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

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Abstract

Root rot of lettuce, which is caused by *Fusarium oxysporum* f. sp. *lactucae* (FOL), is a critical problem in the production of lettuce. FOL-resistant lettuce genetic resources have been identified and used in breeding programs to produce FOL-resistant cultivars. However, the genetic characteristics of resistance genes have not been studied in depth and, therefore, no DNA markers are presently available for these genes. In this study, we analyzed the *RRD2* (resistance for root rot disease race 2) locus, which confers resistance to FOL race 2. Resistance loci were analyzed using two cultivars of crisphead lettuce: VP1013 (resistant) and Patriot (susceptible). The segregation patterns of resistant phenotypes in F_2 indicated a single major locus. To define the positions of resistance loci, a linkage map was constructed using amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) markers. Quantitative trait loci analyses revealed the position of the major resistance locus. A high LOD score was observed for RAPD-marker WF25-42, and this marker showed good correspondence to the phenotype in different cultivars and lines. We successfully developed a sequence characterized amplified region (SCAR) marker from WF25-42.

Introduction

Root rot of lettuce, a soil-borne disease that occurs in many countries, has become a serious problem for lettuce production (Scott et al., 2010). This disease is caused by *Fusarium oxysporum* f. sp. *lactucae* (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. In Japan, FOL is classified into three races, namely 1, 2, and 3 (Fujinaga et al., 2001, 2003). Lettuce genetic resources were evaluated and selected using these races by Tsuchiya et al. (2004a). The 'Shinano Hope' cultivar was bred to be resistant to FOL race 1 (Tsuchiya et al., 2004b), and 'Chouya No. 37' was bred to be resistant to FOL race 2 (Tsuchiya, 2009). These breeding programs to obtain resistant lettuces were carried out using conventional selection, using resistance tests based on pathogen inoculation.

Marker-assisted selection (MAS) is a powerful technique for efficient and rapid selection of certain phenotypes in conventional breeding programs. Disease-resistant candidates can be selected without pathogen inoculation, reducing the time and expense of field work. Genetic markers that are tightly linked to resistance genes, preferably less than 5 cM of genetic distance, are required for MAS in crop breeding (Mohan et al., 1997: Collard and Mackill, 2008). Construction of a detailed genetic map can provide genetic markers that are tightly linked to the locus controlling the character of interest.

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.

The aim of this study was to obtain genetic markers for further breeding programs of FOL-resistant commercial lettuce cultivars. We constructed a linkage map using amplified

fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) markers and performed quantitative trait loci (QTL) analysis to identify FOL resistance loci.

Materials and Methods

Plant materials

We used the crisphead lettuce cultivars VP1013 (resistant to FOL race 2) and Patriot (susceptible) in this study. VP1013 is bred by Nagano Vegetable and Ornamental Crops Experiment Station from commercial cultivar Kikugawa No. 102 bred by Yokohama Ueki Corp, with self pollination (Tsuchiya et al. 2004a, 2009). 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, we used 14–22 plants of the selfed F_3 population to test disease resistance. DNA markers were evaluated using an additional 18 fixed lines and 17 commercial cultivars (see Table 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 incidences and disease severities of F_2 populations were calculated as follows using disease indexes of corresponding F_3 lines:

Disease incidence (%) = number of diseased plants $\times$ 100/number of examined plants.

Disease severity = (3A+2B+C) $\times$ 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 using the cetyl trimethyl ammonium bromide (CTAB) method (Hayashida et al., 2008).

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 EcoRI+AGS (S is G or C) × 8 MseI+CWN (W is A or T and N is A, C, G, or T), EcoRI+ACA × MseI+CTG, EcoRI+ACC × MseI+CTA, EcoRI+AAG × MseI+CTT, and EcoRI+ACT × MseI+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).

Results

Segregation of resistance to FOL race 2

The disease severity of the F_2 progenies derived from the cross between the 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 lines 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 lines, 0–20; 32 lines, 20–80; 14 lines, 80–100. The disease severities of roots were distributed as follows: 11 lines, 0–20; 32 lines, 20–80; 15 lines, 80–100. Regardless of tissue, resistance to FOL race 2 as scored by the disease severity seemed to segregate at the expected ratio of 1:2:1 in the F_2 population (Fig. 1). Results shown in figure 1. suggested resistance to FOL race 2 might be determined by a single major dominant locus, but with minor loci that contribute some modifications. We designated the major locus as *RRD2* (resistance for root rot disease race 2).

Establishment of molecular markers

We carried out AFLP analysis 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 identify polymorphic amplified bands that were tightly linked to *RRD2*. The candidate 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

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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. 2). 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. 2). 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. 3).

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 strongly with the phenotypes of resistance to FOL race 2 (Table 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, Kikugawa No. 102, 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, N8-19, UC-016, 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 be utilized as a SCAR marker. The resultant WF25-42-SCAR showed completely identical signals with the RAPD WF25-42 (Fig. 4).

Discussion

We constructed a linkage map of lettuce 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 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 No. 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 occurred.

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 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 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.

Acknowledgments

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Figure legends

Fig. 1

Disease severities among F_2 plants.

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 increments.

Fig. 2

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. 3

Relationship between RAPD markers and resistance phenotypes.

Disease severities and genotypes of RAPD markers of individual F$_2$ plants derived from VP1013 $\times$ Patriot cross were plotted.   The RAPD markers used were: A) WF25-42, B) WF43-WF45 and C) WF09-WF23.   Dots represent each F$_2$ plant, and indicate disease severity of roots and positive/negative signals of the marker.

Fig. 4

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. V: VP1013, lane P: Patriot.

Table 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	+	-	+	+	+	+	-
Sterray	TS	S	+	-	+	+	+	+	+
Success	TR	S	+	+	+	+	+	+	-
Summer Ace	NF	S	+	-	+	+	+	+	-
VI59	NV	S	+	-	+	+	+	+	+

ᶻ Seed corporation and institusion 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.
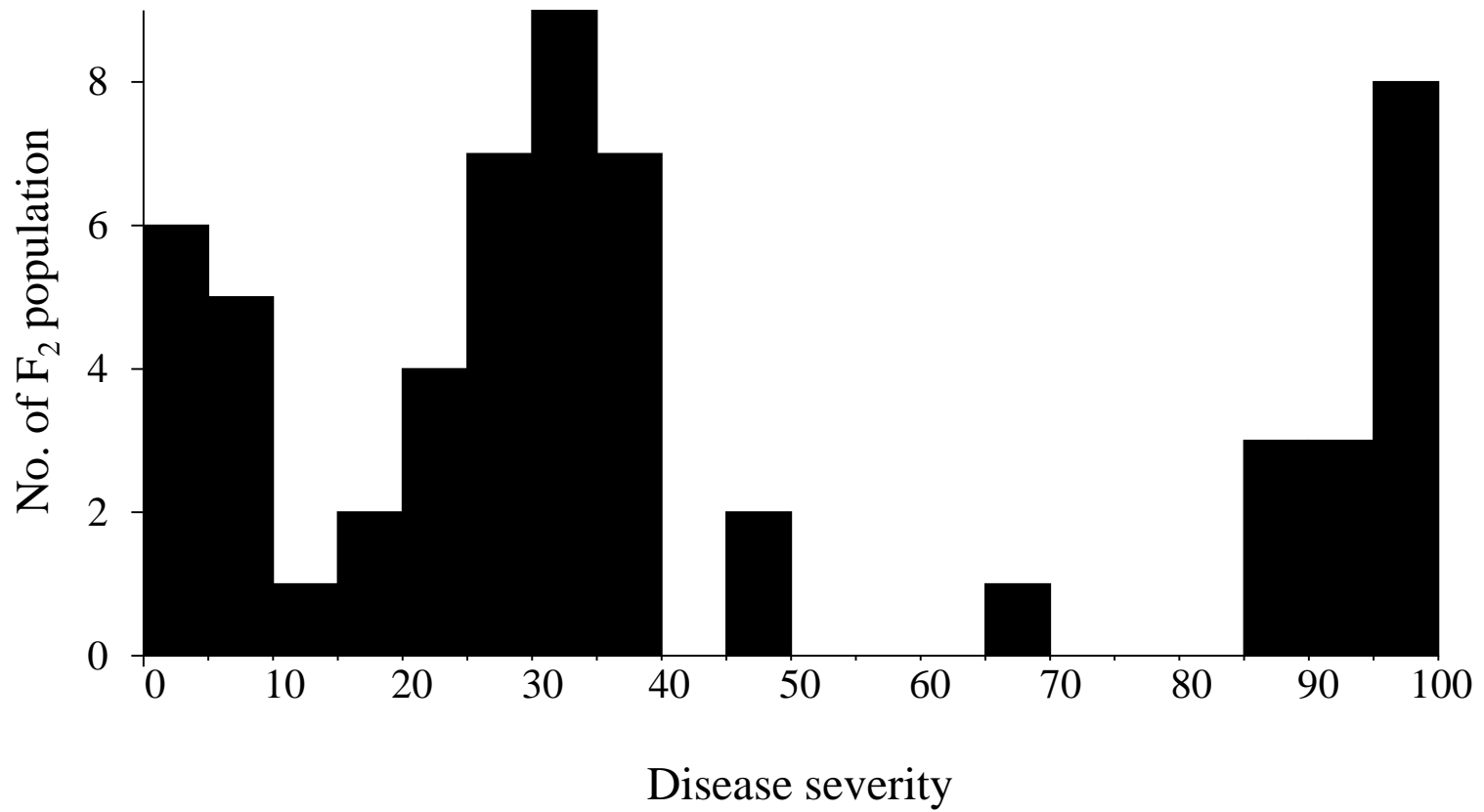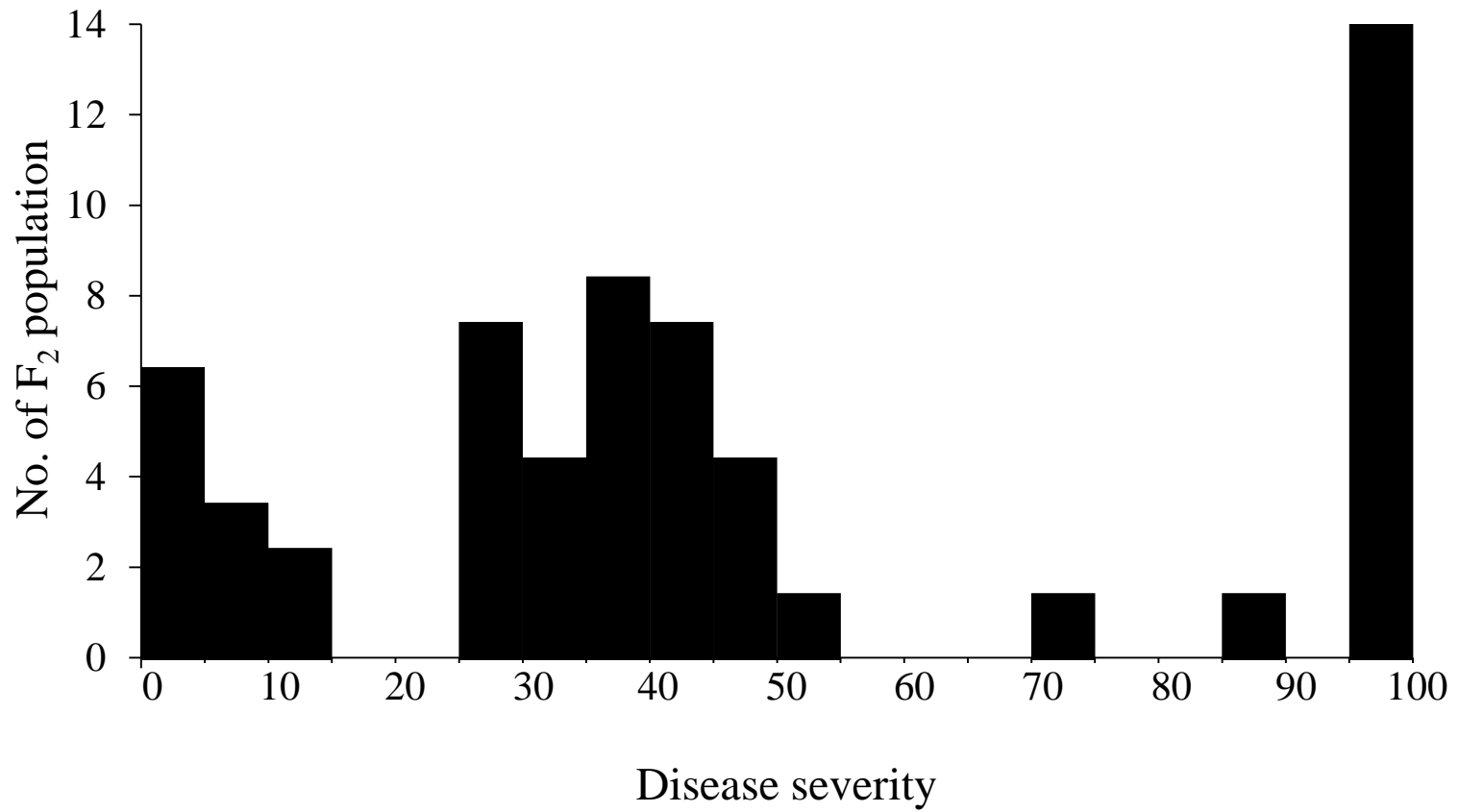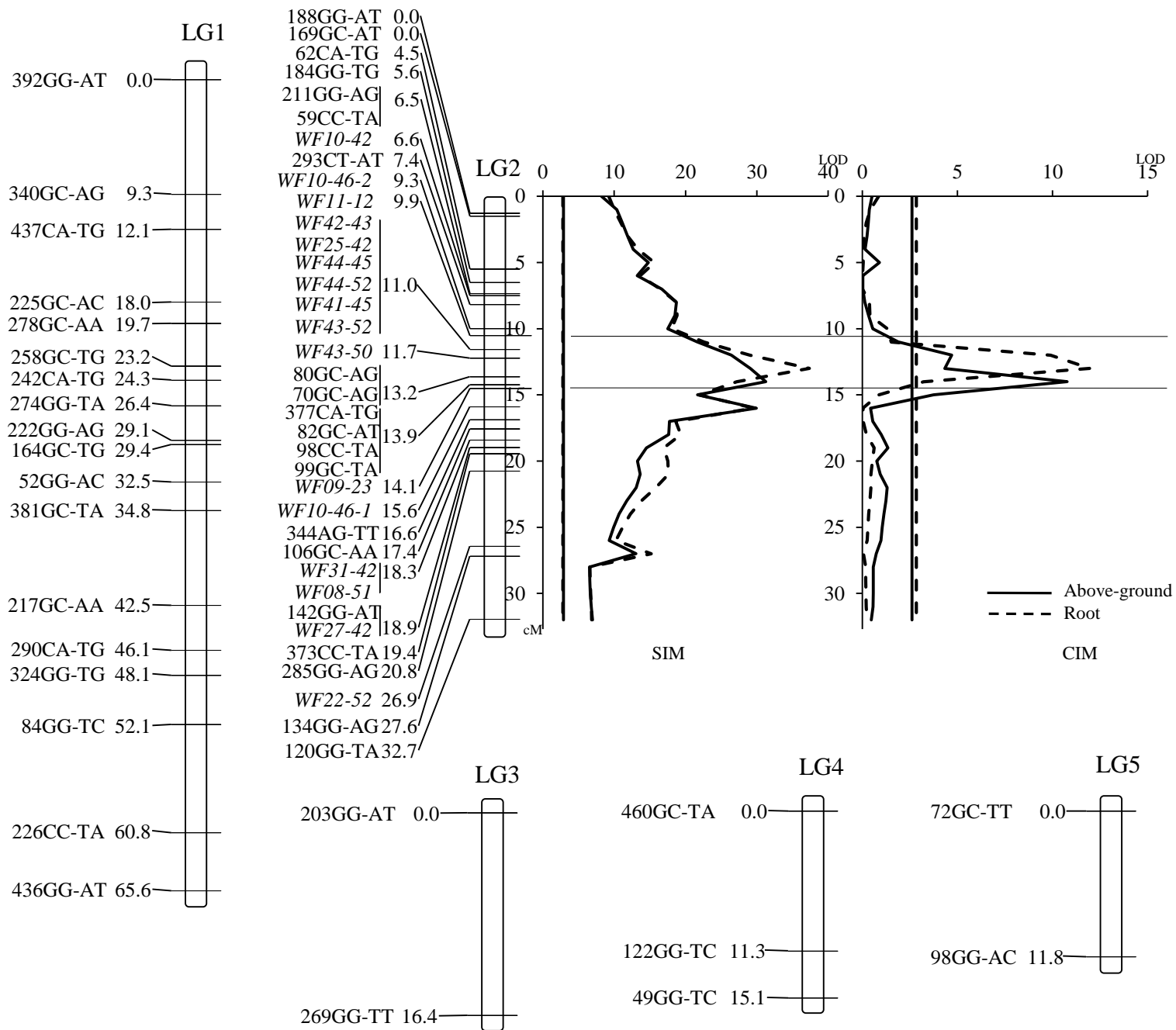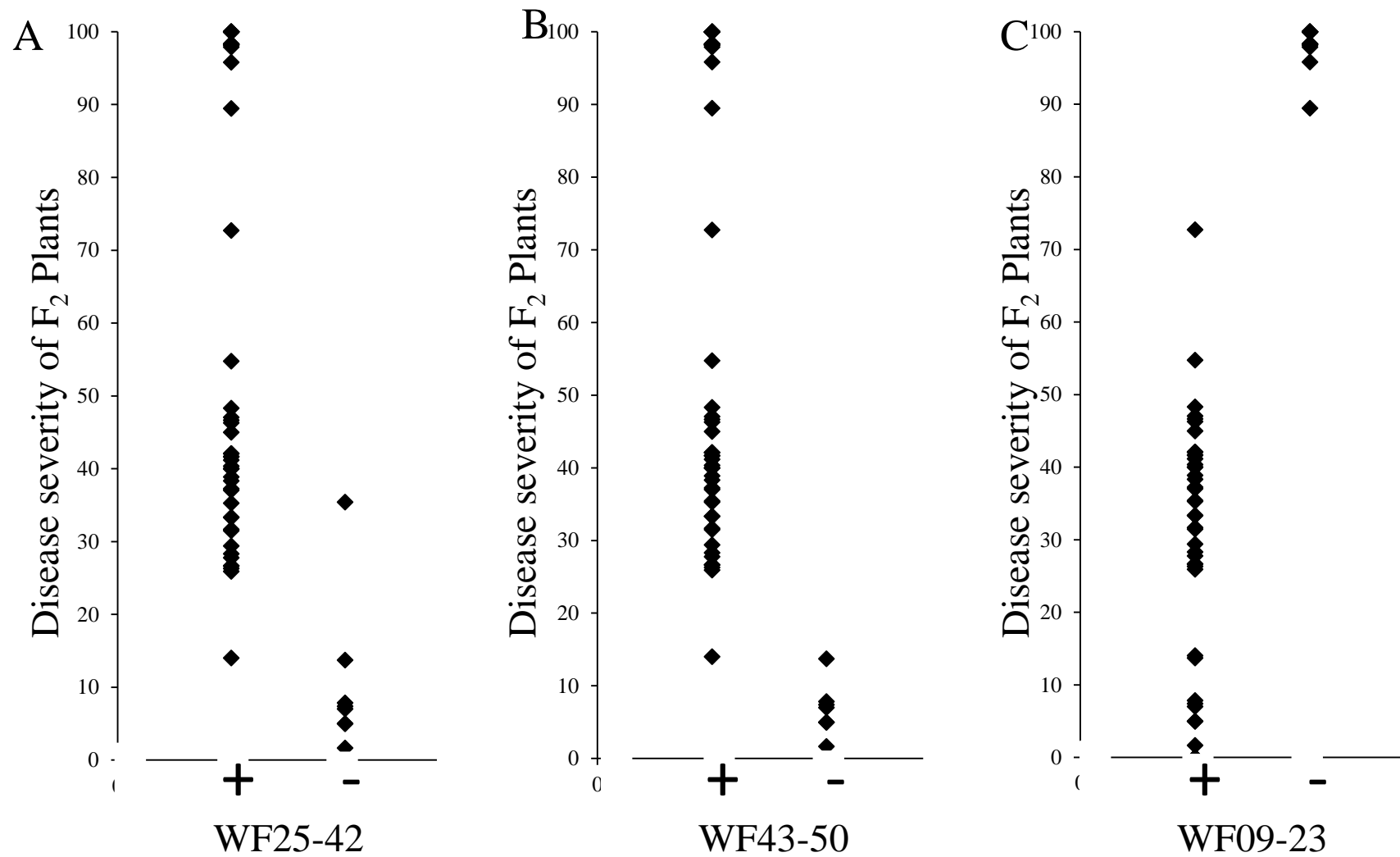
Fig. 1 A

Fig. 1 B

Fig. 2

Fig. 3

A — WF25-42
B — WF43-50
C — WF09-23

Fig. 4