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Microsatellite (SSR) variation in Barley germplasm and its potential use for marker assisted selection in scald resistance breeding

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Abstract

Scald (caused by *Rhynchosporium secalis*) is one of the major plant diseases of barley (*Hordeum vulgare* L.) cultivation in central Anatolia and transitional zones of Turkey. Eighty barley germplasm consisting of 40 resistant and 40 sensitive lines provided by Central Research Institute of Field Crops were screened with six simple sequence repeat (SSR) markers to assess the magnitude and pattern of genetic diversity, as well as to investigate potential use of these markers for efficient scald resistant breeding program in the future. Estimated genetic parameters indicated that scald resistant (H_e=0.43) and sensitive (H_e=0.40) barley lines still maintain large and similar magnitude of genetic diversity. However, genetic variation among the lines was higher in the sensitive lines (46%) than in the resistant lines (39%). Especially, the presence of high genetic diversity among resistant lines (e.g., R25, R39) suggests that this diversity could be an important drive in future scald resistant barley breeding programs. The results of this study also revealed that Bmag6 and Bmag206 as SSR markers could be utilized in pre-screening of large number of barley lines for future scald resistance breeding programs with reduced time, cost and labor.

Keywords: *Hordeum vulgare*, scald, marker assisted selection, simple sequence repeat.

INTRODUCTION

Scald, caused by the imperfect fungus *Rhyncosporium secalis* (Oudem) J. J. Davis, is one of the major diseases of cultivated barley (*Hordeum vulgare* L.). This necrotrophic fungus attacks the leaves and head of barley by producing and secreting toxic compounds that are capable of moving across host's cell walls (Wevelsiep *et al.*, 1993), resulting in considerable yield losses and reduced grain quality.

Barley is cultivated in over 3.6 million ha in Turkey, mainly in central and southeast Anatolia, with a production of about nine million tons per year (Karahocagil and Ege, 2004). The disease is in effect in quite large proportion of barley fields in Turkey (Mamluk *et al.*, 1997) and causes up to 30% yield loses (Kavak, 1998). Given the significant reduction in barley production due to scald in Turkey, Central Research Institute for Field Crops (CRIFC) in Ankara has initiated a breeding program in 1983 to develop scald resistant barley cultivars. The CRIFC has a large collection of barley germplasm for this purpose and determines scald resistance of these collections via phenotypic selection.

Molecular markers are the genetic indicators that flag the presence of genes that control particular traits. They are special segments of DNA -located in or around the gene of interest- that are easier to find. Once a useful marker is known, plant material can be tested for presence or absence of the marker (and thus for the gene of interest). Since simple sequence repeat (SSR) markers are simple PCR based co-dominant markers, which are extremely polymorphic and highly informative due to the number and frequency of alleles, they are the most utilized ones among the molecular markers (Hillel *et al.*, 1990) in many grass species (Budak *et al.*, 2004; Budak *et al.*, 2003; Budak *et al.*, 2005).

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Figure 1: Allelic designations of Bmag603 primer. The segregating bands for each primer were indicated with arrows R: scald resistant, S: scald sensitive (Ladder (L): pUC19 DNA/*Msp*I (*Hpa*II) Marker, Fermentas, Burlington, Ontario, Canada).

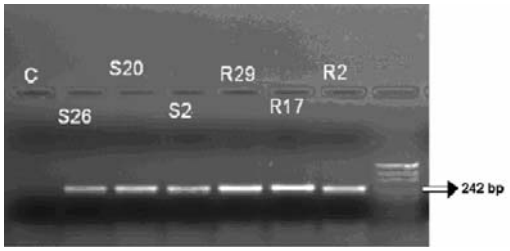

Figure 2: Bands observed with Bmag206 marker for the six individuals that were sequenced. C stands for control and the multiple bands on the right are those of the ladder (pUC19 DNA/*Msp*I (*Hpa*II) Marker, Fermentas, Burlington, Ontario, Canada).

In plant breeding, molecular markers can be employed for many purposes including investigating genetic variation in a given population, screening breeding material, and selecting desired genotypes with marker assisted selection. In the CRIFC's barley breeding program for scald resistance and in genetic characterization and screening barley germplasm, molecular markers have not been utilized previously. Thus, this study was undertaken; 1) to explore magnitude and pattern of genetic variation using SSR markers in the CRIFC's barley germplasm used in scald resistance breeding, and 2) to evaluate the potential use of SSR markers for future marker assisted selection in scald resistance breeding. To meet these goals, 80 barley lines in the CRIFC's germplasm were screened with six SSR markers. Forty of these lines are considered to be scald resistant while the remaining 40 lines are classified as sensitive based on phenotypic scald tests carried out under greenhouse conditions.

MATERIALS AND METHODS

Plant Material

The CRIFC routinely screens its barley germplasm for scald resistance genes which could be incorporated to the ongoing barley breeding program. In this program, barley seedlings are grown and artificially inoculated with *Rhyncosporium secalis* spores as described in Mert and Karakaya (2004). Level of disease severity is evaluated after 18 days from inoculation date, using a modified scales of El-Ahmed (1981) and Ali and Boyd (1974). The genotypes are visually scored for scald resistance on a per plot basis from 0 to 4, where 0 indicates no visible symptoms (highly resistant), 1 indicates small lesions on the tips or on the base of the leaf (resistant), 2 indicates one to two small lesions on the blade and/or a narrow band of lesions extending at the margin of the leaf (intermediate), 3 indicates well-developed lesions on the blade, but without collapse (susceptible), and 4 indicates total collapse (highly susceptible). For this study, a total of 80 lines (40 susceptible with scald scores of 3 or 4, and 40 resistant with scald scores of 0 or 1) were selected from the screening program (Tables 1 *[Supplementary data]* and Table 2 *[Supplementary data]*) (Dizkırıcı, 2006).

DNA Extraction and Molecular Markers

Four seeds from each line were germinated (a total of 320 seedlings). DNA was isolated from young leaves of seedlings with the CTAB method (Doyle and Doyle, 1987). Template DNA from each seedling of 80 lines was screened with six SSR loci (Table 3). PCRs were performed in a total volume of 25 µl and consisted of 5 ng μl^{-1} genomic DNA, 1X PCR Buffer ($MgCl_2$ free), 2.5 mM $MgCl_2$, 500 µM dNTP, 1 µM of each primer and 0.5 units *Taq* DNA polymerase with the following program: 94°C for 3 min; 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; 72°C for 5 min. Seven to 10 µl of each sample were loaded on 7.5% of polyacrilamide (19:1 Acrylamide:Bis) gels with 1 x TBE, which were run under 250 V for 2 h. Gels were stained with 0.5% Ethidium Bromide and visualized under UV light using a Vilber Lourmat gel visualization system (Cedex, France).

Evaluating Genetic Variation in Turkish Barley Lines

Different band patterns were observed in six SSR primers as expected. For each primer pair, amplified DNA product was treated as loci with different alleles (bands with different size) and scored as 1 to 5. While some primer pairs yielded only one band, the others produced more than a single band which are treated as loci with multiple alleles (Fig. 1). After allelic designations in each primer pairs, genotypes of individuals from each of 80 Turkish barley lines were determined and recorded. Number of alleles and type of genotypes produced by SSR primer pairs were provided in table 3 *[Supplementary data]*.

To determine the magnitude of genetic variation in barley lines, POPGENE (Yeh and Boyle, 1997) program was used and allelic richness (A), proportion of polymorphic loci (P), expected (H_e) and observed (H_{obs}) heterozygosities were estimated according to

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Nei (1987). Wright's F-statistics (Wright, 1978; Nei, 1987) were employed to measure heterozygosity in three levels of inbreeding; F_{IT} = Fixation index within population (scald resistant group vs. scald sensitive group), F_{IS} = Fixation index over the total populations, and F_{ST} = reduction in fixation index due to differences among populations.

The estimates of Nei's (1978) standard genetic distance were calculated to show the genetic relationships among Turkish barley lines. To reveal the genetic differentiation pattern of germplasm collections graphically, a cluster analysis was performed using Unweighted Pair Group Method with Arithmetic Averaging (UPGMA) (Nei, 1978).

Identifying Potential SSR Markers for Marker Assisted Selection Purposes

In order to determine the relationship between genotypes yielded by SSR loci on scald resistance, analysis of variance (ANOVA) was conducted. Since scald score is a categorical variable and violates ANOVA assumptions, nonparametric ANOVA was employed. Scald scores first ranked using PROC RANK and analysis of variance (ANOVA) was conducted using PROC GLM of SAS statistical package (SAS Institute Inc., 1990) using ranks instead of actual scald scores. The ANOVA model was: $Y_{ij} = g_i + e_{ij}$

where, Y_{ij} is the rank of scald score of ith genotype in j^{th} line, g_i is the ith genotype, and e_{ij} is random error term.

Statistically significant differences were observed among barley lines with respect to scald resistance for all markers except for Bmac209. For the loci for which genotypes differed significantly for scald scores, all genotypes were classified and tabulated according to their allele and disease types (Table 4 *[Supplementary data]*). Of the molecular markers, the ones that yielded homozygote genotype was selected (Bmag6 and Bmag206) (Table 4, Fig. 2). Then, for each primer pair, the most resistant and the most sensitive three genotypes, that exhibited the homozygote locus, were selected (R2, R17, R29, S2, S20, and S26). The DNA from these individuals was subjected to PCR with the two primer pairs. PCR products were sequenced (REFGEN, Ankara, Turkey) and compared with barley genome's known sequences.

Potential of the SSR markers utilized in this study for marker assisted selection purposes was evaluated by calculating probability of a given plant being susceptible or resistant to scald according to their genotypes with respect to Bmag6 and Bmag206 SSR markers. These two markers were selected because they produced only homozygote banding patterns (three genotypes for Bmag6 and two genotypes for Bmag206, Table 3) and for any given genotypes, number of resistant individuals was almost double the number of sensitive individuals, or vice versa (except for genotype 11 of Bmag6, where numbers of resistant and sensitive are almost equal, Table 4). Thus, for a given individual with known genotypes determined with Bmag6 and Bmag206 SSR markers, probability of being resistant or susceptible can be calculated. Addition rule of probability for not-mutually exclusive events was used to obtain an individual's probability of being resistant (or sensitive) to scald when its genotype is known with respect to Bmag6 and Bmag206 SSR loci.

$$P(A \cup B) = P(A) + P(B) - P(A \cdot B)$$

where $P(A \cup B)$ is the probability of being resistant (or sensitive) for a given individual, $P(A)$ is the probability of being resistant (or sensitive) for a given individual when a certain band observed using Bmag6, $P(B)$ is the probability of being resistant (or sensitive) for a given individual when a certain band observed using Bmag206, and $P(A \cdot B)$ is the probability of being resistant (or sensitive) for a given individual when a certain band observed using both markers. $P(A)$ and $P(B)$ were calculated from Table 4.

RESULTS

Genetic Variation in Barley Lines

Genetic diversity statistics were provided in tables 1 and 2 for resistant and sensitive Turkish barley lines, respectively. The highest number of alleles (5.0) for all lines, regardless of resistant or sensitive to scald, was observed in Bmag603, Bmac67, and Bmag225 loci, while the lowest was detected in Bmag206 (2.0) locus and the mean number of alleles was estimated to be 4.0. Mean number of alleles gives an estimate that is inflated by deleterious genes of which the contribution to genetic variability is small. Thus, the effective number of alleles (n_e) by Kimura and Crow (1964) was estimated and it was between 3.4 (Bmac67) and 2.0 (Bmag206). Observed heterozygosity was highest (1.0) in Bmac67 and Bmag225 and lowest (0.0) in Bmag206 and Bmag6 while expected heterozygosity is highest in Bmac67 (0.7) and lowest in Bmag206 loci (0.5) (Tables 1 and 2).

The mean number of alleles per locus (n_a) ranged from 1.5 to 2.8 for both resistant and sensitive groups. The mean allelic richness (n_a) was 1.93 and 1.81 for resistant and sensitive lines, respectively while n_e was 1.8 for resistant and 1.7 for sensitive lines (Tables 1 and 2).

The proportion of polymorphic loci (P) ranged from 50% to 100% for both resistant and sensitive lines. But the average P was considerably higher in scald resistant lines (74.58%) than in sensitive lines (68.40%). Average observed heterozygosities (H_{obs}) for both groups varied between 0.50 to 0.67, and average H_{obs} values were with similar magnitude in both in resistant (0.63) and in sensitive lines (0.61).

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Expected heterozygosity (H_e) estimates were lower than H_{obs} estimates and ranged from 0.29 to 0.61. Average H_e estimates for resistant (0.43) and sensitive (0.40) groups were similar in magnitude (Tables 1 and 2).

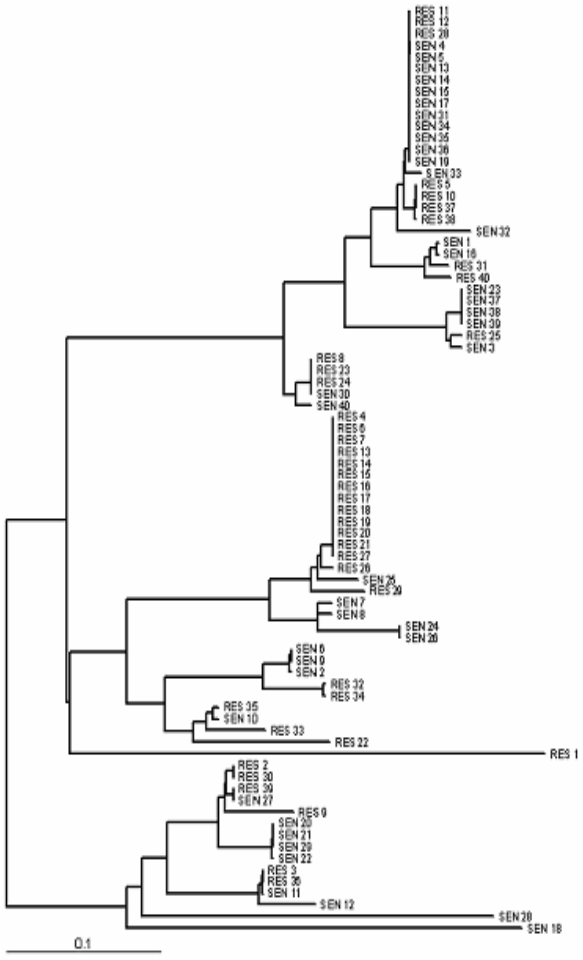

Figure 3: Dendrogram constructed using Nei's (1978) Genetic Distance Statistics showing the clusters of Turkish barley lines (RES: resistant, SEN: sensitive lines). For more information on seed source codes, see Tables 1 and 2

The fixation index within subpopulations (F_{IS}) ranged from -0.93 to 1.00 in resistant lines (mean = -0.67), and from -0.95 to 1.00 for sensitive lines (mean = -0.76). The reduction in fixation index due to differences among subpopulations (F_{ST}) was quite high and ranged from 0.13 to 0.87 in resistant lines (mean = 0.39), and from 0.19 to 0.94 in sensitive lines (mean = 0.46, Table 5).

Genetic distance estimates between studied barley lines revealed that S18, S28, and R1 were genetically the most distant ones. In general, resistant and sensitive lines were clustered in separate groups. However, some sensitive and resistant lines were ended up in the same sub-clusters due to their common genetic background (Fig. 3).

Identifying Potential SSR Markers for Marker Assisted Selection Purposes

The nucleotide sequences for all six individuals (three resistant and three sensitive) were similar for Bmag206 (homology % 83.9, Fig. 4 *[Supplementary data]*). From the genotype frequencies of Bmag206 and Bmag6 loci in resistant and sensitive groups, it appears that these loci are possibly linked to locus or loci conferring scald resistance in barley, but the strength of this link may not be strong because observed band pattern for these markers are not exclusively associated with scald resistance (or sensitivity) (Table 4).

Probabilities of an individual being scald resistant when a certain band pattern combination is observed for Bmag6 and Bmag206 are given in table 6. When genotype "22" is observed with Bmag6, probability of that individual being scald resistant is about 68% in this experimental population. When, however, genotype "22" for Bmag6 and genotype "11" for Bmag206 are observed for an individual, probability of that individual being scald resistance is expected to be as high as 89%.

DISCUSSION

The results of the present study using 80 Turkish barley lines, which were a part of the germplasm collection for scald resistant barley breeding program, indicate the presence of a large amount of genetic diversity existing in them. Estimated overall average allelic richness (4.0 alleles per locus) was lower than that of Malysheva-Otto *et al.* (2007), who reported 8.0 alleles per locus for a large number of diverse European barley cultivars. However, our figure is close to what Ivandic *et al.* (2002) found on wild barley samples from the Fertile Crescent (5.5 alleles per locus). The number and set of SSR markers as well as sampling of lines have also contributed to these observed discrepancies in estimated average allele per locus.

SSR markers employed in this study revealed high proportion of polymorphic loci (75% in resistant lines and 68% in sensitive lines) as well as high heterozygosity values (H_{obs} 0.63 in resistant and 0.61 in sensitive lines). Similarly, high H_e values were found in both resistant (0.43) and in sensitive (0.40) Turkish barley accessions. The detection of high levels of polymorphism clearly reveals that higher heterozygosity rates caused by crossing studies that have been carried out by barley breeders in Turkish barley lines. Intensive artificial selection along with germplasm enrichment for scald improvement practiced by breeders on these populations seems to be favoring the high heterozygosity rates. For instance, Nevo *et al.* (1998) reported that mean expected heterozygosity in wild barley lines in Turkey was much lower (0.15) than those reported in the studies regarding cultivated lines/cultivars (Malysheva-Otto *et al.*, 2007). This estimate is much lower compared to the result from the present study. This means that some lines used in this study are

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Table 3: SSR primers used in the study, and observed number of alleles, size of alleles (base-pair) and observed genotypes of SSR loci in Turkish barley lines

Primers	Repeat type	Number of alleles	Size of alleles (bp)	Observed genotypes
Bmag603[a]	$(AG)_{24}$	5	111, 120, 132, 140, 147	11, 12, 22, 23, 44, 45
Bmag6[a]	$(AG)_{17}$	3	170, 175, 180	11, 22, 33
Bmac67[a]	$(AC)_{21}$	5	145, 160, 165, 170, 175	12, 24, 35
Bmag225[a]	$(AG)_{26}$	5	140, 147, 161, 171, 181	12, 13, 23, 34, 45
Bmac209[b]	$(AC)_{13}$	4	175, 185, 190, 200	11, 12, 13, 23, 34
Bmag206[b]	$(GT)_5(AG)_{14}$	2	250, 999	11, 22

[a] Williams et al., 2001; [b] Macaulay et al., 2001

Table 5: Summary of F-statistics for the six polymorphic loci in 40 resistant (R) and 40 sensitive (S) Turkish barley lines (F_{IS} = the fixation index within subpopulations, F_{IT} = the fixation index over total population, and F_{ST} = the reduction in fixation index due to differences among subpopulations).

Locus	F_{IS}		F_{IT}		F_{ST}	
	R	S	R	S	R	S
Bmag603	-0.75	-0.69	-0.28	-0.002	0.26	0.41
Bmac67	-0.87	-0.95	-0.63	-0.34	0.13	0.31
Bmag206	1.00	1.00	1.00	1.00	0.69	0.81
Bmac209	-0.88	-0.87	-0.43	-0.37	0.24	0.27
Bmag6	1.00	1.00	1.00	1.00	0.87	0.94
Bmag225	-0.93	-0.91	-0.38	-0.55	0.28	0.19
Mean	-0.67	-0.76	-0.01	0.04	0.39	0.46

Table 6: Probability of a barley individual being resistant to scald depending on banding patterns observed using Bmag6 and Bmag206 markers. Probability of an individual being resistant when a specific band observed for each marker is given in parentheses.

		Genotype with Bmag206	
		11 (0.65)	22 (0.36)
Genotype with Bmag6	22 (0.68)	0.89	0.80
	33 (0.35)	0.77	0.58

introduced to diversify the existing germplasm collection for scald disease resistant breeding.

Estimated F_{IS} values were high and negative; -0.67 for resistant and -0.76 for sensitive lines. The sensitive group had higher H_{obs} than expected when it was compared to that of resistant group. This suggests that selection intensity (selection against homozygotes) and crossing practices among scald resistant Turkish barley lines may be higher than those in sensitive ones. As it is expected, estimated F_{ST} values were high both in resistant (0.39) and in sensitive groups (0.46). These results indicate that about 39% of total variation was between, and 61% of the total variation was within resistant groups, while 46% of total variation was between, and 54% of the total variation was within sensitive groups. Turpeinen *et al.* (2003) found that 31% of total variation was between and 69% was within barley populations in Israel. The lower amount of variation among resistant lines comaperd to sensitive lines may be due to higher intensity of selection applied to resistant lines during routine selection studies conducted on these germplasm collections every year.

The dendrogram constructed with genetic distance data yielding an interesting result that some resistant and sensitive lines (R11, R12, R28, R5, R10, R37, R38, S4, S5, S13, S14, S15, S17, S31, S34, S35, S36, S19, S33) were located in the same cluster. The parental origins of most of these lines are proprietary lines such as YEA Eskişehir, Tokak and Antares which were utilized in barley breeding for their valuable agronomic characteristics. Therefore, the reason for having sensitive and resistant lines in the same cluster may be due to common crosses that may have taken place among parental sources through breeding. Although the clustering pattern, for some lines, is not very firm the more markers included, the clearer the pattern of grouping is expected to become.

Grouping which was done using common parental origin revealed that in sensitive lines, there were no significant differences between H_{obs} and H_e because these sources are not preferred for artificial selection, which is expected (Table 7 *[Supplementary data]*). In contrast, in two of the resistant lines (Tokak and Einbull), H_{obs} was higher than H_e (Table 7). This may be due to the fact that these two lines were involved in many crosses due to their valuable agronomic characteristics such as winter hardiness, cold and drought tolerance along with scald resistance (T. Akar, unpublished data). Because of these important properties, these lines might be preferred more often to others as a parental source for development of new scald resistant lines. The previous studies dealing with land races of corn in Turkey have shown that land races maintain considerable genetic variation in morphological and agronomic traits (Ilarslan *et al.*, 2002) as well as neutral isozyme markers (Ilarslan *et al.*, 2001). These studies suggested that those land races with valuable agronomic traits, more frequently moved and planted in places where intensive agriculture (e.g., use of high yielding corn lines) is practiced, had higher genetic diversity.

Current barley screening procedure for scald resistance is time and labor consuming because plants

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Table 7: The population genetic diversity parameters for scald sensitive and resistant Turkish barley lines grouped according to their parental origins. n_a = observed number of alleles per locus, n_e = Effective number of alleles (Kimura and Crow, 1964), *P* = the proportion of polymorphic loci, H_{obs} = the observed heterozygosity, and *He* = the expected heterozygosity (Levene, 1949).

Parental Origin	n_a	n_e	Percent polymorphic loci (P)[a]	Mean heterozygosity	
				Observed (H_{obs})	Expected (H_e)[b]
Sensitive					
Pamir	3.0±0.17	2.4±0.11	100.0	0.63±0.09	0.57±0.02
Anteres	3.0±0.23	2.6±0.24	100.0	0.54±0.13	0.59±0.03
Tokak	2.5±0.27	2.1±0.19	83.3	0.61±0.12	0.48±0.07
Roho	3.3±0.17	2.7±0.16	100.0	0.58±0.10	0.61±0.02
Eskisehir	3.0±0.38	2.6±0.32	83.3	0.61±0.15	0.54±0.09
Yea	3.5±0.27	2.2±0.18	100.0	0.65±0.13	0.53±0.04
Others	3.3±0.12	2.7±0.09	100.0	0.61±0.07	0.62±0.01
Resistant					
Einbull	2.7±0.11	1.9±0.07	100.0	0.66±0.07	0.45±0.02
H272/Deir Alla	3.0±0.34	2.5±0.25	100.0	0.58±0.18	0.63±0.04
Tokak	2.8±0.19	2.4±0.13	100.0	0.67±0.13	0.42±0.02
AVD/Bulbul	2.7±0.40	2.0±0.29	83.3	0.52±0.20	0.45±0.10
4814/132TH	2.2±0.29	1.9±0.22	83.3	0.56±0.18	0.45±0.09
Yea	3.5±0.20	2.7±0.10	100.0	0.62±0.09	0.63±0.02
Others	3.3±0.14	2.7±0.14	100.0	0.61±0.09	0.58±0.02

must be first grown, then inoculated with *R. secalis* spores, and finally visually evaluated after 18 days of inoculation (Mert and Karakaya, 2004). Any reduction in resources allocated to this process would improve efficiency of barley breeding for scald resistance. Of the six SSR markers employed in this study, Bmag6 and Bmag206 seem to have potential to accelerate screening of large number of barley individuals for scald resistance in relatively short time. Almost 90% of the individuals exhibiting the genotypes of "11" for Bmag206 and "22" for Bmag6 will be resistant to scald. Therefore, large number of barley individuals can be pre-screened using these two SSR markers with the above banding pattern (producing "11" genotype of Bmag206 and "22" genotype of Bmag6 loci) for scald resistance.

Marker assisted selection is a promising tool for plant breeders. While there are several major genes and quantitative trait loci are identified in barley for disease resistance and grain quality, it is hard to say that the full potential of marker assisted selection is utilized in barley breeding (Rae *et al.*, 2007). Further research with selected lines created for scald resistance breeding purposes is needed for possible use of Bmag6 and Bmag206 for marker assisted selection in barley breeding.

In conclusion, the results of this study indicated that a set of SSR markers sufficiently characterized the barley lines used the in scald resistance breeding programs and breeders and researchers dealing with barley breeding could use these SSR markers for fingerprinting of genetic resources and screening lines, germplasm collection and genetic material exchange purposes. Of the markers studied, Bmag6 and Bmag206 can be utilized in quickly screening large number of germplasm for scald resistance before they are tested conventionally in greenhouse.

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