

# Mapping candidate QTLs related to plant persistency in red clover

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**Abstract** Red clover (*Trifolium pratense* L.) is a diploid ( $2n = 14$ ), self-incompatible legume that is widely cultivated as a forage legume in cold geographical regions. Because it is a short-lived perennial species, improvement of plant persistency is the most important objective for red clover breeding. To develop a marker-assisted selection (MAS) approach for red clover, we identified candidate QTLs related to plant persistency. Two full-sib mapping populations, 272 × WF1680 and HR × R130, were used for QTL identification. Resistance to *Sclerotinia trifoliorum* and *Fusarium* species, as well as to winter hardiness, was investigated in the laboratory and in field experiments

in Moscow region (Russia), and Sapporo (Japan). With the genotype data derived from microsatellite and other DNA markers, candidate QTLs were identified by simple interval mapping (SIM), Kruskal–Wallis analysis (KW analysis) and genotype matrix mapping (GMM). A total of 10 and 23 candidate QTL regions for plant persistency were identified in the 272 × WF1680 and the HR × R130 mapping populations, respectively. The QTLs identified by multiple mapping approaches were mapped on linkage group (LG) 3 and LG6. The significant QTL interactions identified by GMM explained the higher phenotypic variation than single effect QTLs. Identification of haplotypes having positive effect QTLs in each parent were first demonstrated in this study for pseudo-testcross mapping populations in plant species using experimental data.

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## Introduction

Red clover (*Trifolium pratense* L.) is a diploid ( $2n = 14$ ), self-incompatible legume that is widely cultivated as a forage legume in moist and cool regions of the world, due to its high seedling vigor and adaptability to acidic and humid conditions. Because it is a short-lived perennial species, the use of red clover often results in a reduction in the quality and quantity of forage (Taylor and Quesenberry 1996). Therefore, most red clover breeding programs to date have primarily emphasized persistency, i.e., individual plant longevity (Taylor 2008). However, improvement of plant persistency has been a challenging objective for conventional red clover breeding programs, because it is a complex trait and requires lengthy studies for trait investigation. Marker-assisted selection (MAS) using DNA markers is expected to make an important contribution to red clover breeding programs that aim to enhance persistency.

Although several factors affect plant persistency in red clover, the main factor, which leads to a significant reduction in red clover green matter (from 30 to 80%), is the biotic stress caused by pathogen infections (Novosiolova 2002). One of the most destructive diseases is *Sclerotinia* crown and root rot, which is caused by the fungal pathogen *Sclerotinia trifoliorum* Erikson (Dijkstra 1964). The pathogen infects plants in the autumn and develops in the plant under snow cover during the winter. The disease symptoms rapidly appear on the plant once temperatures rise and the snow melts. Another important disease is *Fusarium* root rot, which is most frequently caused by *Fusarium* species, such as *F. oxysporum*, *F. solani* and *F. roseum* (Pederson et al. 1980). Because the root rot pathogens tend to accumulate in the soil, it is difficult to control root rot in red clover (Taylor and Quesenberry 1996). Once the pathogen infects the plant, root rot develops throughout plant growth.

Along with biotic stresses, abiotic stresses have an impact on plant persistency in red clover. Because red clover is predominantly produced in cold regions, a low temperature in the winter is generally considered the most severe abiotic stress. Plant vigor and/or survival rate after winter is often used in red clover breeding programs as an index of winter hardiness. Winter hardiness is considered to be a composite trait, consisting of a mixture of freezing tolerance and disease resistance under snow (Larsen 1994).

Several studies have analyzed quantitative trait loci (QTL) related to *S. sclerotiorum* resistance in sunflower (*Helianthus annuus*, Bert et al. 2004; Micic et al. 2004; Rönicke et al. 2005; Yue et al. 2008), rapeseed (*Brassica napus*, Zhao et al. 2003, 2006), soybean (*Glycine max*, Kim and Diers 2000; Arahana et al. 2001; Vuong et al. 2008) and common bean (*Phaseolus vulgaris*, Miklas 2007; Park et al. 2001), but no reports have focused on QTLs related to *S. trifoliorum* resistance. Most candidate QTLs identified for *S. sclerotiorum* resistance do not explain the large amount of phenotypic variation, which ranges from 10 to 18% in sunflower, 22–23% in rapeseed, and 10–12% in soybean. Miklas (2007) reported the identification and marker-assisted backcrossing of two QTLs associated with partial resistance to *S. sclerotiorum* in the common bean. Each candidate QTL explained 30–52% of the phenotypic variation for disease symptoms in a greenhouse test and accounted for 10% of the variation in a BC<sub>3</sub>F<sub>4:6</sub> population grown in the field. QTLs for *Fusarium* root rot resistance explained 5–53% of the total phenotypic variability (Román-Avilés and Kelly 2005; Schneider et al., 2001). These reports imply that *Sclerotinia* and *Fusarium* resistance are controlled by multiple genes and pyramiding genes by MAS may therefore be an efficient means to improve QTL identification.

Although QTL identification has been performed in various plant species for the past two decades, investigation of QTLs in self-incompatible species has lagged behind examination of self-compatible species, because of the difficulty in developing inbred lines for segregation analysis. The most popular approach for identifying QTLs in self-incompatible species is to develop pseudo-testcross populations for segregation analysis, and then identify QTLs by interval mapping (Brown et al. 2007; Liebhart et al. 2003; Jansen and Stam 1994; Sim et al. 2007; Studer et al. 2007). In red clover, QTLs have been identified for seed yield, plant persistency, and morphological traits using a pseudo-testcross population (Herrmann et al. 2006, 2008). Herrmann et al. (2008) investigated plant persistency based on seasonal plant vigor and identified several QTLs related to plant persistency by multiple QTL mapping (MQM). The QTL on linkage group (LG)3 which was identified on the basis of one seasonal vigor score (average in the third year) as well as three different indices of persistency was the most potent QTL and explained 7.0–12.2% of the observed phenotypic variability.

Though candidate QTLs have been identified in several self-incompatible species, there have been few attempts to develop strategies to use MAS with the identified QTLs. Complex factors, such as haplotype phase, haplotype combination, high heterozygosity and QTL interactions, may make it difficult to interpret the results of interval mapping and to use the identified QTLs for MAS. Recently, Isobe et al. (2007) developed a new QTL mapping approach, genotype matrix mapping (GMM), which searches for QTL interactions in genetic variation. While this demonstrated that multiple QTL interactions could be identified in unrelated individual populations, we considered that the GMM approach would also be efficient for QTL identification in a family population. The identification of multiple QTL interactions by GMM will greatly advance our understanding of the complex QTL expression in a self-incompatible species.

In the present study, we sought to identify QTLs for three stress tolerance traits related to plant persistency in red clover, namely *S. trifoliorum* resistance, *Fusarium* resistance, and winter hardiness. In order to multilaterally discuss the characteristics of the identified QTLs, we performed QTL detection using three calculation approaches, simple interval mapping (SIM), Kruskal–Wallis (KW) analysis, and GMM. SIM is a parametric mapping approach, while KW analysis and GMM are non-parametric mapping approaches (Lehmann 1975; Isobe et al., 2007). SIM and KW analysis identify single locus effects whereas GMM considers multiple locus effects, as described above. In addition, identification of haplotypes having QTLs with positive effects in each heterozygous parental plant was first demonstrated by this study. The study was

**Table 1** Experimental design of the investigated traits related to plant persistency

Population and trait	Trait name	Test field	Location	Season		No. of tested genotypes	No. of replications
				Transplanting	Investigation		
<i>272 × WF1680</i>							
<i>S. trifoliorum</i> resistance							
Laboratory experiment (damage rate, %)	SRL	Laboratory	WFCRI <sup>a</sup>	–	2003	36	2
Field experiment (extent of plant disease, %)	SRR	Non-artificial	Moscow region	2001 May	2002 April	59	6
<i>Fusarium</i> resistance							
Field experiment (intensity of disease development, %)	FRR	Non-artificial	Moscow region	2001 May	2002 April	59	6
Winter hardiness							
Green part ratio of crown (%)	WHJ	Non-artificial	Sapporo	2001 July	2002 April	139	1
<i>HR × R130</i>							
<i>S. trifoliorum</i> resistance							
Field experiment (extent of plant disease, %)	SRR	Artificial	Moscow region	2004 July	2005 April	96	4–13
Field experiment (1: very sensitive; 9: very resistant)	SRJ	Non-artificial	Sapporo	2003 October	2005 April	177	5
<i>Fusarium</i> resistance							
Field experiment (intensity of disease development, %)	FRR05	Artificial	Moscow region	2004 July	2005 April	96	4–13
Field experiment (intensity of disease development, %)	FRR07	Artificial	Moscow region	2006 June	2007 October	61	4–10
Winter hardiness							
Survival rate after winter (%)	WHR	Artificial	Moscow region	2004 July	2005 Apr	96	1
Winter hardiness (1: very weak; 9: very hardy)	WHJ	Non-artificial	Sapporo	2003 October	2004 Apr	178	5

<sup>a</sup> All-Russian Williams Fodder Crop Research Institute

performed with two pseudo-testcross mapping populations and two phenotypic investigation sites (Moscow region, Russia and Sapporo, Japan), in order to enhance the possibility of capturing various QTLs.

## Materials and methods

### Plant materials for QTL mapping

Two previously reported red clover mapping populations, *272 × WF1680* and *HR × R130*, were used in this study. The *272 × WF1680* population is a BC<sub>1</sub>F<sub>1</sub> population consisting of 167 individuals, in which the ‘272’ parent was a single F<sub>1</sub> plant arising from a cross between ‘1588’, a wild germplasm collected in the Arhangelsk region of Russia, and ‘WF1680’, which originated from a central Russian variety “Srednerusskiy” (Isobe et al. 2003). *HR × R130* is a one-way pseudo-testcross mapping population consisting of 188 individuals, in which the female parent, ‘HR’, originated from the Japanese variety

‘Hokuseki’, and the male parent, ‘R130’, was a progeny of the *272 × WF1680* population (Sato et al. 2005). Each of the mapping progenies was clonally propagated by in vitro culture (for the tests in Russia) or by cuttings (for the tests in Japan), and was transplanted into the field in the All-Russian Williams Fodder Crop Research Institute (WFCRI; Moscow region, Russia) or the National Agricultural Research Center for Hokkaido (NARCH; Sapporo, Japan) to evaluate the traits that relate to plant persistency. The investigated traits and the duration of the experiment, the number of tested progeny, and the number of replications performed are summarized in Table 1.

### Laboratory test on resistance to *S. trifoliorum*

Resistance to *S. trifoliorum* was determined in the laboratory at WFCRI to develop a rapid testing method for early diagnosis of infection in red clover, as described by Razgulayeva et al. (1999). *S. trifoliorum* inoculates were cultured on liquid clover-glucose medium at 16°C to form young, white sclerotiums. After 15–20 days, the mycelia

were filtered, dried, minced, and resuspended in sterile water. The leaves of the test plant clones were placed in Petri dishes on moist cotton wool containing 0.004% benzimidazole. Two drops of the pathogen suspension were pipetted onto each leaflet. Two replicates were performed for each genotype and for the control variety 'VIK-7', which has an intermediate level of resistance to *S. trifoliorum*. The inoculated plant material was then transferred into a growth chamber and maintained at 16°C with a 16 h light/8 h dark photoperiod under fluorescent light (5,000 lux). At 7 days post-inoculation, the degree of damage was assessed by visual inspection of symptoms and rated on a scale of 0–3, with 0 representing healthy leaflets and 3 representing severely damaged leaflets. On the base of this rank scale, the damage rate ( $R\%$ ) was calculated using the following formula, according to Ephimova (1981):

$$R\% = \sum(a \times b) \times 100 / (N \times K)$$

where  $a$  is the number of injured leaves;  $b$  the level of damage;  $N$  the total number of leaves in the analysis; and  $K$  is the highest level of damage.

#### Field experiments in the Moscow region

The field tests on resistance to *S. trifoliorum* in the Moscow region were carried out in the test fields at WFCRI. The 272 × WF1680 population traits were examined in normal field conditions (non-artificial infection), while the HR × R130 population was cultivated in an infected field, in which the dose of inoculum cultivated with malt was 25 g/m<sup>2</sup> of soil. The propagated plants and the control variety 'VIK-7' were transplanted into the field in replicates. The damage caused by *S. trifoliorum* infection was evaluated 2 weeks after spring plant regrowth started. The extent of plant disease ( $P\%$ ) was estimated using the following formula according to Chumackov et al. (1974):

$$P\% = n \times 100 / N$$

where  $n$  is the number of plants damaged by *S. trifoliorum* and  $N$  is the total number of plants evaluated.

In order to investigate *Fusarium* resistance in the infected field, the propagated seedlings of the mapping population and a control variety 'VIK-7' were transplanted into a field infected with a mixture of six *Fusarium* fungus species, which occur widely in the Moscow region, namely *F. oxysporum* Schl. em. Snyder et Hans, *F. culmorum* (Sm.) Sacc., *F. solani* (Mart.) App. Et Wr., *F. avenaceum* (Fr.) Sacc., *F. gibbosum* App. et Wr. em. Bilai, *F. sambucinum* Fuck. The dose of inocula was 60 g/m<sup>2</sup> of soil. For analysis of resistance, the roots of each plant were removed, and the degree of damage was assessed using a rank scale of 0 (no symptoms) to 5 (more than 75% of the root tissue damaged)

at the end of the second year after planting. Based on this scale, the intensity of disease development ( $R\%$ ) was estimated using the following formula (Putsa et al. 1999):

$$R\% = \sum(a \times b) \times 100 / (N \times K)$$

where  $a$  is the total number of injured plants;  $b$  the level of damage;  $N$  the total number of plants examined; and  $K$  is the highest scale of the investigation. The survival rate after winter was estimated in the HR × R130 population using data of estimation in the field in 2004–2005.

#### Field experiments in Sapporo

The progeny of the 272 × WF1680 mapping population were transplanted to a non-artificial field in July 2001 with no replication. No plants showed symptoms of *S. trifoliorum* infection in the early spring of 2002. Each plant was photographed 2 weeks after the disappearance of snow, and the numbers of green color pixels to non-green color pixels of digitized photographs of the crown were evaluated using Microsoft Photo Editor (Microsoft Corporation, Redmond, WA, USA).

To estimate the winter hardiness of the HR × R130 mapping population, plant cuttings were transplanted to the non-artificial field at NARCH in October 2003 with five replications. None of the plants showed symptoms of *S. trifoliorum* infection in the spring of the second year (2004). Winter hardiness was then scored on a scale of 1 (very weak) to 9 (very hardy). Symptoms of *S. trifoliorum* infection were observed on the plants 2 weeks after the disappearance of snow in the spring of the third year (2005), and *Sclerotinia* resistance was scored on a scale of 1 (very sensitive) to 9 (very resistant).

#### Marker analysis and linkage map construction

Combined genetic linkage maps of 272 × WF1680 and HR × R130 were reconstructed based on previously reported restriction fragment length polymorphism (RFLP), microsatellite, morphological, random amplified polymorphic DNA (RAPD), and sequence tagged site (STS) markers (Herrmann et al. 2006; Isobe et al. 2003, 2009; Sato et al. 2005). Markers designated a single 'C' or 'OPB' label represent RFLP and RAPD markers, respectively, while 'RCS' and 'TPSSR' followed by a number indicate microsatellite markers. For map construction, parent-specific maps were first developed using a combination of the color map method (Kiss et al. 1998) and JoinMap<sup>®</sup> 4 (Van Ooijen 2006), as previously reported (Isobe et al. 2003). These data were then combined with a genetic linkage map using the 'Join' module of JoinMap<sup>®</sup> 4.

## Statistical analysis and QTL detection

The normality of the phenotypic data distributions was confirmed using the D'Agostino–Pearson  $K^2$  test with the Data Transformation program ([http://www.tuat.ac.jp/~ethology/Columbo/Stat/Data\\_Trans](http://www.tuat.ac.jp/~ethology/Columbo/Stat/Data_Trans)). If the phenotypic data sets did not have a normal distribution, they were transformed to fit a normal distribution. After data transformation, correlations between phenotypic data were assessed using the Pearson correlation coefficients. The broad sense heritability of each trait was calculated as follows:

$$H = \frac{MS_G}{MS_G + MS_R + MS_E}$$

where  $MS_G$  is the mean square for genotype  $MS_R$  the mean square for replication and  $MS_E$  is the mean square for error.

For identification of candidate QTL regions, interval mapping and nonparametric mapping were performed, and then the corresponding QTLs were confirmed. SIM was performed using MapQTL<sup>®</sup> 5 (Van Ooijen 2004). The significant LOD (logarithm of odds) threshold was determined at 5.0% via a permutation test module (10,000 replications). Although MQM is implemented in MapQTL<sup>®</sup> 5, this mapping method was not employed in this study, because the run could not be completed by the software due to the large number of data points.

Nonparametric mapping was performed following the two approaches: KW analysis (Lehman, 1975) using MapQTL<sup>®</sup> 5, and GMM (Isobe et al. 2007) using the GMM ver. 2.1 program (<http://www.kazusa.or.jp/GMM>). KW analysis identifies markers related to single dose QTLs, while GMM is a method that searches for QTL interactions that have an effect on genetic variation. For KW analysis, significant statistics adjusted for ties, which are indicated by  $K^*$ , were employed, and their error probability was set to 0.005. For GMM mapping, single effect QTLs, as well as double and triple locus interactions, were examined within a default searching range, which was set by the program, based on the input data, and the minimum number of corresponding individuals was set to 1.

Two different approaches were employed to identify the haplotypes contributing to the positive effects of QTLs: one for SIM, and the other for KW analysis and GMM. By performing SIM on MapQTL<sup>®</sup>, phenotypic mean values of 'ac', 'ad', 'bc' and 'bd' genotypes on each locus were estimated in a 'cp' population, i.e. a pseudo-testcross population, in which maternal and paternal genotype were coded as 'ab' and 'cd', respectively. Therefore, the mean values between 'ac' and 'ad', 'bc' and 'bd', 'ac' and 'bc', and 'ad' and 'bd' were calculated for the phenotypic mean value of each haplotype of 'a' and 'b' (two maternal haplotypes), and 'c' and 'd' (two paternal haplotypes),

respectively. Positive effect haplotypes were then identified by comparison of the mean values between maternal haplotypes, 'a' and 'b', and paternal haplotypes, 'c' and 'd'. KW analysis and GMM calculate the phenotypic mean value of each genotype code for which the haplotype phases are unknown. Thus, the haplotype phase of each genotype code on the candidate QTLs was confirmed based on the results of color mapping, which identifies the haplotype phase by graphical genotypes. The positive effect haplotype in each parent was then identified by comparing the mean values of haplotypes.

## Results

### Phenotypic data analysis

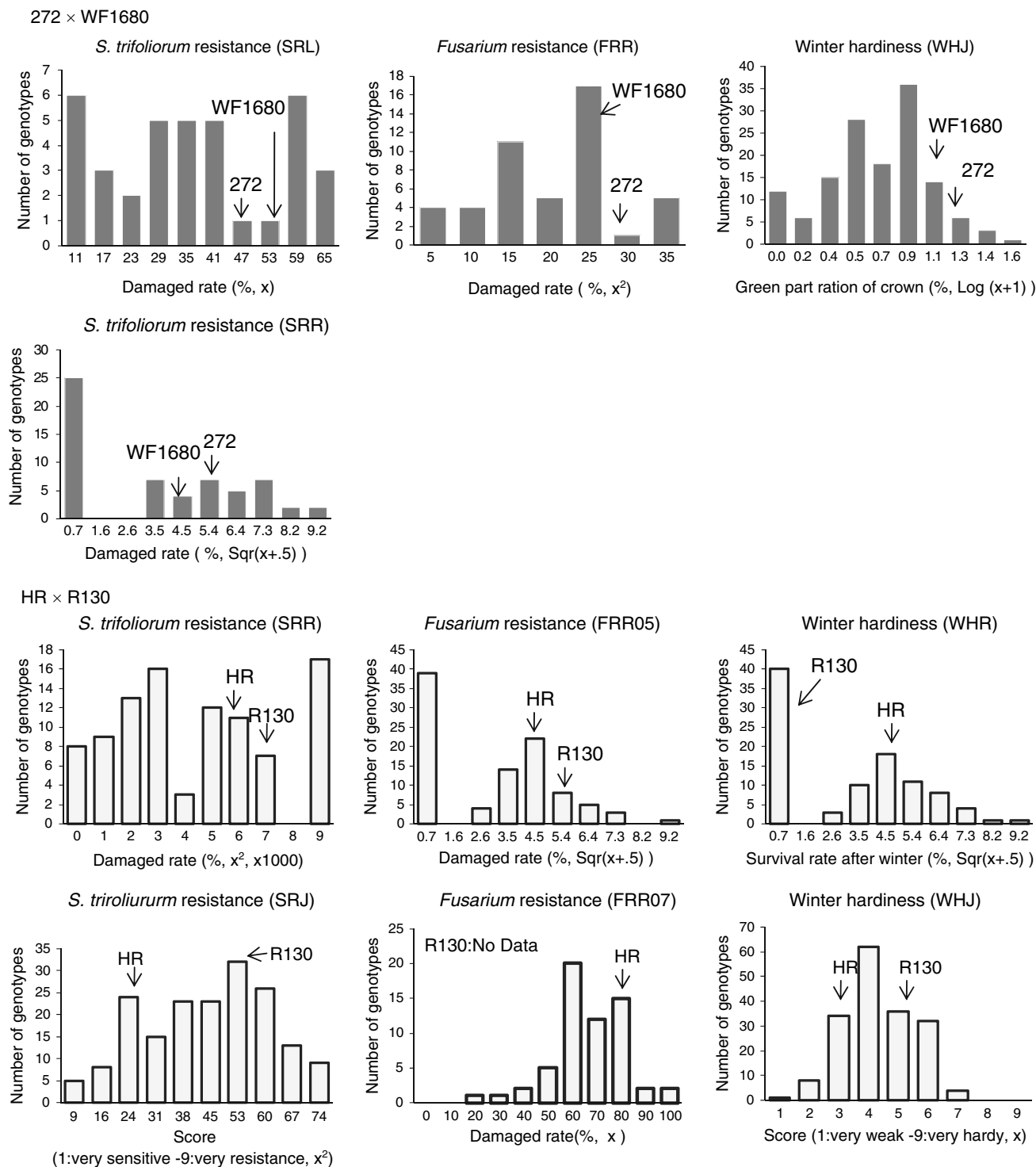
Continuous distributions were observed in all ten of the traits investigated, all of which were related to *S. trifoliorum* resistance, *Fusarium* resistance, and winter hardiness (Fig. 1). According to the D'Agostino–Pearson  $K^2$  test, three of the ten traits (SRL in the 272 × WF1680 population, and FRR07 and WHJ in the HR × R130 population) showed normal distributions, while the seven remaining traits did not have normal distributions (Table 2). These latter seven traits were transformed using square, square root, or Log functions to fit the normal distribution. Broad sense heritability ranged from 0.05 to 0.40, with the lowest and highest heritability values being observed for FRR05 and SRR, respectively, from the HR × R130 population. The coefficient of variance ranged from 5.8% (SRR in the 272 × WF1680 population) to 60.0% (FRR in the 272 × WF1680 population).

The range of phenotypic values of the mapping progenies exceeded the differences between the parents in all traits (Fig. 1). In the 272 × WF1680 population, the 272 parent, showed lower resistance to *S. trifoliorum* in the Russian field tests (SRR) than the WF1680 parent, while no phenotypic differences between the parents were observed in other traits (Table 2). In the HR × R130 population, no significant differences were observed between the parents for all the traits investigated. In the 272 × WF1680 population, a significant correlation was observed only between SRR and SRL (Table 3). In the HR × R130 population, significant correlations were observed between SRR and FRR05, and between SRR and WHR.

### Linkage map construction

A total of 437 loci, including 249 RFLPs, 182 microsatellites, two STSs, four RAPDs and one morphological locus, were mapped onto seven linkage groups of the 272 × WF1680 integrated map. The total length of the





**Fig. 1** Frequency distributions of the traits relating to plant persistence in red clover populations 272 × WF1680 and HR × R130. Parental values of phenotypes are indicated by arrows. The transform

functions used to convert the data to normal distributions are described in parentheses on the X-axis title

linkage map was 817 cM and the average distance between two markers was 1.9 cM. The HR × R130 integrated map consisted of a total of 1502 loci, including 1352 RFLP and 150 RFLP loci, spanning seven linkage groups. The total

length of the map was 801 cM and the average distance between two markers was 0.53 cM. Between the two combined linkage maps, 88 RFLP and 167 microsatellite markers were commonly mapped. The locus orders on the

**Table 2** Mean, CV(%), LSD and heritability of the investigated traits related to plant persistency for the mapping progenies and the parents

Traits <sup>a</sup>	272 × WF1680					
	SRL	SRR	FRR	WHJ		
Transform function	$x$	$\text{Sqr}(x + 0.5)$	$x^2$	$\text{Log}(x + 1)$		
Mean	38.15	2.13	18.53	0.78		
272	42.49	5.78	28.44	1.33		
WF1680	50.00	4.78	25.00	1.11		
CV (%)	38.3	5.8	60.0	–		
LSD (0.05)	29.60	0.47	21.87	–		
Heritability	0.30	0.12	0.13	–		

Traits <sup>a</sup>	HR × R130					
	SRR	SRJ	FRR05	FRR07	WHR	WHJ
Transform function	$x^2$	$x^2$	$\text{Sqr}(x + 0.5)$	$x$	$\text{Sqr}(x + 0.5)$	$x$
Mean	4,136	48.20	4.06	64.10	4.20	4.02
HR	5,098	18.77	3.80	72.00	3.84	3.00
R130	6,400	49.00	4.53	nd	0.70	5.00
CV (%)	14.7	55.2	33.2	41.5	–	56.2
LSD (0.05)	2,061	47.5	ns	ns	–	7.03
Heritability	0.40	0.37	0.05	0.32	–	0.37

CV coefficient of variance, *LSD* least significant difference, *nd* no data, *ns* no significant

<sup>a</sup> Transform function: function for transformation of phenotypic value to fit normal distribution

**Table 3** Correlation coefficients between resistance to *S. trifoliorum*, *Fusarium* and winter hardiness in 272 × WF1680 and HR × R130

	Trait		
	SRR	FRR	WHJ
272 × WF1680			
SRL	0.365*	–0.113	0.015
SRR		–0.160	–0.168
FRR			0.110

	Trait				
	SRJ	FRR05	FRR07	WHR	WHJ
HR × R130					
SRR	–0.015	–0.624**	0.082	–0.698**	–0.165
SRJ		–0.077	–0.004	0.061	0.010
FRR05			–0.203	–0.073	0.164
FRR07				0.059	0.013
WHR					0.077

\* and \*\* indicate significant correlation at  $p < 0.05$  and  $p < 0.001$ , respectively

two maps correspond to those of the accession-specific maps reported by Isobe et al. (2009).

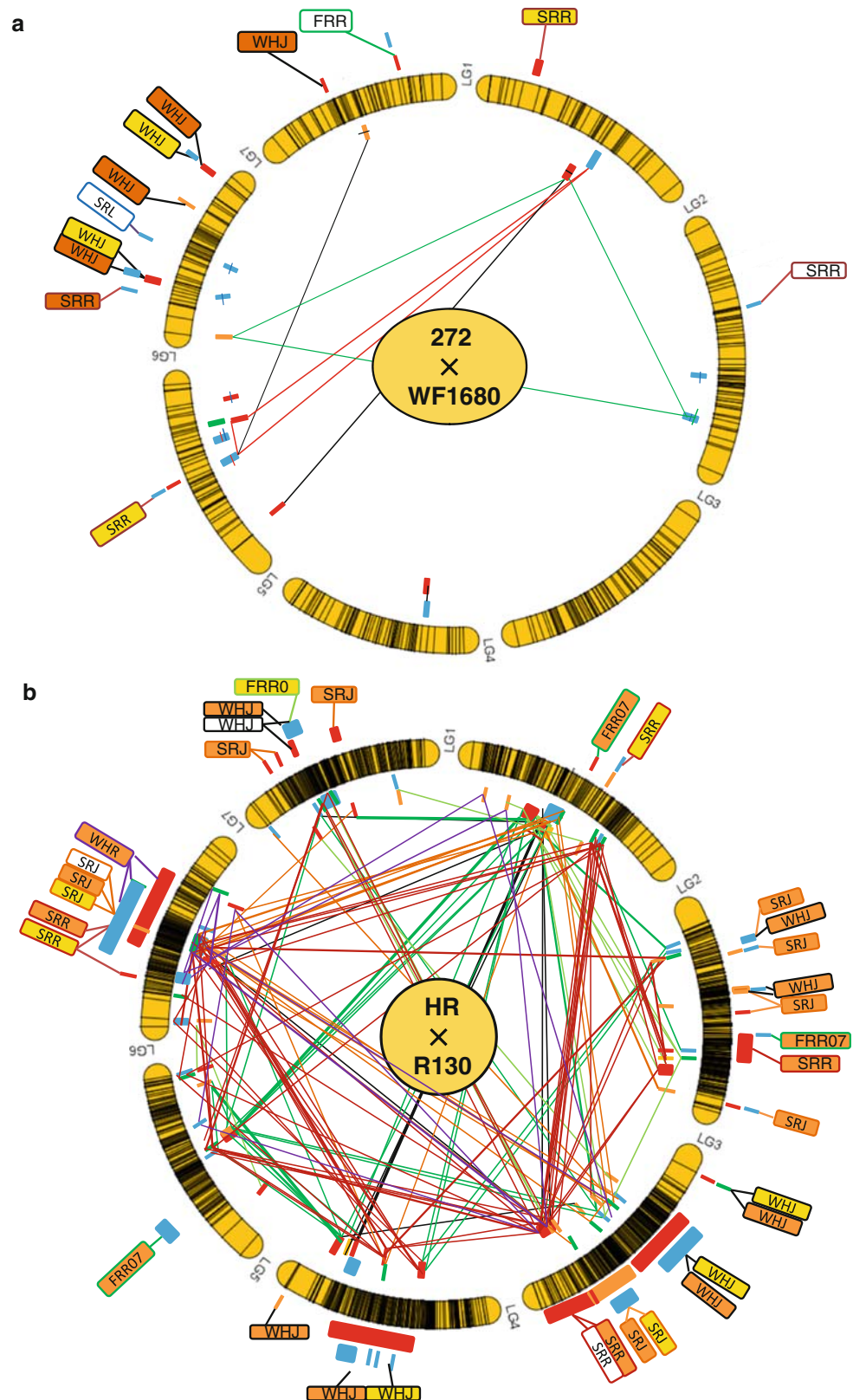
#### QTL detection in the 272 × WF1680 population

A total of 4, 5 and 3 single effect QTLs were detected in the 272 × WF1680 population by SIM, KW analysis, and GMM, respectively (Fig. 2a and Supplementary Table 1). Single QTL were detected for SRL and FRR, while four QTLs were identified for SRR and WHJ. Of the seven linkage groups present in red clover, most QTLs mapped to the LG6. The positive effect QTLs were identified only in

one haplotype in WF1680, but in both haplotypes in 272. The phenotypic variance explained by QTLs detected by SIM ranged from 76.1 to 91.7% for SRR and from 13.5 to 16.2% for WHJ, while variance explained by QTLs, as detected by GMM, was 7.4% for SRL, 10.8% for SRR, and 13.8% for FRR.

The locus combinations representing interacting QTLs were identified by GMM for two- and three-locus combinations (Supplementary Tables 1–3). Significant QTL interactions were observed for each investigated trait: a single two-locus combination for SRL, SRR, and FRR; four-two-locus combinations for WHJ; six-three-locus

**Fig. 2** Mapping position of identified QTLs in the  $272 \times \text{WF1680}$  (a) and the  $\text{HR} \times \text{R130}$  (b) populations. The segments of the outer circle indicate the linkage maps consisting of seven linkage groups and the *black bars* in each segment represent the positions of loci used for QTL detection. *Short bars* outside the segments of the circle indicate the positions of single effect QTLs detected by SIM, KW analysis, and GMM, while *bars inside the circle* represent interacting QTLs detected by GMM (two- or three-locus interactions). *Red and yellow bars* indicate haplotypes with higher resistance or winter hardiness than other haplotypes in 272 or HR, while *blue and green bars* represent haplotypes with a higher tolerance effect in WF1680 or R130. The haplotype phase can only be confirmed within linkage groups, and not across linkage groups. The *letters on boxes* indicate trait names explained in Table 1, and *yellow, orange and white boxes* represent QTLs detected by SIM, KW analysis and GMM, respectively. The *lines connecting short bars* inside the circles represent combinations of interacting QTLs. *Red, green, blue, and black lines* in the  $272 \times \text{WF1680}$  linkage circle represent interacting QTLs for SRL, SRR, FRR, and WHJ, respectively. *Red, orange, light green, green, purple, and black lines* in the  $\text{HR} \times \text{R130}$  linkage circle indicate QTLs for SRR, SRJ, FRR05, FRR07, WHR, and WHJ, respectively



combinations for SRL, two–three-locus combinations for SRR, one–three-locus combination for FRR and three–three-locus combinations for WHJ. The identified loci were

mapped across linkage groups and their combinations were observed both within and between parents (Fig. 2a). In each trait, the highest phenotypic variance explained was



observed in the three-locus combinations (Supplementary Tables 1–3).

#### QTL detection in the HR × R130 population

A total of 10, 191, and 8 single effect QTLs were detected in the HR × R130 population by SIM, KW analysis, and GMM, respectively (Supplementary Table 1). Many QTLs identified by KW analysis and some of the QTLs identified by GMM were sequentially mapped onto LG3, LG4 and LG6 (Fig. 2b). The positive effect QTLs were identified only in one haplotype in R130 with the exception that QTLs for WHJ mapped onto LG3, but in both haplotypes in HR. Most of the QTLs identified in LG3, LG4 and LG6 were detected by multiple approaches, for example, in LG3, WHJ at 5.4 cM (by SIM and KW analysis), WHJ at 27 cM (by SIM and KW analysis), SRJ at 87 cM (by SIM and KW analysis), and SRR at 97 cM (KW analysis and GMM); in LG4, WHJ at 59 cM and 71 cM (SIM and KW analysis); in LG6, SRR at 48 cM (SIM and KW analysis), SRJ at 51 and 61 cM (by SIM and KW analysis and GMM). Co-localized QTLs for different traits were observed in LG2 at 24 cM (by SRJ and WHJ), LG2 at 50 cM (SRJ and WHJ), and LG6 at 45–78 cM (by SRR, SRJ and WHR). QTLs for stress tolerance were mapped to a total of 23 regions, including 5 for SRR, 9 for SRJ, 1 for FRR05, 3 for FRR07, 3 for WHR, and 6 for WHJ. The phenotypic variance explained by QTLs detected by SIM ranged from 70.0 to 94.0% for SRR, 13.8–17.6% for SRJ, 81.6% for FRR05, and 10.7–41.7% for WHJ, while those detected by GMM were 9.1% for SRR, 10.4–11.5% for SRJ, and 7.4–7.7% for WHJ.

Number of significant locus combinations were identified by GMM: for two-locus combinations, 2 were detected for SRR, 10 for SRJ, 7 for FRR05, 10 for FRR07 and 5 for WHJ; for three-locus combinations, 39 were detected for SRR, 10 for SRJ, 7 for FRR05, 11 for FRR07, 6 for WHR, and 2 for WHJ (Supplementary Tables 1–3). The interacting loci were mapped across linkage groups and their combinations were observed both within and between parents (Fig. 2b). The interacting QTLs for multiple traits were identified in LG1 at 31–58 cM (for SRJ, FRR05, FRR07 and WHJ), LG3 at 79–119 cM (for SRR, SRJ, FRR05, WHR and WHJ) and LG6 at 46–60 cM (for SRR, SRJ, FRR07, WHR and WHJ). The phenotypic variance explained of two-QTLs combinations ranged 23.8–24.7% for SRR, 13.4–25.8% for SRJ, 12.9–23.8% for FRR05, 13.7–21.3% for FRR07, and 16.0–23.4% for WHJ, while those of three QTLs combinations were 18.5–47.5% for SRR, 22.6–36.8% for SRJ, 31.3–41.2% for FRR05, 46.0–56.0% for FRR07, 25.4–50.6% for WHR and 25.6–26.57% for WHJ (Supplementary Tables 1–3).

#### Discussion

Controlling *Sclerotinia* and *Fusarium* field resistance, as well as winter hardiness, in red clover is difficult, because the sources of stress develop in the soil and/or beneath snow-covered ground (Dijkstra 1964; Taylor and Quesenberry 1996). The coefficient of variance of the traits investigated in this study was generally high and the broad sense heritabilities were low and variant. Significant QTL interactions were observed between SRL and SRR in the 272 × WF1680 population, and between SRR and FRR05, and SRR and WHR, in the HR × R130 population. However, no other significant interactions were observed. Because vegetative propagation of red clover is difficult, we performed a limited number of phenotypic investigations with a limited number of replications in this study. The difficulty in controlling stress tolerances and the low number of trials may not be sufficient to accurately determine the genotype effect of the populations in this study. However, the low and variant heritability, and the few significant interactions between the traits investigated suggest an absence of major genes controlling the stress tolerances investigated.

A total of 10 and 23 regions were identified where single effect QTLs related to plant persistency mapped in the 272 × WF1680 and the HR × R130 populations, respectively. Because the QTL identification was based on statistical approaches, the possibility of identifying false positive and false negative QTLs for the thresholds and mapping approaches used still exists (McElroy et al. 2006; Mackay and Powell 2007). However, if QTLs were identified by multiple approaches, the reliability of the QTLs is likely to be enhanced. The QTLs observed on LG6 in the 272 × WF1680 population for WHJ, and on LG3, LG4 and LG6 in the HR × WF1680 population for SRR, SRJ and WHJ, were identified by multiple approaches and can be regarded as reliable QTLs. The reliability of QTLs identified for winter hardiness and *Sclerotinia* resistance on LG6 is also suggested by the overlap of QTLs identified in the two mapping populations. Thus, we conclude that LG6 is one of the most significant linkage groups with regard to stress tolerance. Herrmann et al. (2008) recently identified a QTL which was associated with the largest effect on plant persistency in red clover on LG3. Because LG3 contains three candidate QTL regions detected by multiple approaches in this study, we propose that LG3 is also a significant linkage group for red clover plant persistency.

A greater number of QTLs was identified in the HR × R130 population than in the 272 × WF1680 population. Because more DNA markers were used in the HR × R130 population, the power of QTL detection was higher in the HR × R130 population than in the

272 × WF1680 population. Of the three approaches used in this study, KW analysis identified the most single effect QTLs. Along with the differences in algorithms used to detect QTLs in these approaches, the different levels of severity of the thresholds used to identify significant QTLs had a major effect on the number of identified QTLs. The thresholds in SIM (LOD value) and the searching range in GMM were defined in every trait, in consideration of the specific properties of the data, while the thresholds in the KW analysis were uniformly defined for all traits and populations with an error probability of 0.005. Thus, the KW threshold used in this study may be less stringent than the SIM thresholds or the GMM searching range.

The five QTLs identified by SIM (two SRR QTLs in the 272 × WF1680 population, and two SRR QTLs and one FRR05 QTL in the HR × R130 population) were assessed as large effect QTLs that explained more than 70% of the phenotypic variance of each single QTL. However, because the broad sense heritability of the SRR and FRR QTLs was low (0.12–0.40 in SRR and 0.05–0.32 in FRR), and the QTLs were not identified by the other two approaches, the reliability of the estimation of the magnitude of phenotypic variance explained by these QTLs may be questionable. In SIM, significant QTLs are explored at genotyped loci and each ‘mapping step’ point which configured by operators. For the configured ‘mapping step’ points, genotypes are predicted according to the nearest genotyped loci, and then the existence of the QTLs is assessed based on the predicted genotypes. However, when a pseudo-testcross population is used for SIM, the nearest genotyped loci often provide genotype information only for one parent, because the other parent does not have polymorphisms in the loci. Therefore, the reliability of SIM in a pseudo-testcross is generally regarded as low, relative to that of a self-crossing population. Thus, we concluded that the magnitude of QTLs identified by SIM in this study was doubtful and sometimes overestimated.

In this study, combinations of interacting loci that affect the traits investigated were identified by GMM (two and three locus interactions). More interacting QTLs were identified in the HR × R130 population, which may reflect the greater number of genotyped loci. The highest number of interactions was observed for SRR in the HR × R130 population. Although the interacting QTLs were identified across genomes, several ‘hub’ regions for the interactions were observed in the HR × R130 population: for example, LG1 at 31–58 cM (for SRJ, FRR05, FRR07 and WHJ), LG1 at 83–91 cM (for SRR), LG3 at 79–119 cM (for SRR, SRJ, FRR05, WHR and WHJ), and LG6 at 46–60 cM (for SRR, SRJ, FRR07, WHR and WHJ). Interestingly, the numbers of significant QTL interactions identified by

GMM were more than that of single effect QTLs. In addition, the percentages of the phenotypic variance explained by the QTL combinations were higher than those of the single effect QTLs. These results suggest that there were many QTLs for plant persistency that had a small effect as single QTLs, but indicate a large effect with a specific locus combination.

In a pseudo-testcross mapping population, polymorphic QTLs are identified by segregation analysis between two haplotypes in each parent. However, little attention has hitherto been paid to the identification of the haplotype phase associated with positive (or negative) effects of the QTLs. Therefore, the direction of identified QTLs has not been described in past reports that used pseudo-testcross mapping populations for QTL detection. In this study, we first performed an identification of the haplotypes that were associated with positive effect QTLs related to plant persistency. The single dose QTLs that were associated with a positive effect had a tendency to identify with either haplotype of each parent, especially in the HR × R130 population, while the QTL interactions were observed across the four haplotypes. This result implied that it may not be effective to improve plant persistency by pyramiding only single effect QTLs, because the positive QTL interactions would be lost. Therefore, we concluded that the accumulation of both single effect QTLs and interacting QTLs is necessary for the improvement of the traits. However, because the genetic diversity of the breeding populations might be much higher than that of the mapping parents used in this study, there would be other QTL combinations as well as single effect QTLs in each breeding populations. Therefore, the existence of the targeted QTLs in the breeding populations must be confirmed before performing the MAS approach. The QTL regions identified in this study, especially LG1 at 31–58 cM, LG3 at 79–119 cM, and LG6 at 46–60 cM, where interacting QTLs for multiple traits related to plant persistency were located and single effect QTLs also mapped, will be potential candidate regions for the re-investigation of QTLs in red clover breeding populations.

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