



**Mapping and molecular marker development of genes
affecting cold adaptation of cereals**

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1. INTRODUCTION AND THE AIM OF THE STUDY

The enhancement of the winter survival of the cereals is one of the main target of the plant breeding, because the cereals cultivated in Hungary are mainly winter type and their winter survival is critical for the satisfying yield. For the knowing of the mechanism of the winter survival, it is important to know the precise position of the genes responsible for the winter survival using the genetic and physical maps as a tool. Thus, the cloning of these genes is possible and the practical utilization of gene-specific PCR-markers for marker assisted selection (MAS) is also feasible.

In the two chapters of the dissertation, mapping experiments in bread wheat and investigation of PCR-based markers for the rapid selection of frost tolerant barley genotypes are presented.

1.1. Mapping genes affecting flowering time and frost resistance on chromosome 5B of wheat

The bread wheat (*Triticum aestivum* L.) is the most important food-industrial crop in Hungary cultivated on 1.198 hectare in 2001 and 1.106 hectare in 2002. In Hungary, mainly the winter wheat is cultivated because it has 30-40 percent more yield comparing to the spring cultivars.

The winter is the most critical period for the winter cereals. Accordingly, the development of winter surviving cultivars is one of the main target of the wheat breeding. The main components of the winter survival are the frost tolerance and the vernalization requirement. For the satisfying frost tolerance the hardening is necessary, which includes adaptive biochemical and physiological modifications for the survival of frost. In the nature, the hardening occurs during autumn, when the temperature and the light period are going down. Also, the vernalization occurs during autumn, because the low temperature is necessary for the flowering of the winter cereals. In plant growing chambers, these courses can be simulated for the precise investigation of the mechanism of hardening, freezing and vernalization. Although, the frost tolerance is polygenic, there are some determinant genes, which are located on the chromosomes of the homoeologous groups 5 in wheat.

The aim of this study was the mapping the genes affecting flowering time and frost tolerance on the chromosome 5B in wheat.

1.2. Development of PCR-based markers of chromosome 5H for assisted selection of frost tolerant genotypes in barley

The practical utilization of the existing mapping data can be the marker assisted selection using linked markers. The application of PCR-based molecular markers in breeding programs can be not only time saving and cheaper than the traditional selection methods, but also in some cases the only efficient way for selection. In South European winter cereal environments there can be several years without severe cold, and severe winters are an erratic event. During breeding

programs in case of deep freeze in the field (below -12°C without snow) lasting many days all the barley population can be lost in one year especially when they are originated from winter/spring crosses, for inefficient selection of the frost tolerant genotypes. In such cases, it should have been necessary to get molecular markers to avoid field evaluation. Moreover, for selecting spring type cereals tolerant to cold after sowing them in the spring (i.e. in non-stressing conditions), the marker-assisted selection (MAS) is the most effective system.

The position of genes determining stress tolerance was located in the 90's using RFLP. The RFLP is not really proper method for selection because it is a long and expensive procedure, but the transformation of the RFLP markers into PCR-based markers can solve this problem because the PCR is a high throughput method and it is suitable for MAS.

Our goal was the development PCR-based markers starting from RFLP markers mapped on the regions of the chromosome 5 responsible for frost tolerance and the testing and mapping of these PCR based markers in barley.

2. MATERIALS AND METHODS

2.1. Mapping genes affecting flowering time and frost resistance on chromosome 5B of wheat

2.1.1. Mapping populations

Two populations of recombinant substitution lines were studied. The first of 61 lines derived from the cross between the single chromosome substitution line 'Chinese Spring' ('Cheyenne' 5B) and 'Chinese Spring' (CS). The second of 76 lines derived from the cross between the single chromosome substitution line 'Hobbit Sib' ('Chinese Spring' 5BL) and 'Hobbit Sib', a UK semi-dwarf winter wheat.

2.1.2. Determination of flowering time variation

In the absence of vernalization, it can be assumed that differences in flowering time in these materials were mainly attributable to allelic variation at *Vrn-B1*, where the 'Chinese Spring' 5B chromosome carries an allele, *Vrn-B1a*, that is vernalization insensitive (being a spring wheat), and 'Cheyenne' (a winter wheat) is vernalization sensitive and carries the allele *Vrn-B1b*. Ten unvernallized plants of each recombinant, and the parental controls, were grown in pots in a controlled environment room (16h/8h light/dark period; 20 °C) using a randomised block design for each population. In contrast, following full vernalization, differences in flowering time are expected to be attributable to the effect of loci insensitive to vernalization. To detect the presence of such earliness *per se* loci, five vernalized plants (treated at 6 °C for six weeks, 8hr days) of each of the 'Hobbit Sib' ('Chinese Spring' 5B) recombinant substitution lines were grown in pots in a controlled environment room (16h/8h light/dark period; 20 °C) using a randomised block design. Ear emergence time was recorded for each plant when the first tiller headed, measured from the time of sowing.

2.1.3. Freezing test

A test of the frost resistance of the 'Chinese Spring' ('Cheyenne' 5B) and 'Chinese Spring' mapping population was carried out using the procedure described previously by Sutka (1981) using ten plants of each lines in randomized block design. The freezing was carried out on -11, and -12°C. After recovery, the plants were scored from 0 to 5 depending on the recovery rate.

2.1.4. DNS extraction

DNA was extracted from the parents and recombinant lines using standard procedures.

2.1.5. Microsatellite analysis

Genetic maps of the populations were developed using simple sequence repeat (SSR) markers on the 'Chinese Spring' (CS) x CS ('Cheyenne' 5B)

population, and SSR and RFLP markers on the 'Hobbit Sib' ('Chinese Spring' 5BL) x 'Hobbit Sib' population. Primer pairs for SSR polymorphisms known to be on chromosome 5B from a variety of sources were tested on the parental DNA for the two crosses. PCR was performed in 15 µl final volume using 1.5 µl of 10x PCR buffer, 0.45µl of 50mM MgCl₂, 1 µl of 2mM dNTP, 1.5µl of 2µM microsatellite primers, 0.35 unit of Taq polymerase and 100 ng of template DNA. The PCR parameters and the annealing temperatures were applied according to published data (Röder et. al 1998) The separations of the PCR products were done by sequencing gels and visualised by silver staining using a standard protocol.

2.1.6. Map construction and QTL analysis

Linkage maps were constructed using JoinMap. QTL analysis was performed using QTL Cafe (<http://sun1.bham.ac.uk/g.g.seaton>). When putative QTL were found, these locations were further confirmed using MQM mapping using the programme MapQTL.

For the manual analysis of the data colourmapping was performed (Kiss et al. 1998).

2.2. Development of PCR-based markers of chromosome 5H for assisted selection of frost tolerant genotypes in barley

2.2.1. Plant materials

Twenty-eight frost susceptible and tolerant barley cultivars and lines of different origin and belonging to the germplasm collection of the Istituto Sperimentale per la Cerealicoltura (ISC) of Fiorenzuola d'Arda (Italy), with different growth habit were used in this study. The genotypes were selected on the basis of scores of field winter survival recorded in two years, in the growing seasons 2000/2001 and 2001/2002 at the ISC and thus classified as tolerant and susceptible. The growth habit was assigned on the basis of the records of vernalization requirement collected through several years (fall sowing and late spring sowing) at the ISC.

2.2.2. Phenotyping (F_v/F_m measurement)

The frost induced damage was measured in leaves as a decrease of the photochemical capacity of the photosystem II (PSII), using the chlorophyll fluorescence parameter, F_v/F_m , which is the ratio of variable (F_v) to maximal (F_m) fluorescence in dark adapted state. F_v/F_m was measured using a PAM 2000 fluorometer according to Rizza et al., (2001).

2.2.3. DNA extraction

Three hundred milligrams (300 mg) of young leaf tissue of three weeks-old barley plants were ground in liquid nitrogen, and total DNA was extracted with Nucleon PhytoPure Genomic DNA Extraction kit following the manufacturer's instructions.

2.2.4. Primer design and PCR analysis

In the study 16 RFLP markers were selected from barley and wheat maps, from both *Rcg1* and *Fr1* regions of chromosomes 5A and 5H. On the sequence data from the origin of the probes, specific primer pairs (20-27 bps) were designed.

Specific PCR amplification was performed cyclers in 20 µl final volume, containing 1,5 mM MgCl₂, 0,25 mM dNTP, 0,2 µM primer, 0,25 U Taq polymerase, 1x PCR buffer and 20 ng of template DNA. After 6 min. denaturation at 94°C, 35 PCR cycles (94°C, 1 min.; 55° or 58° C, 1 min.; 72°C, 1 min.) were performed, followed by 6 min. final extension at 72°C. The products were separated on 2.5% agarose gel.

2.2.5. RAPD analysis

The RAPD marker *OPA17*, amplified by the sequence 5'-GACCGCTTGT-3', was chosen because known to belong to the long arm of barley chromosome 5H. The PCR amplifications were performed in 20 µl final volume containing 2 µl of 10x PCR buffer, 0.6 µl of 50 mM MgCl₂, 0.2 µl of 10 mM dNTP, 0.3 µl of 10 µM *OPA17* RAPD primer, 0.2 µl of 5U/µl Taq polymerase and 20 ng of template DNA. Following 2 min. of denaturation at 94°C, 46 cycles of amplification were performed (94°C, 1 min.; 30°C, 1 min.; 72°C, 2 min.) and 10 min. at 72°C for the final extension. The PCR products were separated as described before.

2.2.6. Genetic mapping of PCR products

The two STS markers *wg644* and *psr637* and the RAPD *OPA17* were added to a barley map (Francia et al. accepted), to confirm their map position on chromosome 5H. For this purpose, the segregations of the amplified products were scored in a 136 barley F₁-derived doubled-haploid population from the cross 'Nure' x 'Tremois'. Linkage analysis was performed by the DOS 3.0 version of the package MAPMAKER; a LOD threshold of 3.0 was used to accept linkage with neighbouring markers.

3. RESULTS

3.1. Mapping genes affecting flowering time and frost resistance on chromosome 5B of wheat

3.1.1. Mapping of *Vrn-B1*

The mean flowering times of the two parents of the first cross, raised under non-vernalization conditions, were 70.6 ± 2.97 days for 'Chinese Spring' and 75.6 ± 1.08 days for 'Chinese Spring' ('Cheyenne' 5B). The range of flowering times of the recombinant substitution lines was 66.4 to 88.4, where the transgressive segregation suggests the presence of two loci in repulsion.

Nine SSR markers were found to be polymorphic on the long arm of chromosome 5B and tested on the recombinant lines of this mapping population. The map covers 92 cM with an average of 12 cM distance between the SSR markers.

The map positions of two QTLs affecting flowering time were localised using QTL Café, and confirmed by composite interval mapping. The main one was situated distally on the long arm of chromosome 5B (78 cM from the centromeric markers), closely linked to the SSR locus *Xgwm604* and distal to *Xgwm408*. This had an additive effect of 1.76 days and accounted for 10.7% of the phenotypic variation, the late allele coming from Cheyenne 5B. Previously, Röder et al. (1998) have shown that both these SSR loci are closely linked (9.7 cM, and 4.5 cM, respectively) to *Xcdo504-5B*, a RFLP locus on the long arm of the group 5 chromosomes. A homoeologous RFLP locus, *Xcdo504-5A*, was found to be closely linked to *Vrn-A1* on the long arm of chromosome 5A (Galiba et al. 1995). Taking account the synteny among the homoeologous group 5 chromosomes, the QTL found here on 5B should thus equate to *Vrn-B1*.

3.1.2. Mapping of *Eps* loci

The other QTL segregating in the 'CS/Cheyenne' 5B population had a less pronounced, but still significant effect on flowering time, with an additive effect of 1.49 days, accounting for 8.2% of the phenotypic variation. This was linked to the SSR locus *Xwmc73*, close to the centromere. Previous mapping data in barley showed the existence of an *Eps* QTL at a homoeologous position (Laurie et al. 1995). Consequently, we hypothesise that this QTL represents an *Eps* locus. To examine this hypothesis further, the flowering time of the fully vernalized population from the 'Hobbit Sib' x 'Hobbit Sib' ('Chinese Spring' 5BL) cross was analysed.

The mean flowering times of the two parents were 88.5 ± 2.6 days for 'Hobbit Sib' and 86 ± 3.7 days for 'Hobbit Sib' ('Chinese Spring' 5BL). The range of flowering times of the recombinant substitution lines was 77.6 to 102.7, again transgressive segregation being displayed, suggesting the segregation of at least two loci in repulsion.

Nine microsatellite and five RFLP markers were found to be polymorphic on the parents, and the 'Hobbit Sib' x 'Hobbit Sib' ('Chinese Spring' 5BL) recombinant lines were genotyped for these. The map developed covers 68 cM and the average distance between the markers was 6.8 cM.

Although the presence of transgressive segregation suggests that probably two loci in repulsion were segregating, QTL analysis by interval mapping and composite interval mapping only detected the existence of a single QTL for flowering time, close to the centromere (approx 16 cM away), near to the marker *Xgwm499* and the QTL is probably an *Eps* locus. This QTL had an additive effect of 1.2 days but only accounted for 5.7% of the variation in this cross.

3.1.3. Mapping of *Fr-B1*

The means of the frost scores of the parents were 1.8 for 'Chinese Spring', 2.8 for 'Chinese Spring' ('Cheyenne' 5B) at a -11°C test temperature, and 0.5 for 'Chinese Spring', 2.0 for 'Chinese Spring' ('Cheyenne' 5B) at a -12°C test temperature. The range of frost scores of the recombinant substitution lines was 0.9 to 3.4 at -11°C and 0.05 to 3.0 at -12°C . For the QTL analysis the same map was used as for the mapping of *Vrn-B1*, except for 4 missing lines.

Using the mapping data and the frost scores, a single QTL was localised 40 cM from the centromeric marker using interval mapping, marker regression and composite interval mapping, and this mapped at the same position at both test temperatures. This QTL was closely linked to the SSR locus *Xgwm639*, with additive effects of 0.53% and 0.33% at -11°C and -12°C , respectively. At -11°C , this QTL accounted for 31.4% of the variation, so clearly is a major effect.

3.2. Development of PCR-based markers of chromosome 5H for assisted selection of frost tolerant genotypes in barley

The practical utilization of the existing mapping data can be the marker assisted selection using linked markers. Our goal was the development PCR-based markers starting from RFLP markers mapped on the regions of the chromosome 5 responsible for frost tolerance and the testing and mapping of these PCR based markers in barley.

3.2.1. Phenotyping

The genotypes were chosen in order to have two clearly separated groups of barley lines tolerant vs. susceptible to frost, on the basis of scores of two years of winter survival (rated 0 to 9) recorded at Fiorenzuola (Northern Italy).

The results obtained support the attribution of genotypes to the groups of contrasting tolerance: the average F_v/F_m scores of the two groups are dramatically different. The genotypes classified as 'tolerant' showed an average value of F_v/F_m of 0.62, close to 0.80, that is a typical value of a healthy leaf after cold acclimation. The range of variability was between 0.42 and 0.78. The genotypes classified as 'susceptible' had an average value of F_v/F_m of 0.14, ranging from 0.02 to 0.32.

3.2.2. Development and validation of STS markers for selection of frost tolerance

The primers developed from the RFLPs in a first time were tested for their ability to amplify polymorphic STSs on 'Nure' and 'Tremois'. PCR products of the expected size were obtained using 12 primer pairs out of 23, both at 55° and 58°C annealing temperatures. Polymorphisms among 'Nure' and 'Tremois' were detected only for 2 STSs out of 20, derived from *wg644_3* and *psr637F* probe sequences, respectively from their 3'- and 5'- ends.

The expected size of the PCR product amplified by STS primers of *psr637* probe was 282 bp. The annealing polymorphism of the STS is a band clearly identifiable in the majority of frost tolerant barley cultivars, and not amplified in the frost susceptible ones. The polymorphism is not in agreement with tolerance classification only in one case out of 13 frost susceptible cultivars, but in several (7 out of 15) frost tolerant ones.

The expected size of the PCR product using the primer pair designed from the 3'-end sequence of the *wg644* RFLP probe was 215 bp. An intense band slightly smaller than 500 bp, common to most tolerant genotypes, and absent from most susceptible was amplified. Using this primer pair the pattern of the tolerant genotypes is different from that of the susceptible ones, simple, with the predominant absence of the polymorphic band of 500 bp. The amplification pattern of the susceptibles was more complex, with many additional fragments, without the intense amplicon of 500 bp. This marker is not universal because exceptions in both groups can be noticeable.

3.2.3. Testing of the *OPA17* RAPD marker

It can be clearly seen that the *OPA17* RAPD marker separates tolerant genotypes, possessing a lower (~650 bp) band, from susceptible ones, possessing one or two different fragments of about 700-750 bp. There is one tolerant genotype possessing a susceptible allele, while three susceptible lines possess the tolerant allele of 650 bp.

3.2.4. Mapping of the developed PCR-based markers

The STS locus *psr637* was located on the long arm of chromosome 5H, proximal to the *Frl-Vrn-H1* region, at about 16 cM from this. Its position is consistent with the known position for the RFLP marker from which it was derived (Galiba et al 1995).

Two polymorphic fragments obtained by the amplification of *wg644_3* segregated and were mapped in the 'Nure' x 'Tremois' doubled haploid population. The first one (of about 500 bp), indicated as *wg644c*, was mapped on the long arm of chromosome 5H close to the centromere. A second polymorphic amplicon of the developed STS, corresponding to a faint band of approximately the expected size of 215 bp, was segregating independently by the former, and thus mapped in the

'Nure' x 'Tremois' population on the long arm of chromosome 2H, between SSR markers *Bmag0125* and *EBmac0415*. It was named *wg644b*.

The RAPD marker *OPA17* was mapped more proximal respect to the *Fr1-Vrn-H1* region, at 7.1 cM from *psr637*.

3.3. New scientific results

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- a) New genetic maps were developed from the long arm of chromosome 5B in wheat using mapping populations derived from 'Chinese Spring' x 'Chinese Spring' ('Cheyenne' 5B) and 'Hobbit' x 'Hobbit' ('Chinese' 'Spring' 5BL) crosses.
- b) A new locus responsible for flowering time was mapped on the distal portion of the long arm of 5B chromosome using mapping population derived from the 'Chinese Spring' x 'Chinese Spring' ('Cheyenne' 5B) cross. This locus is linked to the *Xgwm604* SSR locus and it is probably corresponds to the *Vrn-B1*.
- c) A new locus responsible for flowering time was mapped close to the centromere on the long arm of 5B chromosome using mapping population derived from the 'Chinese Spring' x 'Chinese Spring' ('Cheyenne' 5B) cross. This locus is linked to the *Xwmc79* SSR locus and it is probably corresponds to an earliness *per se* locus (*Eps-5BL*).
- d) A new locus responsible for flowering time was mapped close to the centromere on the long arm of 5B chromosome using mapping population derived from the 'Hobbit' x 'Hobbit' ('Chinese' 'Spring' 5BL) cross. This locus is linked to the *Xgwm499* SSR locus.
- e) A new locus responsible for frost tolerance was mapped on the long arm of 5B chromosome using mapping population derived from the 'Chinese Spring' x 'Chinese Spring' ('Cheyenne' 5B) cross. This locus is linked to the *Xgwm639* SSR locus and it is probably corresponds to the *Fr-B1*

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- f) The *wg644_3* and the *psr637* RFLP probes were converted to PCR-based STS probes.
- g) The STS markers derived from the RFLP marker, furthermore, the *OPA17* RAPD marker are suitable with some limitation to the selection of frost tolerant and susceptible barley genotypes.
- h) The *psr637* STS marker and the *OPA17* RAPD marker were mapped on the long arm of the 5H chromosome of barley, with 7.1 cM distance from each other.
- i) The *wg644_3* STS marker was mapped on the 2H chromosome of barley between the *Bmag0125* (12,4 cM) and the *Ebmac0415* (27,7 cM) SSR markers and this STS was also localized in a new position on the 5H chromosome tightly linked with the *Bmac0113* SSR marker (1,2 cM).

4. CONCLUSIONS AND SUGGESTIONS

4.1. Mapping genes affecting flowering time and frost resistance on chromosome 5B of wheat

In the present study, vernalization requirement, earliness *per se* and frost tolerance loci were mapped using two mapping populations. Our maps covered the long arm of chromosome 5B, and the similarity in the order of the markers with the map published by Röder et al. (1998) is obvious.

Using flowering time data earliness *per se* loci were mapped in both populations close to the centromere. In barley, previous mapping data suggest the existence of earliness *per se* locus in similar position (Laurie és mtsai. 1995) and taking account the colinearity, the existence of similar locus close to the centromere on the chromosome 5 of wheat was predictable. Moreover, Sarma et al. (2000) reported the physical mapping of two loci responsible for flowering time on the chromosome 5B and one of them located close to the centromere. The positions the two *Eps* loci mapped on two different mapping populations are different but taking account the small difference between them, probably the same loci were mapped in both cases.

A comparison of the maps of chromosome 5B to other maps of chromosomes 5A and 5D was difficult due to the lack of the common markers, but the similar localisation of the *Vrn* loci on the long arms distal from the centromeres is clearly noticeable. *Vrn-B1* was mapped closely linked to the SSR locus *Xgwm604*. Although, this marker was not mapped on the 5A chromosome previously (Galiba et al. 1995) the *Xcdo504* RFLP locus links both to *Vrn-A1* and *Xgwm604*. Additionally, Sarma et al. (2000) characterised the chromosomes of homoeologous group 5 of wheat in terms of rice linkage blocks. RFLP analysis shows that both 5A and 5B *Vrn* regions map to syntenous regions on rice chromosome 3.

In the case of the *Fr* genes, there is less obvious similarity concerning the positions on chromosomes 5A and 5B. However, comparative mapping with rice again suggests a link. These results reveal that rice chromosome 9 is syntenous to a large part of the long arms of the wheat homoeologous group 5 chromosomes, proximal to the centromere. In our case, the *Fr-B1* locus was mapped closely linked to the SSR locus *Xgwm639*. The *Xgwm639* was also linked to the RFLP locus *Xpsr120* on the map developed from *T. turgidum ssp. durum*, 'Messapia' x *T. turgidum ssp. dicoccoides*, 'MG4343' (Korzun et al. 1999). This RFLP locus was localised on the 5B chromosome region, which is syntenous with rice chromosome 9. Using deletion lines of 'Chinese Spring', Sutka et al. (1999) also showed that *Fr-A1* on 5A mapped to a region syntenous to rice chromosome 9, proximal to the rice chromosome 3 region. So, although *Vrn-B1* and *Fr-B1* are not as tightly linked on the long arm of chromosome 5B as *Vrn-A1* and *Fr-A1* are on 5A, nevertheless, they both appear to be homoeoallelic. The most recent results of Vágújfalvi et al. (2003) showed that there are a second frost tolerance locus on the chromosome 5A

of *Triticum monococcum* L. namely *Fr-A2*. This locus is tightly linked to the RFLP locus *psr637* and this region seems to be syntenous to the region, where the *Fr-B1* has been mapped. Thus, it can't be excluded the other hypothesis that the *Fr-B1* is orthologous to the *Fr-A2*.

This study thus completes the mapping of the homoeoallelic series of vernalization requirement genes and frost resistance genes on the chromosomes of the homoeologous group 5 in wheat.

4.1.1. Manual analysis of the mapping data: colourmapping

It can be useful to verify the results calculated by computer softwares using a simple manual data analysis, and for this, the colourmapping is an efficient tool. (Kiss et al. 1998).

The linkage map of the long arm of chromosome 5B was verified using colourmapping and this method confirmed the results of the computer analysis.

By the analysis of the flowering time data, the recombinant lines showed parental genotype were investigated and we found that the phenotypic data of these lines showed differences from the phenotypic data of the parents. The lines carried recombinations only on the 5B chromosome and the other part of the genome was identical. Thus, this finding can be explained only with the existence of other major loci affecting flowering time on the short arm of the 5B chromosome, which was not mapped in this study. These major loci acting together with the loci mapped on the long arm are responsible for the phenotypic data obtained. This hypothesis is confirmed with the finding that despite of the transgressive segregation the QTLs mapped can explain only few percent of the phenotypic variance. In our experiments, several markers were tested to build up the map of the short arm of 5B chromosome but we could not find polymorphic markers. The marker saturation of the short arm can elucidate this question.

Applying colourmapping, not merely linkage map can be constructed but using the phenotypic data of the freezing test on -12°C , we could map the frost tolerance locus. It was possible because the phenotypic variance was occasioned by one locus, thus the phenotypic data of the recombinant lines was convertible to parental genotypic data and the frost tolerance locus was easily mapped as a single locus attribution using colourmapping. The advantage of this method was that the recombinations between the linked markers and the phenotype can be easily detected and the map based cloning is performable.

The phenotypic data of the freezing test on -11°C and of the flowering time were not suitable for this type of mapping procedure.

4.2. Development of PCR-based markers of chromosome 5H for assisted selection of frost tolerant genotypes in barley

4.2.1. Development of STS markers from RFLP markers

In the present study, RFLP markers mapped on the chromosomes 5A and 5H playing key role in the frost tolerance of wheat and barley were investigated

and PCR-based markers for MAS of frost tolerant genotypes were developed, tested and mapped. Using the sequences of 16 RFLP probes 23 primer pairs were designed and tested on two barley cultivars ('Nure' and 'Tremoisi') differed characteristically in their frost tolerance. The amplification with 12 primer pairs out of the 23 resulted products with the expected size, and two of them showed annealing polymorphism between the two cultivars. The polymorphic STS markers were tested on 28 barley genotypes with different frost tolerance (15 frost tolerant vs. 13 frost susceptible).

The STS marker developed from the RFLP probe showed good efficiency to differentiate the frost tolerant and susceptible barley genotypes. The *psr637* RFLP marker has been mapped proximal to the *Fr-Vrn* region (Galiba et al. 1995), and the STS derived from it was mapped in similar position. The distance of the *psr637* STS and the *Fr-Vrn* region is too large to explain the different pattern on the gel between the tolerant and susceptible genotypes. Winter survival and artificial freezing tests were carried out with our Italian collaborator to map QTLs affecting frost tolerance on the Nure x Tremois dihaploid mapping population (Francia et al. accepted for publication). The presentation of the data from this experiment is necessary to explain our results, but these data are not a part of the new scientific results of this dissertation. A new frost tolerance QTL was mapped on the 5H chromosome with 7.6 cM distance to the *psr637* using the winter survival test and a significant QTL was mapped on the 5H chromosome with 9.9 cM distance to the *psr637* following the freezing test. The distance between the new QTLs and the STS marker can explain the different pattern on the gel between the tolerant and susceptible genotypes and also can explain the few exceptions because there can be recombinations between the marker locus and the QTL.

The case of the STS marker developed from the *wg644* RFLP is more complicated. The locus was mapped on the chromosome 2H is probably identical with the RFLP locus, *Xwg644b* RFLP locus has been mapped in *Hordeum bulbosum* by Salvo-Garrido et al. (2001). The second polymorphism was mapped on the chromosome 5H but more proximal from the position of the original RFLP locus and this can be a new locus of the *wg644*. Since, no frost tolerance QTLs were mapped in these positions, they can't explain the different pattern between the tolerant and susceptible genotypes. We hypothesize, that the fragments corresponding the original RFLP marker were not polymorphic between the parents of the mapping population but they can be are identical with the fragments existing, non-mapped in the frost susceptible genotypes. The direct sequencing and the analysis of these fragments on other mapping population can help to confirm this hypothesis.

4.2.2. Other developed markers: RAPD OPA17

This RAPD marker resulted 3 polymorphic fragments. The QTL analysis using the winter survival and freezing test data showed the existence of two loci affecting frost tolerance on the long arm of chromosome 5H. One of them was

mapped on the *cbf1-OPAI7a* interval (4,1 cM) and accounted for the 46% of the phenotypic variation with 13.4 LOD value. The winter survival and frost tolerance QTLs were tightly linked to the *OPAI7* because the distance between them was 2.8 cM and 0.5 cM, respectively.

The *OPAI7* out of the three markers tested, seems to be the more effective because it is located linked to the frost tolerance QTL.

This is the first example in barley where three PCR-based, user-friendly, agarose gel-detected markers, *psr637*, *wg644* and *OPAI7*, were developed for selecting frost tolerant genotypes. With one step of PCR, without digestion using restriction enzymes, tolerant genotypes can be selected in a barley breeding program. Although one or two loci of barley explain the majority of variation for the trait, frost tolerance is a complex trait. For this reason the markers will be validated on different segregating progenies and breeding lines for the calculation of the increased tolerance that they will be able to introduce into genotypes. The three markers developed showed to be consistently associated with the trait in the germplasm surveyed, rather independently from the growth habit. None of the three markers resulted to be universal, and frost resistant as well as susceptible genotypes with opposite marker alleles were found.

The frost tolerance is a complex quantitative trait and the tolerance is formed by more loci. The key role of the chromosome 5H in the frost tolerance is proven (Hayes et al. 1993), thus the markers mapped in this region can be suitable for the following of the segregation of region affecting frost tolerance.

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