ANALYSIS OF QUANTITATIVE TRAIT LOCI ASSOCIATED WITH FREEZING TOLERANCE IN RAPESEED (BRASSICA NAPUS L.)

A. Asghari1, S.A. Mohammadi2, M. Moghaddam2, M. Toorchi2, A. D. Mohammadinasab2
University of Mohaghegh Ardabili, Faculty of Agriculture, Department of Crop Production and Breeding, Ardabil, Iran1
University of Tabriz, Faculty of Agriculture, Department of Crop Production and Breeding, Tabriz, Iran2
Correspondence to: Seyed Abolgasem Mohammadi
E-mail: mohammadi@tabrizu.ac.ir

ABSTRACT
Freezing tolerance is a major component of winter survival and is the ability of plant to survive subfreezing temperatures. In order to identify the molecular markers linked to chromosomal regions governing freezing tolerance in rapeseed, an F2:3 population of 199 plants derived from crossing between cv. ‘SLMO46’ (winter type and cold resistant) and cv. ‘Quantum’ (spring type and susceptible to low temperature) were used. The LT50 (the temperature at which 50% of plants were killed) was measured as a cold resistance index in F1 families. The parental polymorphism was assessed using 350 SSR primer pairs and 250 RAPD primers. The 32 microsatellite and 47 RAPD markers, polymorphic between parental lines were used to screen F2 individuals. Linkage map was constructed using polymorphic markers. The markers were assigned into 14 linkage groups with total length of 1199.1 cM and an average distance of 17.13 cM between adjacent markers. The relationship between LT50 and genotypic data was analyzed using single marker analysis (SMA), interval mapping (IM) and composite interval mapping (CIM) methods and four putative QTLs were detected. These QTLs explained 24% of the LT50 total phenotypic variance.

Keywords: Brassica napus, canola, freezing tolerance, microsatellites, QTL mapping, RAPD, rapeseed

Introduction
Freezing tolerance, the ability of plants to survive subfreezing temperatures, is the major component of winter survival and important characteristic necessary for optimum seed yield of both winter and spring rapeseed varieties (16, 24). Freezing tolerance may be assayed in both winter and spring rapeseed varieties (16, 24). Freezing tolerance can also be up-regulated by low temperature (2, 8, 21, 22). Freezing tolerance is also strongly correlated with the capacity for maintaining high photosynthesis during cold acclimation, because it is indispensable to ensure an energy source during cold acclimation. Cessation of growth during cold acclimation is also necessary to reach the resistance (17, 22).

Rapacz and Janowiak (17) pointed out that prehardening of winter rape, by keeping the plants at +12/20°C day/night from sprouting until the beginning of the 1st stage of cold acclimation is essential to reinforce the effect of cold acclimation and increase its effectiveness. During prehardening, plants formed leaf rosette and increased the photosynthetic efficiency at chilling temperatures. During 1st stage of cold acclimation, the water content of prehardened plants decreases and soluble sugars and free proline of leaves increase (16).

The genetic regulation of freezing tolerance and winter hardiness is complex in most or all crop species (10, 13, 25). The reported gene action for freezing tolerance has varied from recessive to partially dominant in winter wheat (7), largely additive (4) to partially dominant (15) in alfalfa and partially recessive in potato (23).

In recent years, molecular markers, as useful complementary tools for classical breeding methods, were used in selection programs for quantitative traits such as freezing tolerance. Various molecular markers were developed and applied for mapping QTLs and MAS (Marker Assisted Selection). The objective of the present study was to identify microsatellite and RAPD markers linked to cold resistance genes in rapeseed.

Materials and Methods
A set of 199 rapeseed (Brassica napus L.) F2:3 families derived from crossing between cv. ‘SLMO46’ (winter type and cold resistant) and cv. ‘Quantum’ (spring type and susceptible to low temperature) were used. This population was used for QTL analysis of winter survival in rapeseed (1). Plants were grown in greenhouse with photoperiod of 14/10 (day/night) and leaf samples from rosette stage were harvested from...
individual F2 plants for DNA extraction. F2 individual plants were selfpollinated for development of F3 families.

Parental lines and 199 F2 derived F3 families were assayed for their freezing tolerance. For prehardening, fourteen plants from each family were grown in pots (15x35x25 cm diameters) and with five centimeters space between adjacent plans under controlled conditions (12/20°C day/night and 14 h day length) for five weeks in greenhouse. The prehardened plants were transferred to growth chamber for an additional three weeks to cold acclimation under controlled condition (4/2°C day/night, 260 μmol/m²/s light and 14 h day length). Then, all of the pots were kept at -2°C in the dark condition for 24 h. After this period, the temperature was decreased at a rate of 2°C per hour to reach -4, -8, -12 and -16°C as test temperature using programmable freezer. After completion of freezing test, the temperature was raised to 1°C at a rate of 2°C per hour. The plants were kept at 1°C for another 24 h, and then were transferred to greenhouse with similar condition to the period before cold acclimation. After three weeks the percent of dead plants in each family was counted and the LT50 values were calculated based on the probit method (5).

The DNA of parental lines and individual F2 plants was extracted using the CTAB procedure according to Saghai-Maroof et al. (19). The quality and quantity of DNA samples were assessed using spectrophotometer (Biophotometer Eppendorf, Germany) and 0.8% agarose gel electrophoresis. All of the DNA samples were diluted to 25 ng/μl and used in PCR reactions. A total of 350 microsatellite primer pairs (The BBSRC primers, for which the sequences were obtained from http/ukcrop.Net/perl/ace/tree/brassicaDB) and 250 RAPD primers (NAPS unit standard primers: set # 5, set # 6 and set # 7) were used to analyze polymorphism in the parents and the polymorphic primers were used to genotype 199 F2 individuals.

Microsatellite analysis was carried out using PCR reaction of 10μl volume containing 50ng of DNA template, 3mM MgCl2, 0.25 mM each dNTP, 0.25 μM each primer, 1U Taq DNA polymerase and 1x reaction buffer. PCR reaction for RAPD analysis was performed in a volume of 15μl contained 50ng of DNA template, 2mM MgCl2, 0.05mM each dNTP, 0.132 μM primer, 1U Taq DNA polymerase and 1x PCR buffer.

The amplification profile for microsatellite analysis consisted of an initial 3-min denaturation step at 94°C followed by 35 cycles of 1-min at 94°C, 1-min for annealing at 55-65°C (depending on each primer pair), 2-min extension at 72°C and a final 7-min extension step at 72°C. For RAPD primers the amplification profile consisted of a 3-min initial denaturation step at 94°C followed by 40 cycles of denaturing at 94°C for one min, annealing at 37°C for one min, extension step at 72°C for two min and a final extension step at 72°C for seven min.

In the microsatellite analysis, PCR products were detected using 6% denaturing polyacrylamide gels and silver staining method based on CIMMYT protocols (3), whereas RAPD amplified products were analyzed using 2% agarose gels and ethidium bromide staining.

After the testing of segregation distortion in each marker locus, the linkage map of polymorphic markers were constructed using map manager/QTL v. 2, considering of a LOD score of 3.0 and a maximum distance of 50 cM between adjacent markers. The Kosambi (11) mapping function was used for converting recombination frequencies into genetic distances in centiMorgans. QTL analysis was performed using QTL Cartographer v. 2.5. SMA, IM and CIM methods were used to identify the QTLs and to estimate their effects.

**Results and Discussion**

LT50 of the resistant parent, ‘SLMO46’, and the susceptible parent, ‘Quantum’, were –13.33 and –8.62°C, respectively and LT50 of the F3 families ranged from –16.87 to –4°C with an average of –11.86°C. Forty families (20%) had lower LT50 than the resistant parent, ‘SLMO46’, while twenty families (10%) had greater LT50 than the susceptible parent, ‘Quantum’ (Fig. 1).

**Fig. 1.** Relative freezing tolerances of parental lines ‘Quantum’ (Q), ‘SLMO46’ (S) and F3 families

**Fig. 2.** LOD profile in LG10 for a QTL controlling cold resistance in F3 population from the Brassica napus L. cross ‘SLMO46’ × ‘Quantum’. This profile computed using Win QTL Cartographer v. 2.5. The additive effects are displayed with simple lines under the LOD profile of QTLs.
In SMA method, three microsatellite and three RAPD markers showed association with chromosomal regions involved in cold tolerance in rapeseed. Four QTLs were confirmed using IM and CIM methods and at least one of the flanking markers of detected QTLs by these methods, was correlated with LT50 at P≤ 0.01 based on SM method. The other two detected QTLs using SMA method were not confirmed by IM and CIM methods. The first confirmed QTL was situated on the Linkage group 3 (Fig. 2) between the RAPD markers 528a and 528b and explained 3% of the phenotypic variance of LT50. This QTL had an additive and dominant effects of 0.55 and 1.82, respectively (Table 1). The second QTL was located on Linkage group 8 between RAPD marker 593c and microsatellite marker Ol10-G06 with additive and dominant effects of 0.25 and 0.41, respectively explaining 6% of the phenotypic variance. The third QTL was located on the Linkage group 9 between Na10-B08 (microsatellite marker) and 430b (RAPD marker) with positive additive effect of 0.97 and negative dominant effect of –0.46, which explained 7% of the phenotypic variance. In a separated study, we found a QTL for winter survival near the 650a and 430b RAPD primers and this can be showed the relationship between winter survival and LT50 in rapeseed (1). The fourth QTL was located on the Linkage group 10 between RAPD markers 474a and 430b, showing additive and dominant effects of –1.05 and –1.32, respectively. This QTL explained 8% of the LT50 phenotypic variance (Table 1). The position of third and fourth QTLs is on chromosomes 15 and 14 of *B. napus* based on BBSRC linkage maps, respectively.

### Table 1

<table>
<thead>
<tr>
<th>QTL</th>
<th>Linkage group</th>
<th>Peak position (cM)</th>
<th>LOD</th>
<th>Additive effect</th>
<th>Dominant effect</th>
<th>Explained Phenotypic Variance %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>20</td>
<td>2.7</td>
<td>0.55</td>
<td>1.82</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>102</td>
<td>2.4</td>
<td>0.25</td>
<td>0.41</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>34</td>
<td>2.7</td>
<td>0.97</td>
<td>-0.46</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>28.6</td>
<td>2.2</td>
<td>-1.05</td>
<td>-1.4</td>
<td>8</td>
</tr>
</tbody>
</table>

Transgressive segregation for LT50 in this study revealed the polygenic control for freezing tolerance in rapeseed. Teutonico et al. (25) reported transgressive segregation for freezing tolerance and acclimation ability in *B. rapa* and *B. napus* populations. They concluded that freezing tolerance is a trait controlled by many genes and the positive effects come from both parents. Fuller (6) suggested that the basis of Cauliflower varietal differences in frost resistance is the quantitative accumulation of frost resistance genes.

The four detected QTLs explained 24% of the total phenotypic variance of LT50 and the effects of each QTL ranged between 3 and 8%. The QTL on the linkage group 10 had maximum effect on cold tolerance. In other studies, QTLs...
The major and minor effects on freezing tolerance had been detected. In an acclimated F$_2$ population of *B. rapa*, four QTLs for freezing tolerance ability were detected and their effects ranged from 3.0 to 20.5% (25). Kole et al. (10) reported 16 QTLs for winter survival and related traits in a *B. napus* population of haploid lines where their effects ranged between 3 and 35.9. In wheat, one QTL on chromosome 5B explaining 31.5% of the freezing tolerance phenotypic variance was detected (26). In the F$_2$:3 population of maize, forty QTLs were identified under cold stress were associated with the shoot, root and seed traits. The QTLs effects ranged from 0.4 to 30.1% (9).

The QTLs on linkage group 3 and 8 had positive additive and dominant effects (Table 1), indicating that resistant alleles of ‘SLMO46’ at these loci increased freezing tolerance of the F$_2$ plants and that the heterozygotes had greater tolerance than the resistant parent ‘SLMO46’. The QTL on linkage group 9 had positive additive but negative dominant effect. This showed that the allele increasing freezing tolerance in F$_2$ plants, had been transmitted from ‘SLMO46’ and the heterozygotes had lower freezing tolerance as compared to ‘SLMO46’. The QTL on linkage group 10 showed negative additive and dominant effects. This indicates that in this chromosomal region, the recessive allele increasing freezing tolerance in F$_2$ plants, come from susceptible parent ‘Quantum’ and that freezing tolerance of heterozygotes was lower than ‘SLMO46’. Three QTLs had smaller additive effects as compared to their dominant effects and in forth QTL the additive effect was greater than the dominant effect. These results revealed that freezing tolerance is controlled by genes having both dominant and additive effects but the dominant effects was predominant in controlling freezing tolerance of rapeseed. Teutonico et al. (25) reported that the additive effects were quite small and dominant effects were more significant in freezing tolerance of rapeseed. They observed heterosis for freezing tolerance in F, and suggested that some QTLs should have dominant or over dominant gene action for freezing tolerance. Kole et al. (10) found that QTLs with positive and negative additive effects come from both parents to double haploid lines.

**Conclusions**

In this study four QTLs with positive and negative additive and dominant effects were detected which explained only 24% of the freezing tolerance phenotypic variance in F$_3$ families. Therefore, additional QTLs are probably involved which we did not detect in this program. If the identified QTLs are confirmed in the next generations after several meiotic cycles, they could be used more efficiently in MAS for freezing tolerance.

**Acknowledgements**

The study was funded by the Center of Excellence for Molecular Plant Breeding, Department of Crop Production and Breeding, Faculty of Agriculture, University of Tabriz, Iran.

**REFERENCES**