polymorphism in the parental lines were tested using individual F_2 plants, and 63 markers showed almost 3:1

segregation. RAPD analysis using 432 primer combinations was carried out with resistant and susceptible bulks to survey polymorphic amplified bands that were tightly linked to *RRD2*. The candidates bands were subsequently validated on individual F_2 plants. Finally, 16 markers were selected that showed almost 3:1 segregation. The segregation of all 16 RAPD-markers in 58 F_2 plants was tested with the chi-squared test ($p > 0.05$) and did not deviate from expected ratios of 3:1.

Construction of linkage map and QTL analysis

Linkage analysis was carried out with 63 AFLP markers and 16 RAPD markers, and in total these 61 markers formed five linkage groups (numbered LG1 to LG5 in descending order of length), and 18 markers were independent of these groups (Fig. 1-3). More than half of the AFLP markers were located in two major linkage groups, namely LG1 and LG2 (65.6 cM and 32.7 cM, and 29% and 32% of all markers are incorporated, respectively).

QTL analysis of resistance to FOL race 2 was performed on the constructed linkage map with the data sets of disease severities from both leaves and roots (Fig. 1-3). Only the LOD score on LG2 showed large peaks that exceeded the LOD thresholds, as calculated by QGene in both tissues. The strongest QTL peak was located in the region between the two markers WF11-12 and WF09-23.

Within this major QTL region, three RAPD markers were located (WF25-42 (11.0 cM), WF43-50 (11.7 cM) and WF09-23 (14.1 cM)). According to the genotyping data, WF25-42 and WF43-50 were associated with the susceptible phenotype, whereas WF09-23 was associated with the

resistant phenotype (Fig. 1-4).

RRD2 genotypes of different cultivars and lines

The three markers in the largest QTL peak and four linked markers were used to genotype 17 cultivars and 18 lines. Of these 35 cultivars/lines, 21 were resistant and 14 were susceptible. The genotypes of *RRD2* and markers were considered to be homozygous in these cultivars/lines because lettuce is a self-pollinator. The resultant genotypes of the DNA markers correlated with the phenotypes of resistance to FOL race 2 (Table 1-1). In particular, WF25-42 closely predicted the susceptible phenotype.

For six of the DNA markers, some exceptions in the correlations between phenotypes and genotypes were observed. WF10-46-2, WF42-43, WF41-45, and WF43-50 were associated with the susceptible phenotype, but signals of these markers were detected in some resistant cultivars/lines (Chouya No. 37, N8-19, UC-016, UC-023, and YL216). Signals of the markers WF11-12 and WF09-23, which were associated with the resistant phenotype, were inconsistent with the phenotypes of Banchu Red Fire, NEL05, UC-016, YL216, Shinano hope, Ster Ray, Success, and VI59. Because of its good predictability, the amplified fragment of WF25-42 was sequenced in order to design a specific primer set so that it could utilized as a SCAR marker. The resultant WF25-42-SCAR showed completely identical signals with the RAPD WF25-42 (Fig. 1-5).

1-4. Discussion

A linkage map of lettuce was constructed using F_2 plants from the cross between VP1013 and Patriot, and identified the major root rot

resistance locus *RRD2*, which was positioned between WF11-12 and WF09-23 in LG2. Particularly the RAPD marker WF25-42 was tightly associated with the susceptible allele of *RRD2*. Thus, this marker will provide efficient selection of candidates that are resistant to FOL race 2 in breeding programs. FOL is categorized into three races, namely 1, 2, and 3 (Fujinaga et al., 2001; 2003). To date, only single races of FOL have been found in infected areas, but it is possible that fields will be contaminated by multiple races in the near future. In lettuce, it is presumed that individual resistance genes exist for each of the FOL races. Pyramiding of those resistance genes is desirable to produce new cultivars that are resistant to several races. The DNA markers identified in this study could help to achieve this goal.

In previous studies, linkage maps of lettuce were constructed using crosses between materials that were genetically distinct. Kesseli et al. (1994), Witsenboer et al. (1997) and Waycott et al. (1999) used a population from an intraspecific cross between crisphead and butterhead lettuce (*L. sativa*). Jeuken et al. (2001) used a population from the interspecific cross between *L. saligna* and *L. sativa,* and Syed et al. (2006) and Truco et al. (2007) used a population from an interspecific cross between *L. serriola* and *L. sativa*. Unfortunately, the previously reported genetic maps are not directly applicable to Japanese lettuce, as they were constructed using American/continental lines. The cultivars VP1013 and Patriot used in the present study were regarded as genetically distant materials. VP1013 is a cultivar derived from Kikugawa 102 (Salinas-type) and Patriot is a commercial cultivar bred from Empire-type materials in Japan. These cultivars have many different characters, such as disease

resistance and flowering time.

Lactuca sativa has nine chromosomes, but only five linkage groups were constructed in this study. Markers were concentrated on a few limited linkage groups. It was thought to be due to poor polymorphisms in ice berg lettuce (Hu et al., 2005)

Many markers were identified around the *RRD2* locus, which suggests partial introgression of genome regions that contain *RRD2* into VP1013 from a genetically distant ancestor.

Prior to the present study, it was assumed resistance to FOL race 2 was regulated by a single dominant locus in VP1013. The distribution ratio of disease severities supports this hypothesis, because they seemed to be to the expected ratio of 1:2:1 (resistant:intermediate:susceptible) in the F_2 population. Individuals in the intermediate class in the F_2 population were supposed to be heterozygous for *RRD2* locus.

The results of RAPD analysis using different cultivars and lines showed the defined RAPD markers reliably identified plants that carry the *RRD2* locus, and predicted the resistant phenotype. Especially, markers WF25-42 and WF25-42-SCAR showed a perfect association with the phenotype in all materials analyzed. On the other hand, markers mapped near the *RRD2* locus showed some exceptions. This could be caused by chromosomal recombination accumulated during the breeding programs that produced the cultivars and lines used in this study, thus providing supportive information on the region surrounding the *RRD2* locus. Thus, WF25-42 must be closely linked to *RRD2*, which are probably conserved among different cultivars or lines. WF25-42 will be applicable for selection of the *RRD2* locus derived from a wide range of genetic resources.

It is expected that further exploration of molecular markers would facilitate identification of the *RRD2* gene and elucidate molecular mechanisms of root rot resistance in lettuce. In addition, further research to develop new markers for other FOL resistance genes would be useful to increase our knowledge of FOL resistance and to exploit this trait in novel cultivars.

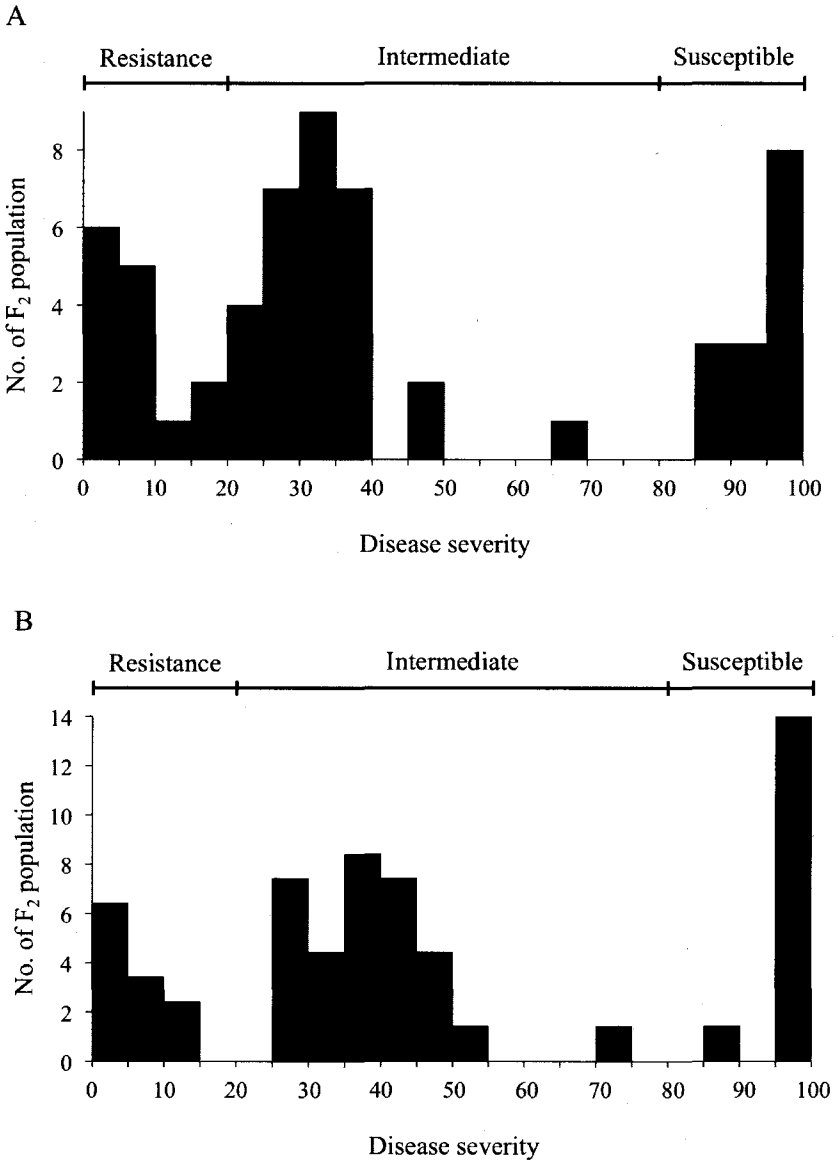
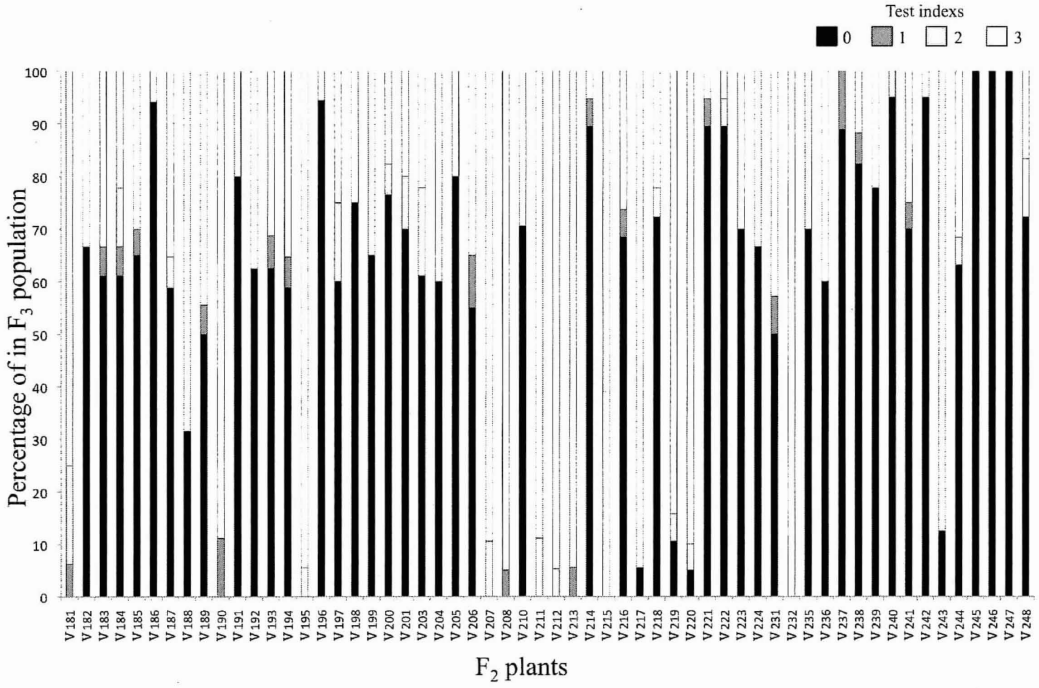


Fig. 1-1

Disease severities among F_2 plants.

The histogram shows the number of F_2 plants showing each disease severity score. Disease severity was calculated as described in the Materials and Methods. A) Disease severity scores for leaves. B) Disease severity scores for roots. The horizontal axis indicates disease severity in 5-point

A


Test indexs
0
1
2
3
Percentage of in F3 population
F2 plants

B

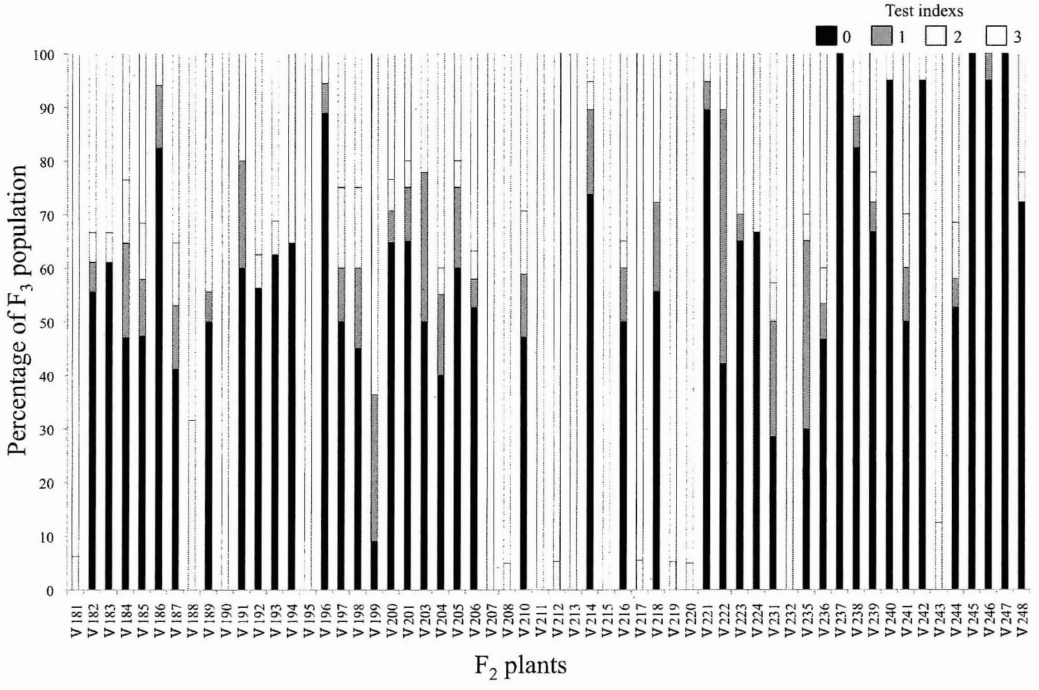

Test indexs
0
1
2
3
Percentage of F3 population
F2 plants

Fig. 1-2

Segregation of FOL resistance phenotype in F_3 population.

Resistance was evaluated as disease indexes (0 to 3 as gray-scale). Percentages of each index in the F_3 population derived from each F_2 plant are indicated on the vertical axis. Individual F_2 plants are represented on the horizontal axis. Panel A shows data for leaves, and data for roots are shown in panel B.

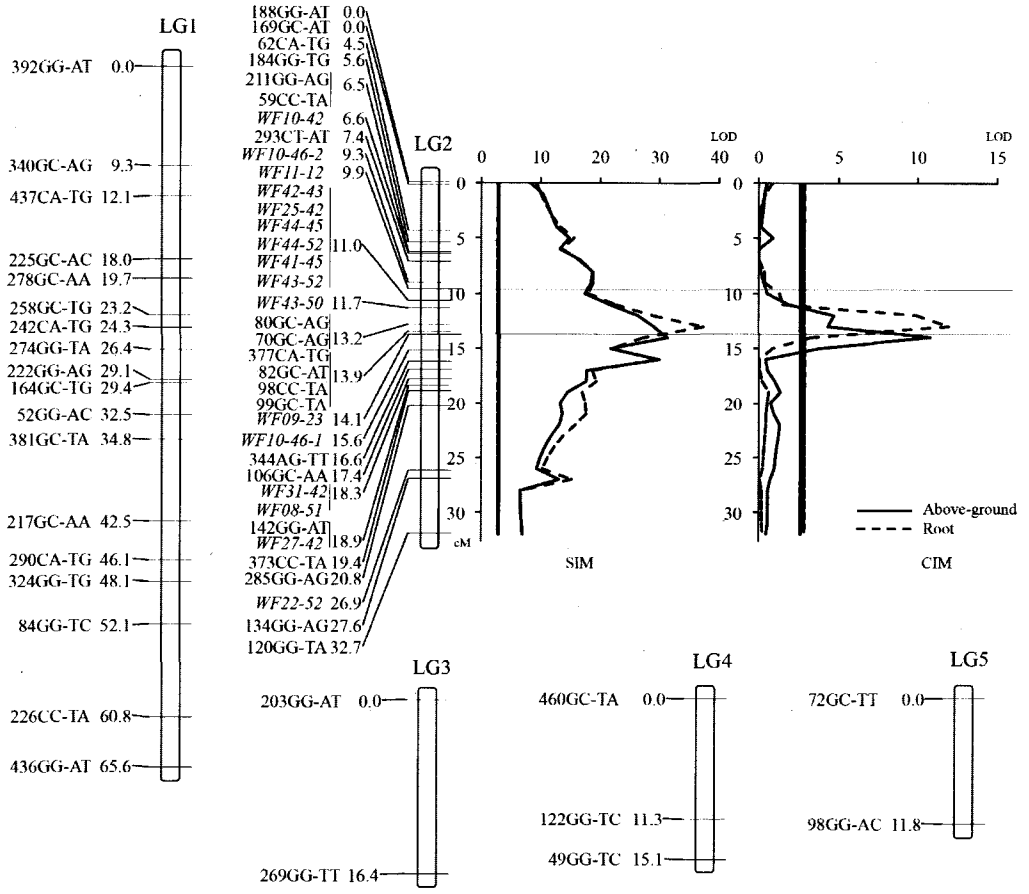


Fig. 1-3

Linkage map, LOD scores, and identification of the *RRD2* locus.

Join Map 4.0 and QGene software were used for the analysis as described in the Materials and Methods. The marker distances and LOD scores are illustrated at the same scale. LOD scores less than thresholds were omitted. Locus names and map distances (cM) are indicated on the left of the linkage groups (LGs). AFLP markers are named as XXX NN-NN format, whereas RAPD markers are WFXX-XX format (where X means numeric, and N means G, A, T, or C). Roman letters indicate AFLP markers and italic letters indicate RAPD markers. In the QTL chart, a solid line indicates the LOD score calculated from the leaves data set, and a broken

line indicates the LOD score calculated from the roots data set. Vertical lines indicate LOD thresholds. LOD thresholds in SIM were 2.9 (leaves) and 2.8 (roots). LOD thresholds in CIM were 2.6 (leaves) and 2.8 (roots).

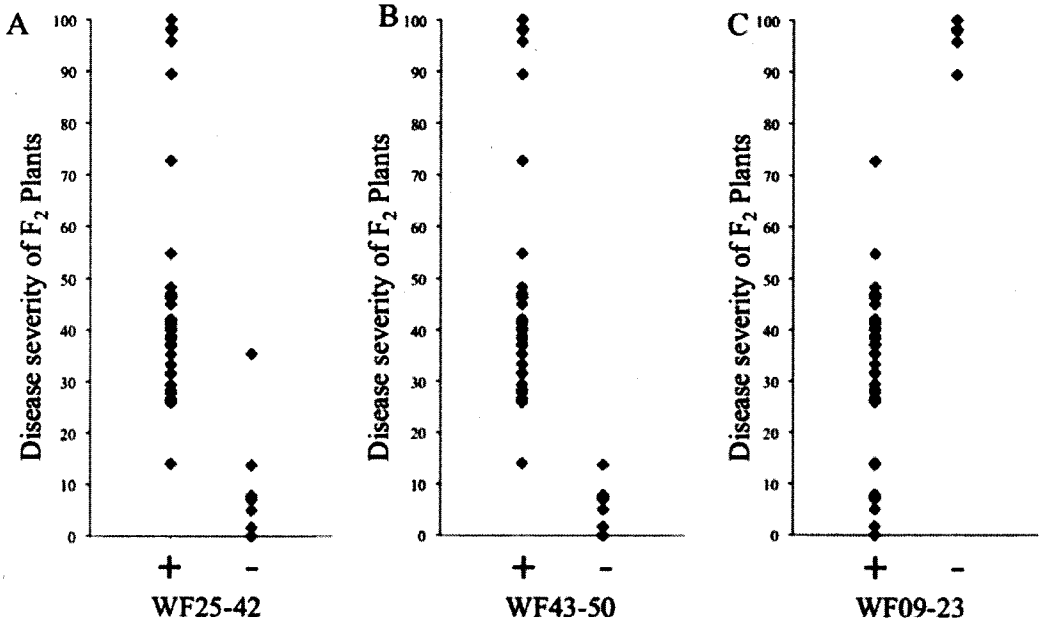


Fig. 1-4

Relationship between RAPD markers and resistance phenotypes.

Disease severities and genotypes of RAPD markers of individual F_2 plants derived from VP1013 × Patriot cross were plotted. The RAPD markers used were: A) WF25-42, B) WF43-45 and C) WF09-23. Dots represent each F_2 plant, and indicate disease severity of roots and positive/negative signals of the marker.

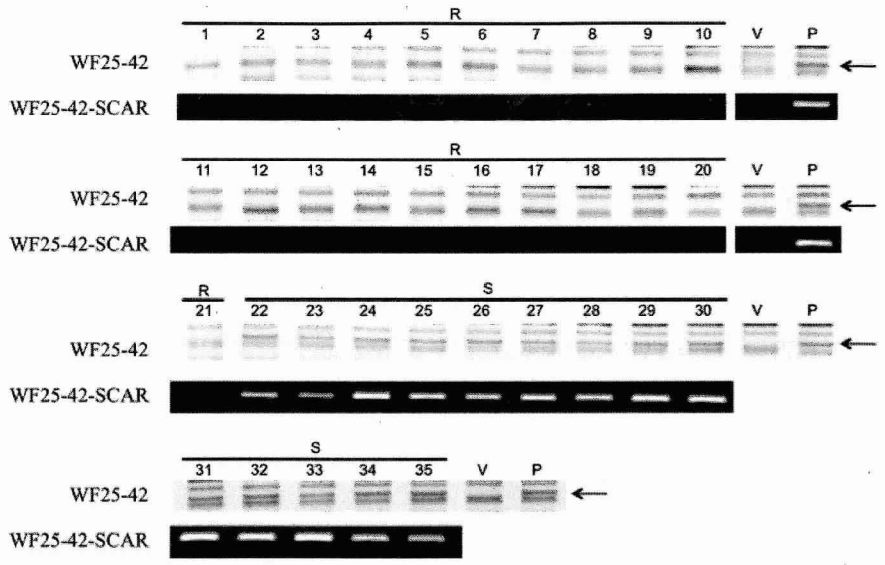


Fig. 1-5

Detection of WF25-42 RAPD marker and WF25-42-SCAR SCAR marker. Agarose gel electrophoresis of the PCR product using RAPD primers for WF25-42 visualized using an image scanner. The specific band at 260 bp marked by an arrow indicates the position of the WF25-42 marker. Photo images of the WF25-42-SCAR PCR product stained with ethidium bromide are presented. Lanes 1–21 are samples from resistant plants. Lanes 22–35 are samples from susceptible plants. The names of the cultivar/line in each individual lane are listed in Table 1-1. V: VP1013, lane P: Patriot.

Table 1-1 Genetic analysis for resistance to FOL race 2 of various cultivars and lines.

Cultivar or line	Source[z]	Resistance to FOL race 2	WF10-46-2	WF11-12	WF42-43	WF25-42	WF41-45	WF43-50	WF09-23
VP1013	NV	R	-	+	-	-	-	-	+
Patriot	NN	S	+	-	+	+	+	+	-
Banchu Red Fire	TS	R	-	-	-	-	-	-	+
Chouya No. 37	NF	R	+	+	+	-	-	-	+
D206	NV	R	-	+	-	-	-	-	+
Kikugawa No. 102	YN	R	-	+	-	-	-	-	+
N8-19	KS	R	+	+	+	-	-	-	+
NLE05	TS	R	-	-	-	-	-	-	+
Rusina66	TS	R	-	+	-	-	-	-	+
T-0566	TS	R	-	+	-	-	-	-	+
T-0567	TS	R	-	+	-	-	-	-	+
UC-002	YN	R	-	+	-	-	-	-	+
UC-003	YN	R	-	+	-	-	-	-	+
UC-006	YN	R	-	+	-	-	-	-	+
UC-007	YN	R	-	+	-	-	-	-	+
UC-008	YN	R	-	+	-	-	-	-	+
UC-016	YN	R	-	+	-	-	+	+	-
UC-017	YN	R	-	+	-	-	-	-	+
UC-018	YN	R	-	+	-	-	-	-	+

UC-023	YN	R	+	+	-	-	-	-	+
Wide View 6	YN	R	-	+	-	-	-	-	+
Wold Mans Green	TS	R	-	+	-	-	-	-	+
YL216	SA	R	+	-	-	-	-	-	+
D156	NV	S	+	-	+	+	+	+	-
D179	NV	S	+	-	+	+	+	+	-
Derosa	SA	S	+	-	+	+	+	+	-
Gokuwase Sisco	TS	S	+	-	+	+	+	+	-
Lalaport	YN	S	+	-	+	+	+	+	-
Olympia	MK	S	+	-	+	+	+	+	-
Raptor	YN	S	+	-	+	+	+	+	-
Shinano Green	NF	S	+	-	+	+	+	+	-
Shinano Hope	NF	S	+	-	+	+	+	+	+
Smart	TR	S	+	-	+	+	+	+	-
Ster Ray	TS	S	+	-	+	+	+	+	+
Success	TR	S	+	+	+	+	+	+	-
Summer Ace	NF	S	+	-	+	+	+	+	-
VI59	NV	S	+	-	+	+	+	+	+

[z] Seed corporation and institution was represented by abbreviation. Long form was as follows: NV; Nagano Vegetable and Ornamental Crops Experiment Station, NN; Nitto Nosan Seed, TS; Takii Seed, NF; Nagano Foundation Seed Center, YN; Yokohama Nursery, KS; Kaneko Seed, SA; Sumika Agrotec, MK; Mikado Kyowa Seed, TR; Tsuruta Seed.

Chapter 2

Construction of a practical SCAR marker linked to clubroot resistance in Chinese cabbage, with intensive analysis of HC352b genes

Chapter 2. Construction of a practical SCAR marker linked to clubroot resistance in Chinese cabbage, with intensive analysis of HC352b genes

2-1. Introduction

Clubroot is one of the major diseases of *Brassicaceae*. It is caused by the parasitic infection of *Plasmodiophora brassicae*, a soilborne fungus, in the stem root of plants (Ludwig-Müller, 1999). Chinese cabbage (*Brassica rapa* L. ssp *pekinensis*) is highly susceptible to the disease and frequently suffers destructive damage in infected fields. Several breeding programs have been successfully carried out to develop CR cultivars of Chinese cabbage using European turnips (*B. rapa* ssp. *rapifera*) as sources of resistance (Hirai et al., 2004; Matsumoto et al., 1998). CR in turnips is considered to be qualitative and is grouped into several genotypical categories corresponding to the pathotype of *P. brassicae* (Matsumoto et al., 2005). CR cultivars of Chinese cabbage are considered to have only a part of turnip CR genes, resulting limited CR reaction to the variety of pathotypes. For the stronger CR breeding, it is important to monitor the inheritance of these resistance genes using DNA makers that are linked to respective CR loci in the breeding of Chinese cabbage (Kuginuki et al., 1997; Matsumoto et al., 2005; Suwabe et al., 2003).

CRa is the responsible locus for CR in a dominant manner. HC352b was reported to be the most useful DNA marker linked to *CRa*, together with $E49_{380}$ (Matsumoto et al., 2005). The RAPD marker $E49_{380}$ is located 4.6 cM from *CRa* with a coupling linkage. HC352b was mapped at 2.9 cM from *CRa* on the opposite side of $E49_{380}$, and appeared to have a repulsion linkage. HC352b was established as an RFLP marker with a probe derived

from cDNA clone HC352 (Matsumoto et al., 1998). Although specific HC352b signals detected by cDNA show tight linkage to clubroot resistance, many unlinked signals were detected simultaneously. The appearance patterns of non-linked signals were complicated and confused, obstructing the practical use of HC352b in breeding programs.

In this chapter, the genomic information of the HC352 loci was analyzed intensively in two doubled haploid (DH) lines, T136-8 (*CRa*) and K10 (a susceptible allele of *CRa*), to construct a SCAR marker with the characteristics of usefulness, clarity and reproducibility on HC352b locus.

2-2. Materials and Methods

Plant materials

As source material with *CRa*, a DH line T136-8 (*B. rapa* L. ssp *pekinensis*) was used (Matsumoto et al., 2005). DH lines of *B. rapa* L. ssp *pekinensis*, K10 and Q5, were used as the parent as sources of a susceptible allele of *CRa*. K10 originated from the "CR Kanko" and does not have *CRa*. Q5 is a typical clubroot-susceptible line. The original resistance source of the parental line T136-8 is a member of the European Clubroot Differential (ECD) series, ECD 02 (*B. campestris* ssp. *rapifera* line AAbbCC).

Fourteen and 90 individuals of F_2 progeny between T136-8 × K10 and T136-8 × Q5 were used for DNA analysis and CR tests. F_1 between T136-8 and Q5 was crossed with Q5 to produce back-crossed (BC) populations. Each F_2 and BC individual was selfed to produce the next generation for segregation analysis.

DNA extraction

DNA was extracted from the leaves of parental lines, F_2 and BC_1 plants, according to the cetyl trimethyl ammonium bromide (CTAB) method (Murray and Thompson, 1980).

RFLP analysis

RFLP analyses were performed as previously described using the cDNA clone HC352 (Matsumoto et al., 1998) and its subclones composed of *Eco*RI-digested fragments. Conditions of electrophoresis, membrane transfer, probe preparation, hybridization, washing and signal detection were described previously (Matsumoto et al., 1998).

Cloning and sequencing

Regular procedures were used for cloning and sequencing DNA fragments (Nakanishi et al., 2005). A cloning vector pXcm, the vector for the TA cloning of PCR fragments, was used with a Ligation Kit (Nippon Gene, Tokyo, Japan). Sequencing was performed according to the manufacturer's instructions (ABI PRISM 310, Applied Biosystems, CA, USA). Analysis of sequence information was performed using the Genetyx software package (Software Development, Tokyo, Japan) and the BLAST search with DDBJ (http://blast.ddbj.nig.ac.jp/top-j.html). The sequence data produced in this report were submitted to DDBJ.

PCR

The standard protocol was used as follows: In a 10 μL reaction solution were mixed 3 ng of template DNA, 0.5 mM of forward and

reverse primer, respectively, 0.2 mM dNTPs, 1 μL of 10 × reaction buffer, 1.0 mM $MgCl_2$, and 0.5 unit of Taq DNA polymerase (Promega Corporation, Madison, USA). Amplification was performed in a thermal cycler programmed as follows: 94°C for 4 min, then 40 cycles at 94°C for 30 s, at an annealing temperature for 1 min and at 72°C for 1 min, followed by a final extension of 7 min at 72°C. The annealing temperature for F7-R11 was 38°C, and for F16-R13 it was 46°C. Sequences of F7 and R11 were "5'-TGCTACACCAAAAGATTCGAGAT-3' and 5'-GGATCTCTTCTGAATGTGATG-3'". F16 and R13 were designed as "5'-CTTTATAATGGCTACCTATTTA-3' and 5'-TGCTCATGAGTGTATAACTA-3'".

CR tests

Inoculation tests for resistance to clubroot were as previously reported (Matsumoto et al., 1998). As an inoculation source, we used the M85 isolate of *P. brassicae*, which was identified as race 2 according to Williams's classification (Williams, 1966). Selfed progeny of 14 and 90 F_2 plants derived from T136-8 × K10 and T136-8 × Q5, and 21 BC_1 plants produced from F_1 × Q5 were used for CR tests. Twenty to 40 seeds of each F_3 and the BC_1S_1 population 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). F_2 and BC_1 individuals that segregated no susceptible plants in their selfed progeny were scored as homozygotes (A) for *CRa*, while those that segregated both resistant and susceptible plants were assessed as heterozygotes (H). F_2 and BC_1 individuals whose selfed progeny were thoroughly galled were scored

as homozygotes (B) for susceptibility.

2-3. Results

Genomic information from RFLP analysis

Hybridization patterns of HC352 on the genomic DNAs of T136-8, K10, and their F_2 progeny were analyzed. T136-8 has *CRa*, only one locus with exclusive responsibility for CR in the cultivar. As shown in Fig. 2-1A, HC352 detected several fragments that were both in the lanes of K10 and T136-8.

For detailed analysis, HC352 was divided into two subclones at the *Eco*RI site located in the cDNA (Fig. 2-1B), and hybridization patterns were also developed using these subclones (Fig. 2-2 and data not shown). Using subclone 2 as a probe, 4 kb and 5 kb signals are clearly segregated, suggesting physical maps of HC352 genes. A summary of signals detected in parental lines is interpreted as in the scheme shown in Fig. 2-1B. It was necessary to assume that two or more loci were detected by HC352 in order to explain all of the hybridized signals. Here we judged one of these loci as HC352b that was found using other lines of Chinese cabbage (Matsumoto et al., 2005). The other locus should be HC352a.

Signals of 0.8 kb and 5 kb in length would reflect the existence of the HC352b-T allele. Unfortunately, however, if we use the full length of the HC352 cDNA clone, the signal at 0.8 kb is somewhat faint, and the signal at 5 kb is overlapped by another signal derived from HC352a-T.

Sequence information from cDNA clone HC352

The cDNA clone HC352 and two subclones were successfully

sequenced and submitted to DDBJ (AB302983). Database searching analysis showed high homology with genes encoding RNase L inhibitor proteins. Two genes, At4g19210 and At3g13640, of *Arabidopsis thaliana* have the highest homology, and sequence AY185357 was recorded from Chinese cabbage. Since AY185357 was 3' partial EST, unfortunately it was not sufficient to be referred for the primer design. In the *A. thaliana* genome, two genes coding the RNase L inhibitor protein are located separately, i.e., in chromosomes IV and III. This coincides with our scheme of the HC352 regions.

Sequence information from the HC352 regions of genomic DNA

Primers F7 and R11 were designed on the positions of highly conserved sequences with two introns in the target sequence. This primer set brought a couple of fragments with PCR using genomic DNA (Fig. 2-3). The discrete fragments in Fig. 2-3 were tentatively named $T\text{-}_L$, $T\text{-}_S$, $K\text{-}_L$, and $K\text{-}_S$, and were sequenced after cloning ($T\text{-}_L$: AB302979; $K\text{-}_L$: AB302980; $T\text{-}_S$: AB302981; $K\text{-}_S$: AB302982). All of these fragments had good homology to part of the cDNA clone HC352, with two extra sequences considered to be introns. $T\text{-}_L$ has higher homology to $K\text{-}_L$ (98.8%) than to $T\text{-}_S$ (84.7%). Namely $T\text{-}_L$ and $K\text{-}_L$ appear allelic, and $T\text{-}_S$ and $K\text{-}_S$ could be allelic. $T\text{-}_S$ and $K\text{-}_S$ were assumed to be *CRa* linked (see Discussion), and were used for the further design of primers.

Construction of the HC352b-SCAR marker

HC352b-SCAR was designed to be amplified by $T\text{-}_S$ specific primer set F16-R13, on the assumption that the diversity of $T\text{-}_S$ vs $K\text{-}_S$ is

the substantial polymorphism of HC352b. PCR with F_2 from T136-8 × K10 successfully amplified targeted fragments only from genomic DNAs of F_2 individuals that have the HC352b signal in RFLP analysis (Figs. 2-2 and 2-4). Finally, linkage of the HC352b-SCAR marker to the *CRa* phenotype was demonstrated using the F_2 population from T136-8 × Q5 and the BC population from F_1 × Q5 (Table 2-1). The HC352b-SCAR positiveness and the CR against M85 isolate (*CRa* phenotype) were indicated to be tightly linked.

2-4. Discussion

In this chapter, the co-dominant SCAR marker HC352b-SCAR tightly linked to *CRa* was established with intensive analysis of HC352-related genes. On the other hand, RAPD marker $E49_{380}$ was positioned at 4.6 cM from *CRa* with coupling linkage (Matsumoto et al., 2005). HC352b-SCAR would be positioned at 2.9 cM from *CRa* and 7.5 cM from $E49_{380}$, respectively, if it correctly detected "HC352b" as we expected. This result means that *CRa* was closely put between dominant and co-dominant markers, providing efficient selection of CR plants in CR breeding programs.

The hybridization patterns of RFLP analysis coincided with reported data using segregated populations from crosses among several *B. rapa* cultivars and lines, including fodder turnips with a slight change in the size of fragments (Matsumoto et al., 2005). The 4 kb fragment in Fig. 2-1 and 2-2 correspond to the 4.4 kb fragments detected in the susceptible lines as reported (Matsumoto et al., 1998, 2005). The scheme of restriction sites proposed in this report could explain those reported RFLP patterns,

with the possibility that the scheme would also be applicable to other cultivars of Chinese cabbage and to turnips.

HC352 cDNA-specific primers were designed at several positions referring to sequences of the *A. thaliana* homologue. Amplified products using cDNA-specific primers are single fragments in every combination, with no polymorphism between T136-8 and K10 (data not shown), indicating that these primers could amplify only one of the loci detected by RFLP analysis. *Eco*RI digestion experiments with amplified fragments suggested that HC352a (Fig. 2-1B) had been amplified exclusively. The sequence of the cDNA clone HC352 coincides almost completely with that of $T\text{-}_L$ and $K\text{-}_L$, indicating that the cDNA and $T\text{-}_L$ / $K\text{-}_L$ could be derived from allelic genes. Therefore, the cDNA clone HC352 and PCR fragments $T\text{-}_L$ and $K\text{-}_L$ were possibly derived from HC352a. We concluded that a simple effort to make direct conversion of the sequence information derived from the HC352 cDNA clone to the SCAR marker is not fruitful.

Analysis of the genomic sequence brought more insight into the constitution of HC352-related genes, supporting the scheme in Fig. 2-1B. Here, the genomic region HC352b-T in Fig. 2-1B may correspond to the $T\text{-}_S$ fragment in Fig. 2-3, and HC352b-K may correspond to $K\text{-}_S$, HC352a-T to $T\text{-}_L$ and HC352a-K to $K\text{-}_L$. $K\text{-}_L$ and $T\text{-}_L$ appear to be derived from allelic genes. $K\text{-}_S$ is also a counterpart of $T\text{-}_S$. In contrast, "HC352a" and "HC352b" genes are diverse genes that are related to each other as paralogs, having originated from the duplication of an ancestral gene. To confirm this possibility, the full sequences of HC352 genes are required.

Recently, large-scale duplications or multiplications in the genome of *B. rapa* were indicated in linkage map analysis (Kim et al., 2006).

Synteny analysis with *A. thaliana* also provides evidence of multiplication in the Chinese cabbage genome (Saito et al., 2006). This information makes us cautious regarding the cross-detection of paralogical genes in studies of marker-assisted breeding. To overcome this problem, re-sequencing of homologous genes might be a powerful tool, as in this work. The employment of divergence and polymorphism uncovered by intensive sequencing could become a stable and secure strategy.

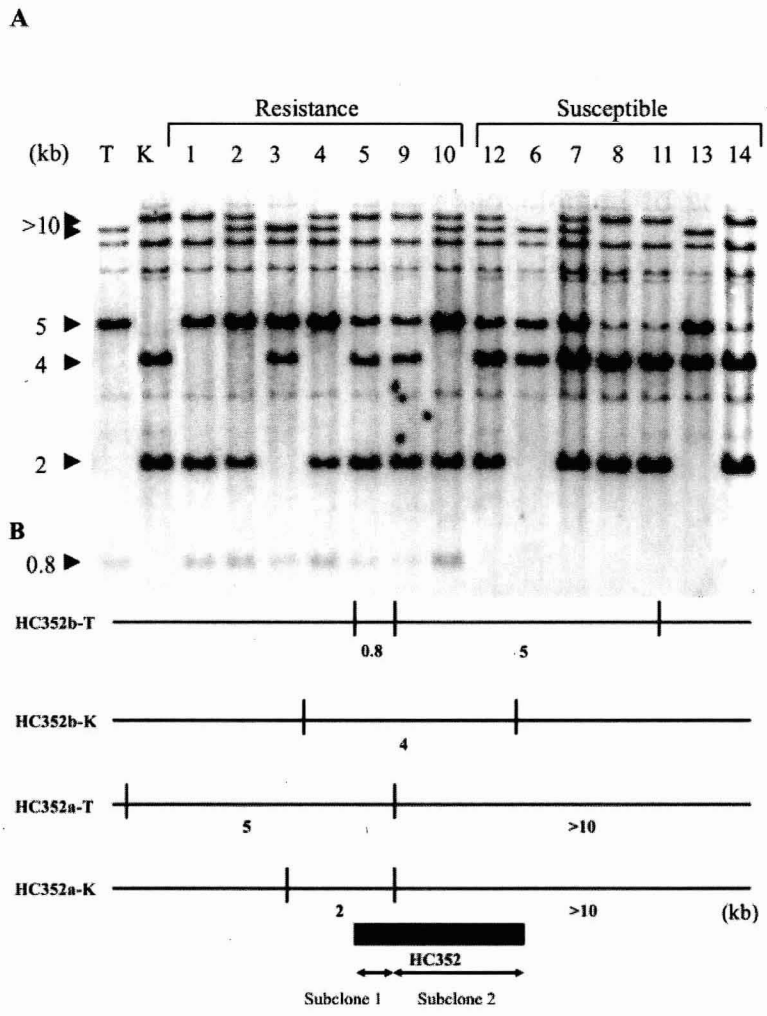


Fig. 2-1

Hybridization patterns of cDNA clone HC352 with the F_2 population of T136-8 × K10.

A) The full length of the clone was used for the probe. Lanes T, K, 1~5, 9, and 10 (resistance), and 6-8 and 11-14 (susceptible) indicate the genomic DNA of T136-8, K10, and the F_2 individuals showing CR and non-CR, respectively. Each genomic DNA was digested by *Eco*RI. B) Scheme of

the physical map of the HC352 genomic region. Horizontal lines indicate the genomic region containing the HC352 gene. The vertical line indicates *Eco*RI sites. Numbers correspond to the sizes of fragments detected in the hybridization. The filled box represents the position corresponding to the HC352 probe. Solid lines with arrowheads indicate positions of the subclones.

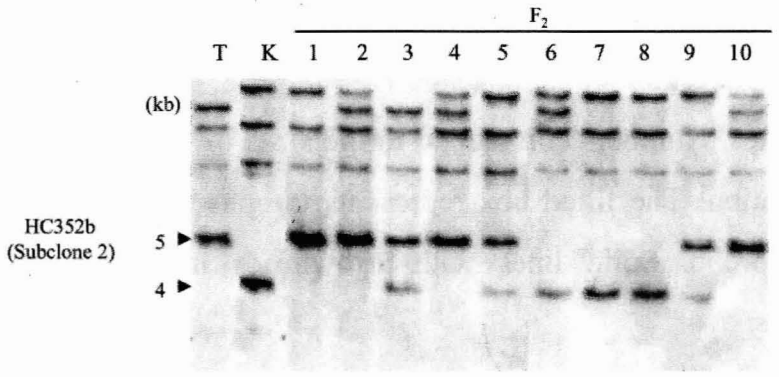


Fig. 2-2

Hybridization patterns of HC352 using subclone 2 of HC352 cDNA.

RFLP was detected as in Fig. 2-1A except that subclone 2 of HC352 cDNA was used as the probe. T: T136-8; K: K10; F_2, 1-10: individuals of F_2 progeny of T136-8 × K10.

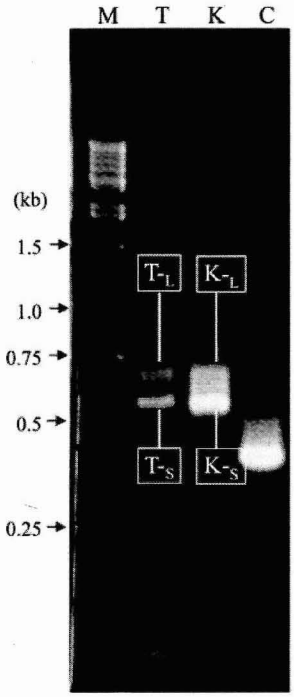


Fig. 2-3

PCR results using F7-R11 primers.

Templates are T: genomic DNA of T136-8; K: genomic DNA of K10; C: HC352 cDNA clone. Fragments indicated as $T\text{-}_L$, $T\text{-}_S$, $K\text{-}_L$, and $K\text{-}_S$ are recovered from agarose gel for further analysis (see text). M: 1 kb DNA Ladder.

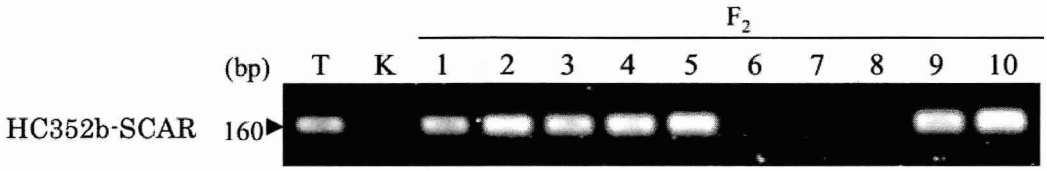


Fig. 2-4

Detection of HC352b-SCAR.

HC352b-SCAR was detected with PCR using F16-R13 primer set. Material plants are the same as in Fig. 2-2.

Table 2-1 Linkage between HC352b-SCAR and *CRa* phenotype.

Populations	HC352b-SCAR	*CRa* phenotype		
		A	H	B
F_2	+	23	60	0
	-	0	0	7
BC_1	+	-	15	0
	-	-	0	6

F_1 and F_2 plants were derived from T136-8 × Q5. BC_1 plants were produced from F_1 × Q5. *CRa* phenotype was scored with CR tests using each F_3 and BC_1S_1 populations. A: *CRa* homozygous; H: *CRa* heterozygous; B: Susceptible allele of *CRa*.

Chapter 3

Distribution of *CRa* in commercial
clubroot resistance cultivars of Chinese cabbage

Chapter 3. Distribution of *CRa* in commercial clubroot resistance cultivars of Chinese cabbage

3-1. Introduction

HC352b was used for further exploration of the gene responsible for the *CRa*. Finally it was identified producing a DNA marker, CRaim, designed to the *CRa* gene itself (Ueno et al., unpublished). The total length of *CRa* (8kb) and many paralogous genes in the Chinese cabbage genome make trouble for the direct isolation and sequence identification of full-length *CRa* genes in individual plants.

Clubroot resistance (CR) cultivars have been bred by several plant breeders in seed companies and public organizations. CR cultivars vary in several characters: growth rate, cold tolerance, head formation and timing of bolting. Such variety is preferable to adapt to the range of field conditions in Japan. However, CR genes in these cultivars are poorly understood. They have been bred without molecular knowledge of the CR genes, as breeding material further applications are also difficult when lacking the molecular information. In this chapter, commercial CR cultivars are tested for the presence or absence of *CRa*.

3-2. Materials and Methods

Plant materials

Forty-one commercial CR cultivars were collected from several breeding companies and used for DNA preparations. All were F_1 hybrid. The S_1 populations were produced by self-pollination of the cultivars and subjected to the CR test and DNA analysis. Two doubled haploid Chinese

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

CR test

Inoculation tests for resistance to clubroot were performed as previously reported (Matsumoto et al., 1998). As an inoculation source, we used the M85 isolate of *P. brassicae*, which was identified as race 2 according to Williams's classification (Williams, 1966). Eleven to 32 seeds of each S_1 population were sown in the pathogen-inoculated soil. M85 isolate of *P. brassicae* was used for the pathogen. Disease infection was rated 30 days after sowing. Evaluation of CR was based on the previous report (Matsumoto et al., 2005).

DNA extraction

DNA was extracted from leaves. A small piece of leaf, approximately 100 mg, was frozen at -80 °C for 2 hours 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 minutes, and then centrifuged at 18,000 g for 10 minutes. The supernatants were transferred to new tubes, and purified with a PCI treatment, chloroform treatment and ethanol precipitation. The purified DNA was resuspended in 50μL of TE buffer.

PCR

CRaim uses two combinations of primers to detect resistance, presence of *CRa*, and susceptibility, the absence of *CRa*. One combination of primers, Resistance-FW (5′-TATATTAATGATAAAGCAGAAGAAGAAA -3′) and Resistance-RV (5′- AATGCGACTGAGAAAGTTGTAG -3′), was used to detect *CRa* (resistant type). Another set of primers, susceptible-FW (5′-TGAAGAATGCGGGCTACGTCCTCTGAAATC-3′) and susceptible-RV (5′- GAAGTAGATGAACGTGTTTATTTTAGAAA-3′), was used to detect the susceptible (not *CRa*). The PCR reaction solution contained 3 ng template DNA, 0.5 μM primers, 0.2 mM dNTPs, 1 μL of 10 × reaction buffer, 4 mM $MgCl_2$, and 0.25 unit of Taq DNA polymerase (Bioline) in a total volume of 10 μL. For the resistant allele, thermal cycler (ThermoGen) was programmed as follows: 94°C for 1 min; 35 cycles of 94°C for 30 s, 63°C for 30 s, and 72°C for 1 min; and 72°C for 7 min. For the susceptible allele, the program was as follows: 94°C for 1 min; 38 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 2 min; and 72°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 photo image instrument (ATTO).

3-3. Results

CR test and CRaim detection

The genetic locus *CRa* was originally identified in the T136-8 line by resistance to the M85 isolate of *P. brassicae*. Twelve cultivars were

selected by the CR test using their S_1 populations infected by the M85 isolate. PCR detection using the DNA marker CRaim indicates they all have *CRa* gene (Table3-1). This result showed that the presence of *CRa*, as judged by M85 resistance, coincided with the results of the CRaim detection.

Detection of *CRa* in CR cultivars by CRaim

Further detection of *CRa* was carried out in various CR cultivars (Table 3-2). Among the 41 cultivars, 21 showed heterozygous genotypes. Eleven cultivars appeared to have the *CRa* in a homozygous genotype indicating both paternal and maternal lines of the F_1 seed bore the *CRa*. Nine cultivars were indicated to have no *CRa* homozygote of the susceptible allele.

3-4. Discussion

In this chapter, genotyping of the *CRa* locus in commercial CR cultivars was identified by CR test and the DNA marker CRaim. Thirty-two out of 41 commercial cultivars retained the *CRa* gene, indicating that the *CRa* is widely distributed in CR cultivars of Chinese cabbage, in both homozygous and heterozygous forms. Homozygotes could be derived from a breeding strategy that selected CR lines repeatedly to achieve the appropriate parents for F_1-seed production. Information on *CRa* produced by this study will be helpful for breeders in seed companies because, they will no longer need duplicated selection of CR candidates. *CRa* works dominantly and could be introduced (or retained) in cultivars that have original, desired features by monitoring only CRaim. *CRa* could

be bred into cultivars which can adapt to a variety of conditions in the field, and the orders of farmers.

Among the 41 CR cultivars, 9 cultivars were indicated to retain no *CRa* (the susceptible *CRa* allele). These cultivars could possess other CR loci. At present a total of 7 CR loci, *CRa*, *CRb*, *CRk*, *CRc*, *Crr1*, *Crr2* and *Crr3*, have been reported for Chinese cabbage (Hirai et al., 2004; 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 genes. These CR cultivars are widely used in major production areas, but do not always have consistent results due to the breakdown of resistance. Because these cultivars have only one or a few of all the CR genes, they are fragile against the invasion of a new race of the pathogen. Pyramiding of the resistant genes, or multiline technique are effective ways to avoid resistance breakdown. Pyramiding is the breeding technique that accumulates multiple genes of similar function in a line, and multiline is the horticultural technique of planting many lines (each line bears single resistance) in a field. For both pyramiding and multiline, information on individual genes is necessary. Although *CRa* emerged as the representative CR gene in this work, it is not sufficient, and information on the other CR genes is required to achieve strong CR resistance, as in the fodder turnip. To identify the genotypes of the other CR loci, DNA markers that detect the respective CR loci are needed. Some of them have been developed already, but the genetic distances are still too large to use. It is expected that DNA markers, like CRaim, will be developed and applied, thus opening a new stage of CR breeding in Chinese cabbage.

Table 3-1 Genotypes of CRa locus in selfed progenies derived from commercial CR cultivars

Cultivar	Total number of S_1 progeny	Resistance to M85[Z]	Number of plants for CRaim genotype Resistant allele / Susceptible allele[Y]		
			A	H	B
CR Akogare	11	R	2	3	0
		S	0	0	6
CR Kyotakara	27	R	4	16	0
		S	0	0	7
CR Ohken	12	R	2	7	0
		S	0	0	3
CR Strong 75	27	R	9	7	0
		S	0	0	11
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
Kunki 70	12	R	0	0	4
		S	0	0	8
Masashige	15	R	2	8	0
		S	0	0	5
Ryutoku	28	R	10	14	0
		S	0	0	4
Super CR Shinrisoh	24	R	8	6	0
		S	0	0	9
T136-8	5	R	5	0	0
Q5	5	S	0	0	5

[Z] 'R' indicates resistance to M85 isolate of *P. brassicae* and 'S' indicates susceptible to M85 isolate of *P. brassicae*.

[Y] 'A' indicates resistant homozygote, 'H' indicates heterozygote and 'B' indicates susceptible homozygote.

Table 3-2 Genotypes of CRa locus in commercial CR cultivars

Number	Cultivar	Source[Z]	CRaim genotype[Y]	Number	Cultivar	Source[Z]	CRaim genotype[Y]
1	Chiyobuki 85	SS	A	23	Kiraboshi 65	TK	H
2	CR Akogare	NN	H	24	Kiraku 60	TS	H
3	CR Gekka	KS	A	25	Kiryoyoshi	KA	H
4	CR Kikoma	KS	H	26	Kougetsu 77	KA	H
5	CR Kisaku 80	MA	H	27	Kunki 70	WS	B
6	CR Kyotakara	MA	H	28	Masashige	WS	H
7	CR Ohken	KY	H	29	Minebuki 505	SS	A
8	CR Seiga	IS	H	30	Moegi	KA	H
9	CR Senmai 65	TS	B	31	Muso	TK	B
10	CR Sensyuu 65	TS	B	32	Ryutoku	WS	H
11	Strong CR 75	WS	H	33	Saegi 90	IS	A
12	CRREmperor	KS	A	34	Satobuki 613	SS	A
13	Daifuku 234	TS	B	35	Shinseiki	TS	B
14	Gokigen 65	TA	B	36	Super CR Hiroki	KB	B
15	Haregi 65	TK	A	37	Super CR Kimi 85	KB	B
16	Fuyusae	IS	H	38	Super CR Shinrisoh	NN	H
17	Kaname	WS	H	39	Syouki	KA	A
18	Kiai 65	WS	A	40	Yumebuki502	SS	H
19	Kigokoro 65	TK	A	41	Yuuki	TK	H
20	Kifuku 65	NN	H		T136-8	NV	A
21	Kikou 65	WS	H		Q5	NV	B
22	Kinami 90	IS	A				

[Z] Seed corporation and institusion was represented by abbreviation. Long form was as follows: SS; Sakata Seed, NN; Nippon Norin Seed, KS; Kobayashi Seed, MA; Marutane, KY; Kyowa Seed, IS; Ishii Seed Growers, TS; Tohoku Seed, WS; Watanabe Seed TA;Takeyama Seed, TK; Takii Seed, KA; Kaneko Seed, KB; Kakinuma Breeding Center, NV Nagano Vesitable and Ornamental Crops Experiment Station.

[Y] 'A' indicates resistant homozygote, 'H' indicates heterozygote and 'B' indicates susceptible homozygote.

General discussion and Conclusion

In this thesis, development of DNA markers that enable the MAS breeding in lettuce and Chinese cabbage, was performed.

In chapter 1, FOL race 2 resistant locus *RRD2* was identified by analysis of its heredity pattern, construction of linkage map and QTL analysis. Detection pattern of RAPD marker WF25-42 was demonstrated to represent the phenotype of *RRD2* locus perfectly, throughout the various cultivars and lines. In addition, WF25-42 was converted into SCAR marker.

In chapter 2, RFLP marker HC352b which is linked to clubroot resistant locus *CRa* was analyzed and converted into a PCR based SCAR marker. The developed HC352b-SCAR detected the *CRa* locus easily and accurately more than the original HC352b.

In chapter 3, distribution of *CRa* gene was analyzed in the commercial CR Chinese cabbage cultivars. Retention of the *CRa* locus was identified by CR test and the genotype of *CRa* gene was judged by DNA marker designed on the *CRa* gene. It was turned out that *CRa* was found in more than 78 % of cultivars tested, indicating it is the major CR gene in the commercial cultivars.

The DNA markers developed in this study will enable efficient MAS selection for breeding programs. WF25-42-SCAR marker in lettuce will be offered to Nagano Vegetable and Ornamental Crops Experiment Station. The marker is planned to be used in breeding programs to develop new resistant cultivars of lettuce. HC352b-SCAR in Chinese cabbage enabled to perform a more detailed linkage analysis against the *CRa* region. In the collaborative research, more tightly linked marker was developed

through the application of HC352b-SCAR, and was used to create a pyramiding line possessing 3 individual CR loci (Matsumoto et al., 2012). The information of *CRa* distribution in the CR cultivars also will be offered to and used by the breeders in the seed companies.

It is important to accumulate DNA markers linked to the various traits in the single crop using broad genetic resources. With the huge number of markers that cover major genes responsible for all agronomically important traits, individual characters could be picked up from the resource pool, and combined freely to develop novel cultivars as desired. To open the path for this strategy, called "order-made breeding", massive work should be needed to design DNA markers. Recently developed high-throughput DNA sequencing systems will help the massive work providing whole genome sequence (WGS) of the genetic resources of the crop. Combination of WGS would work as most detailed linkage map and enable easier design of the DNA markers than the currently used method such as RFLP, RAPD and AFLP. Together with acceleration of DNA marker design, agronomically important genes should be identified and analyzed its function and role in the crop.

Of this perspective, progression of the breeding science is expected to provide fertile and sustainable agricultural production for human kind in the future, and I hope this work would be a part of the foundation of the progression.

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Quantitative trait loci analysis for resistance against Turnip mosaic virus based on a doubled-haploid population in Chinese cabbage. Plant Breed. 127: 82-87

List of Publications

Daisuke Aruga • Nobuaki Tsuchiya • Hideo Matsumura • Etsuo Matsumoto • Nobuaki Hayashida (2012) Analysis of RAPD and AFLP markers linked to resistance to *Fusarium oxysporum* f. sp. *lactucae* race 2 in lettuce (*Lactuca sativa* L.) Euphytica DOI: 10.1007/s10681-012-0665-5

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