TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2016) 40: 488-495

doi:10.3906/bot-1508-21

Validation of RAPD markers associated with frost tolerance in winter faba bean (*Vicia faba* L.)

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Received: 25.08.2015 • **Accepted/Published Online:** 24.04.2016 • **Final Version:** 19.07.2016

Abstract: A set of 189 single seed lines (validation set, v-set), derived from a natural crossing crossing between 11 founder lines, were used as a different genetic background to validate markers associated with frost tolerance and leaf fatty acid composition content in the winter faba bean. These lines were evaluated in 20 replications with lattice design under three freezing temperatures (–16 °C, –18 °C, and –19 °C). Two morphological traits, regrowth after frost (REG) and area under symptom progress curve (AUSPC), were scored. In addition, leaf fatty acid composition content was analyzed after hardening conditions (without frost). High significant correlation was found between REG and AUSPC (r = –0.58**). AUSPC showed positive significant correlations with C18:0 and C18:2, and negative significant correlations with C18:3. Three RAPD markers were validated in a v-set and were found to be associated with REG and fatty acid contents (C18:0, C18:2, and C18:3). Of these markers, two were found to be associated with more than one trait. The results of the present study indicate that improving the winter faba bean using marker-assisted selection (MAS) is feasible and effective in accelerating breeding programs for producing cultivars with high frost tolerance.

Key words: Faba bean, frost tolerance, QTL validation, marker-assisted selection

1. Introduction

Frost stress is one of the abiotic stresses that limit winter faba bean production in cool-temperate regions. Due to insufficient winter hardiness genotypes, the faba bean in these regions is mainly sown as a spring crop, which has lower yield than the winter type (Arbaoui, 2007). In the faba bean, frost tolerance is a highly heritable trait and controlled by many genes with additive effects (Herzog, 1988; Arbaoui et al., 2008; Sallam and Martsch, 2015). Therefore, improving the frost tolerance of the winter faba bean is needed due to its agronomic features.

The current progress in genetic map development offers a new approach for improving resistance to frost tolerance. DNA molecular markers are of great interest to breeders and geneticists for analyzing quantitative resistance by targeting associated genomic regions or QTLs as well as genetic makers tightly linked to the trait of interest. The development of marker-assisted selection (MAS) techniques is promising for the accelerating breeding program to improve target traits (Díaz-Ruiz et al., 2010). The first study to map QTL controlling frost tolerance in *V. faba* was conducted by Arbaoui et al. (2008). They reported five and seven putative QTLs for frost tolerance and fatty acid content, respectively, using random amplified polymorphism DNA (RAPD) markers. Recently, Sallam and Martsch (2015) found 74 putative QTLs for frost tolerance and fatty acid content using 156 SNP markers in the faba bean. Moreover, a total of 26 putative QTLs were detected for winter hardiness and yield attributes by Sallam et al. (2015a). All these studies are considered primary studies. The results of these studies should be confirmed and validated by so-called replication studies (Kolb et al., 2001).

The next step after targeting the important putative QTLs is to validate and verify these QTLs in a different genetic background in order to maximize the usefulness of MAS (Kolb et al., 2001). Replication studies confirm that the detected QTL is real. A QTL validation study can be conducted from (I) a new mapping population derived from the same parents or closely related parents, and/or (II) the same population that was previously used as a primary study to address putative QTL for the target traits (Singh, 2015).

Validation of QTL controlling frost tolerance in additional winter faba bean breeding populations is necessary for testing the consistency of QTL effects across

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experiments and/or environments. Unfortunately, only a few studies have been conducted on improving frost tolerance in the faba bean using the MAS approach.

The objective of the current study was to validate several RAPD makers associated with frost tolerance and fatty acid composition in different genetic backgrounds.

2. Materials and methods

2.1. Plant materials

The plant materials of this study consisted of 189 single seed descent (SSD) lines from the Göttingen winter bean population (GWBP), which is derived from a natural crossing crossing between 11 founder lines: six lines from Germany (Webo, Wibo, Hiverna/1, L79/79, L977/88, and L979/S1), two from France (Côte d'Or/1 and Arrisot), and three from the UK (Banner, Bourdon, and Bulldo). The 189 SSD lines were used in QTL validation and named the validation set (v-set). Four additional spring faba bean lines were used as checks in the frost experiments: Limbo/7, Mélodie/7, Hedin/2, and Minica. The production of the 189 SSD was previously described in Sallam et al. (2015b).

2.2. Frost experiments

All lines were tested at the seedling stage in ten experiments with two replications (r = 20, as a final). The plants in each replication were exposed to hardening conditions at 5 °C for 10 continuous days. Then frost stress took place for 3 nights under –16 °C, –18 °C, and –19 °C. The experiments were conducted in a frost growth chamber (FGCh) according to Sallam et al. (2015b). After frost stress, all plants were chopped off at the second internode of the main stem to test their regrowth after frost. After 4 weeks, all regrowing juvenile plants were chopped off again and weighed to measure their regrowth (REG, g), as described in Sallam et al. (2015b) (Figure 1). The area under symptom progress curve (AUSPC) was calculated to reflect the symptoms of frost stress on faba bean leaves (Arbaoui and Link, 2007).

2.3. Fatty acid analysis experiment

In this experiment, all juvenile faba bean genotypes were exposed only to hardening conditions at 5 °C. Then the leaves of hardened genotypes were used in order to analyze fatty acid composition (Arbaoui and Link, 2007; Sallam et al., 2015b).

2.3.1. Selection of putative QTL associated with frost tolerance

A number of 12 putative QTLs for frost tolerance and fatty acid composition were reported by Arbaoui et al. (2008) using randomized amplified polymorphism DNA (RAPD). These putative QTLs were detected in biparental population (BPP, 101 inbred lines) derived from a crossing between two frost tolerant parents: Côte d'Or (one of the founder lines used to produce GWBP) and BPL4628 (a Chinese inbred line from the ICARDA germplasm collection). Of these QTLs, four RAPD markers (C06_768, F15_474, I10_661, and E20_1556) associated with frost tolerance and FAC were selected based on their proportion of phenotypic variation (R^2) and their association with at least two traits. Therefore, these markers were used for validation in GWBP.

2.4. DNA isolation and marker data

Leaf tissue from the v-set, founder lines, and the parents of BPP were collected and freeze-dried. Then the genomic

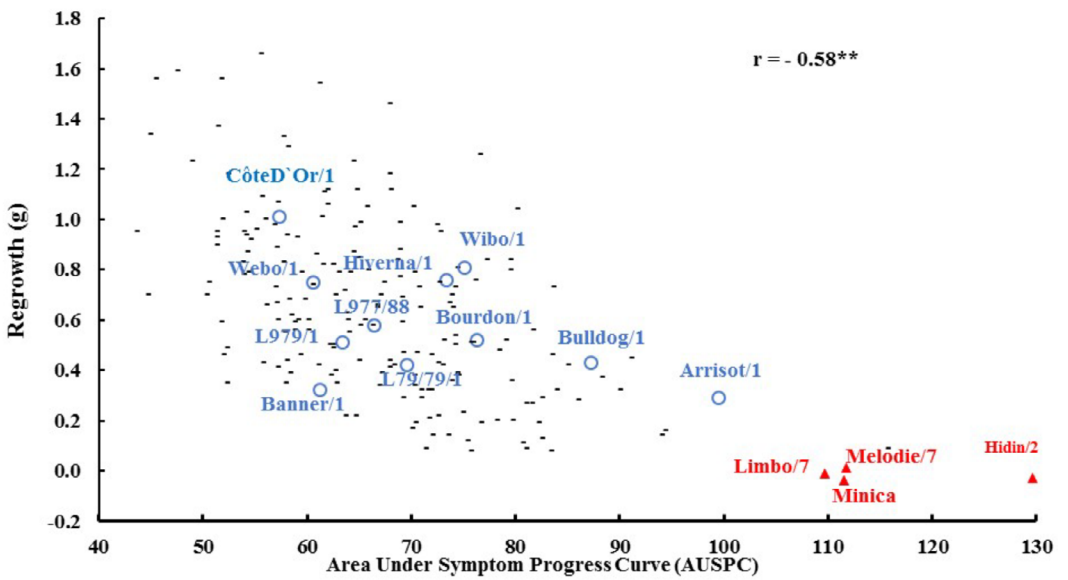

Figure 1. Spearman rank correlation between AUSPC and regrowth after frost. Blue circles refer to the 11 founder lines, while the red triangles refer to the four spring genotypes.

DNA was extracted using illustra Nucleon Phytopure Genomic DNA extraction kits (GE Healthcare Ltd, UK). The RAPD analyses were conducted as described in the experimental protocol of Williams et al. (1990), with some modifications according to Torres et al. (1993). The data were scored as 1 (presence of the target band) vs. 0 (absence of the band).

Moreover, the DNA from the founder lines and PBL4628 (12 parents as a total) was extracted using the aforementioned protocol. The DNA of each sample was analyzed by KBiosciences (Cambridge, UK), using 189 SNP markers to study the genetic relatedness between the 12 parents.

2.5. Statistical analysis

2.5.1. Phenotypic data

The artificial frost tests and fatty acid analyses were performed using lattice design, as extensively described by Sallam et al. (2015b). Spearman rank correlation was used to estimate the correlation between traits. Microsoft Office Excel 2013 was used to illustrate the graphical correlation.

2.5.2. QTL validation

The association between phenotypic data and RAPD marker data in the v-set was tested by single marker analysis (SMA) using the following model:

$$Y = \mu + f(\text{marker}) + \text{error},$$

where Y is equal to the trait value, μ is equal to the population mean, and f(marker) is a function of the molecular marker. The analysis was performed using STATISTICA 10 and confirmed with SAS 9.1.

Phenotypic effects at the marker loci were calculated as differences between the means of the marker classes. A positive value indicates that the specified marker allele increases the trait, whereas a negative value indicates that this allele is associated with a decrease in the trait. The phenotypic variance, explained (R^2) by the significant markers, was determined by multiple regressions. The box and whisker plot was analyzed using STATISTICA 10 to present the genotypes that possessed the band of interest (1) and the genotypes that did not have the band (0).

2.5.3. Principle coordinate analysis

The PCoA based on genetic distance was used to reveal the possible groups for the 12 parents. The genetic distance was calculated using simple match coefficient by R package 'ade4' (Chessel et al., 2004). The analysis was performed with the help of R software (R Development Core Team, 2008).

3. Results

The ranges (maximum and minimum), means, analysis of variance, and repeatability estimates for REG and fatty acid composition in v-set were discussed in Sallam and Martsch (2015). High genetic variation was found among all genotypes for AUSPC (F value = 12.08**). The AUSPC in the v-set ranged from 43.26 to 115.65. Among all traits, AUSPC showed the highest repeatability estimate (h^2 = 0.92).

The phenotypic correlation between REG and FAC was studied by Sallam and Martsch (2015). The Spearman rank correlation between AUSPC and other traits is presented in Figure 2. The highest positive correlation was found between AUSPC and REG (r = –0.58**). The most frost-tolerant parent was Côte d'Or/1, while the most frost susceptible was Arrisot/1. The AUSPC showed positive significant correlation with C18:0 and C18:2, while it was negatively and significantly correlated with C18:3.

The summary of QTL validation in the v-set is presented in Table 1. Of the four RAPD markers used in this study, three were validated in the v-set. F15_467 was found to be associated with three of the fatty acid composition contents (C18:0, C18:2, and C18:3). The I10-661 was associated with REG and C18:0. The third marker, E20_1556, was found to be associated with C18:0.

In order to verify some important frost tolerant QTLs that had been previously detected within the biparental population (101 lines), a different genetic background, consisting of 189 highly homozygous lines, was used. The single maker analysis for the validated QTL is presented in Table 2. The threshold of $P \leq 0.05$ was used in order to declare the presence of a significant QTL for each trait and to handle nonnormality in both phenotypic and genotypic data. As a result, the ANOVA analysis exhibited significant effects for only four traits: C18:0, C18:2, C18:3, and REG.

Three markers, F15_467, I10-661, and E20_1556, were found to be significantly associated with four traits: C18:0, C18:2, C18:3, and REG. No markers were found to be associated with AUSPC scored in this study. Marker F15_467 explained about 2.27%, 4.11%, and 4.57% of total variation in C18:0, 18:3, and C18:2, respectively. Moreover, the visible allele of this marker was associated with increased C18:2 and decreased C18:0 and C18:3. Two traits (REG and C18:0) were significantly associated with the I0-660 marker. The phenotypic variation explained by this marker was 3.41% and 3.91% for both C18:0 and REG, respectively. The visible allele of this marker was associated with increased REG (after frost) and decreased C18:0. Finally, the E20_1556 marker showed a significant association with C18:0. The phenotypic variation explained by this marker was 4.86 of the total variation. The visible allele of this marker was associated with decreased C18:0. The graphical analyses of the two groups of genotypes (1 vs. 0) for all marker-trait associations are presented in Figure 3.

The 189 SNP markers were used to perform the PCoA, based on genetic distance for all the 11 founder lines and BPL4628 (Figure 4). PCoA1 and PCoA2 accounts for

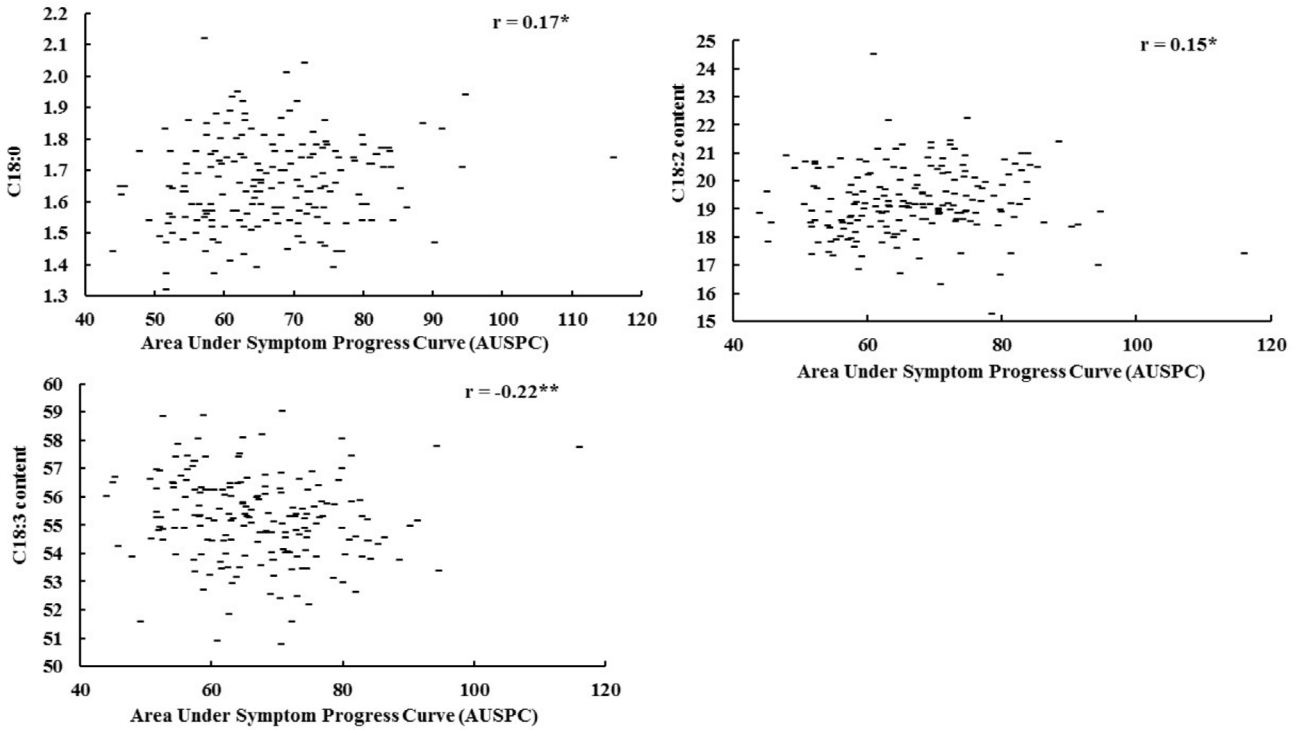

Figure 2. Spearman rank correlation between AUSPC and (a) C18:0, (b) C18:2, and (c) C18:3 contents.

Table 1. List of RAPD markers validated in validation set (v-set) and their QTL in biparental population (BPP) mapped by Arbaoui et al. (2008).

Marker	QTL in v-set	QTL in BPP	Source in BPP
F15_467	C18:0	U_AUSPC-2	Côte d'Or 1
	C18:2		
	C18:3		
I10_661	REG	H_AUSPC-2	Côte d'Or 1
	C18:0		
E20_1556	C18:0	D_C18:2	BPL 4628

Table 2. Single marker analysis for maker-trait association found in v-set.

Marker	QTL	LG[(1)]	*P value*	R^{2} [(2)]	Allele effects[(3)]	LSD
F15_476	C18:0	LG10	0.042003	2.27	–0.051130	0.05
	C18:2		0.004384	4.57	0.620694	0.43
	C18:3		0.020438	4.11	–0.625215	0.53
I10_661	Regrowth (REG)	LG10	0.049314	3.91	0.107847	0.11
	C18:0		0.021395	3.41	–0.048458	0.04
E20_1556	C18:0	LG03	0.003095	4.86	–0.106094	0.05

[(1)]Linkage group (Arbaoui et al., 2008); [(2)]Phenotypic variation explained by marker; [(3)]Allele effects of the visible allele.

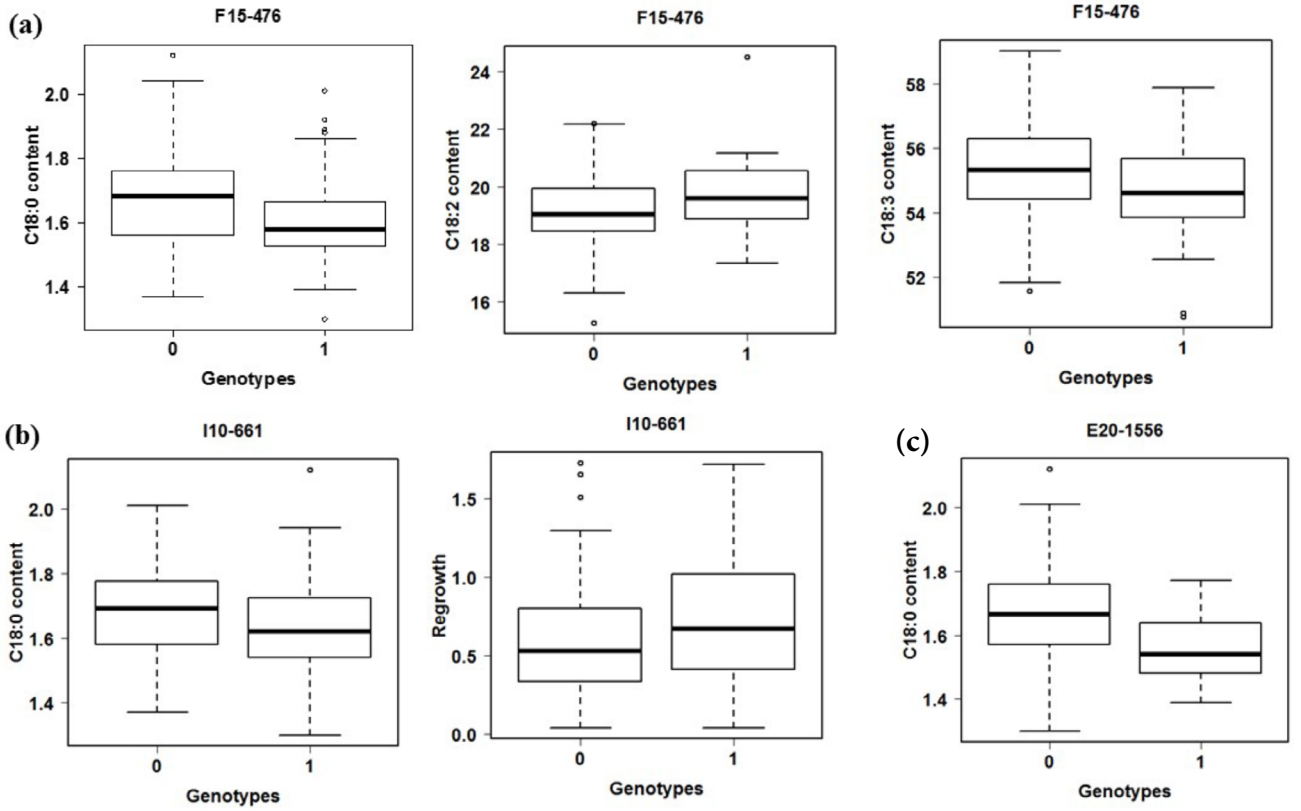

Figure 3. Phenotypic variation between genotypes carrying target allele (1) and not carrying target allele (0) for traits showing association with markers (a) F15_146 (b) I10_661, and (c) E20_1556.

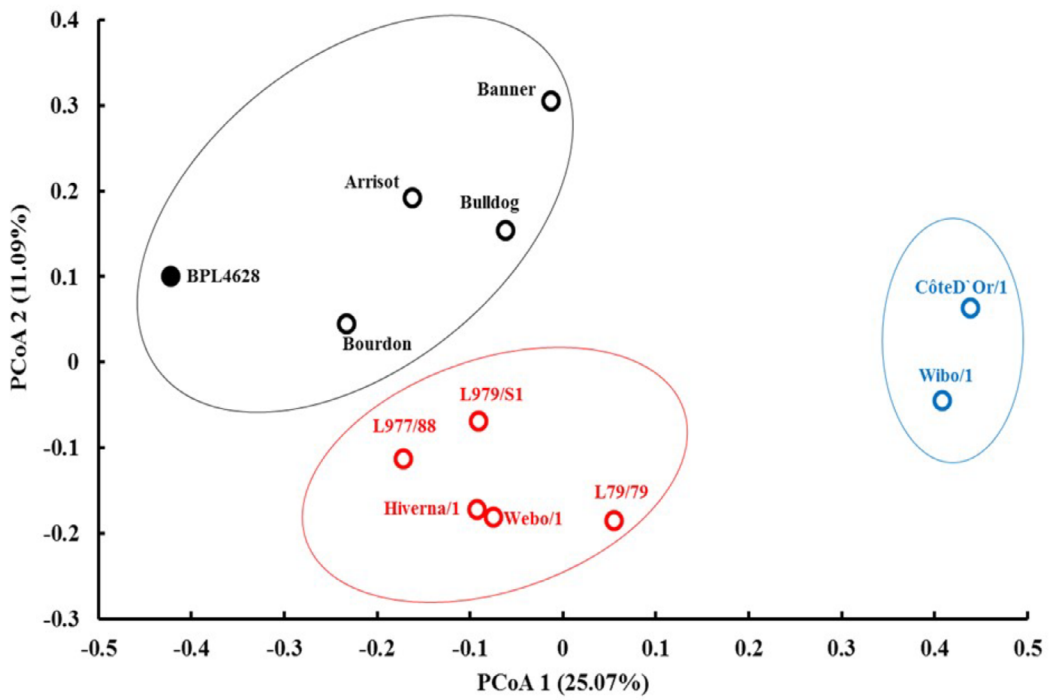

Figure 4. Principal coordinate analysis (PCoA) for the parents of verification set (v-set, 11 founder lines) and BPP4628 using 189 SNP markers. BPL4628 was marked by a filled-black circle.

25.07% and 11.09%, respectively. Notably, three possible groups can be distinguished, namely Groups I (Côte d'Or/1 and Wibo/1), II (L979/S1, L977/88, Hiverna/1, and L79/79), and III (Banner, Arrissot/1, Bulldog, Bourdon, BPL4628).

4. Discussion

A high genetic variation was found between all genotypes in the v-set for C18:0, C18:2, C18:3, and REG (Sallam et al., 2015b). Moreover, high significant differences were found among all genotypes for AUSPC. In this study, REG after frost and AUSPC were considered as frost tolerant traits, because these traits reflected the symptoms of frost on the juvenile faba bean. The founder lines also showed high variation in their frost tolerance. The most tolerant parent was Côte d'Or/1, while Arrisot/1 was the most susceptible parent. Côte d'Or/1 was previously reported as a frost tolerant faba bean genotype (Arbaoui et al., 2008). This high genetic variation found between genotypes for all traits (especially REG and AUSPC) could be used by plant breeders to improve frost tolerance in the faba bean. Moreover, high repeatability estimates were reported for C18:0 ($h^2 = 0.88$), C18:2 ($h^2 = 0.75$), C18:3 ($h^2 = 0.64$), and REG ($h^2 = 0.63$) by Sallam and Martsch (2015). Moreover, AUSPC showed the highest repeatability estimates ($h^2 = 0.92$). A high repeatability estimate was found for AUSPC ($h^2 = 0.89$) (Arbaoui et al., 2008). Such high repeatability estimates allow selection under frost stress (REG and AUSPC) and after hardening (FAC) to be feasible and effective for improving the winter faba bean. The four spring beans genotypes (checks) did not survive after frost in any frost experiment.

The highest phenotypic correlation was found between AUSPC and REG after frost ($r = -0.58^{**}$). This indicates that the fewer the symptoms of frost in faba bean leaves, the higher its likelihood to survive after frost. The AUSPC showed a positive significant association with C18:0 (one of the saturated fatty acids) and a negative significant correlation with C18:2 (one of the polyunsaturated fatty acids). Williams et al. (1988) and Palta et al. (1993) found a high accumulation of unsaturated fatty acid in the membrane lipids of chilling-resistant plants during acclimation to cool temperatures, and a high amount of saturated fatty acid in chilling-susceptible plants. The AUSPC was also negatively and significantly correlated with C18:3. This was expected due to the negative significant correlation between C18:2 and C18:3 ($r = -0.74^{**}$), according to Sallam et al. (2015b). Additionally, low correlations were reported between fatty acid content and frost tolerance by Arbaoui et al. (2008). These low-significant correlation values indicate that fatty acid composition cannot be used as a tool to directly select for frost tolerance.

Important putative QTLs associated with frost tolerance and FAC in the faba bean were previously published by Arbaoui et al. (2008), to be used in marker-assisted selection to improve the winter faba bean. These putative QTLs were detected in BPP, consisted of 101 lines, and derived from a crossing between two frost-tolerant parents (CôteD'Or/1 × BPL4628). An important step in MAS is to verify these QTLs in an independent population with a larger population size (Collard et al., 2005; Hassan 2013). In this study, we used an interesting genetic background consisting of 189 winter faba beans (v-set). Our material derived from a natural crossing of 11 founder lines originating from three different parts. CôteD'Or/1 was the common parent in both BPP (used for QTL mapping) and v-set. QTL validation is commonly conducted using an additional mapping population derived from parents other than those used in the preliminary mapping (Singh 2015).

The association between the genotypic data (selected RAPD markers) and all phenotypic data scored by Sallam et al. (2015b) was tested using SMA (Table 2). As a result, four traits (C18:0. C18:2, C18:3, and REG) were found to be associated with three markers (F15_476, I10_661, and E20_1556). In Arbaoui's study, F15_476 was found to be associated with decreased U_AUSPC (frost symptoms without hardening conditions). In our study, this marker was found to be associated with three FACs (C18:0, C18:2, and C18:3), which showed significant correlation with AUSPC (frost tolerance with hardening, Figures 2b–2d). Marker I10_661 was previously reported as a marker associated with decreased H_AUSPC (frost tolerance with hardening conditions). In the current study, it was associated with REG after frost (frost tolerance with hardening) and C18:0 (FAC). Finally, E20_1556 was putatively linked to D_C18:2 (FAC after hardening) in BPP, whereas it was associated with C18:0 (FAC after hardening) in the current study. Although some studies considered RAPD as anonymous markers, these markers are still used in developing genetic maps, QTL mapping, and genome-wide association studies (Gupta et al., 2012; Xu et al., 2012; Novalina and Sagala, 2013; Cheng et al., 2015).

The phenotypic variation explained by each marker for all traits scored in the v-set was lower than those reported (9.7% by E10_661, 11.5% by F15_476, and 18.7%

by E20_1556) for frost tolerance and FAC by Arbaoui et al. (2008). This may be due to the large number of lines used in this study compared to those used in BPP. Another reason is the nature production of the v-set. The low phenotypic variation explained by these markers in this study and in Arbaoui's study confirmed that these QTLs have little effect on frost tolerance. In the populations with a size of 200, the proximity of detecting QTL with $R^2 > 3\%$ is low, regardless of how many markers are screened (Li et al., 2010). In order to have QTL with $R^2 > 5\%$, a slightly smaller population is required to achieve similar detection power when more makers are used (Li et al., 2010). Although highly significant phenotypic differences were found among genotypes in the v-set, only a limited proportion of frost tolerance was explained. This may be due to the fact that frost tolerance in a polygenic trait could be affected by few major QTLs (Arbaoui et al., 2008).

The visible allele of the three markers showed different effects. For instance, the visible allele of F15_474 was associated with decreased C18:0 and C18:3 and increased C18:2. This allele was associated with decreased symptoms of frost (U_AUSPC) in BPP. Likewise, the visible allele of I10_660 was associated with decreased C18:0 and increased REG after frost. In BPP of Arbaoui et al. (2008), the same allele was found to be associated with decreased symptoms of frost (H_AUSPC). Finally, the visible allele of E20_1556 was found to be associated with decreased C18:0 in v-set, whereas it was associated with increased C18:2 (D_C18:2) in the BPP.

QTL for different traits may occur at linked loci in the genome (Zhao et al., 2007; Amar et al., 2008; Basunanda et al., 2010). These QTLs seem to have pleiotropic effects at one locus. Notably, F15_476 and I10-661 were mapped in the same linkage group (LG10), while E20_1556 was mapped in LG03 (Table 2). The linkage disequilibrium (LD), as a correlation between alleles at two loci mapped in the same linkage group, was estimated between F15_476 and I10_661. The LD was 0.002. These two markers were associated with C18:0. The nonsignificant LD indicates that each marker is a single QTL for C18:0.

In BPP, the visible alleles of F15_476 and I10_661 were inherited from Côte d'Or/1, which is one of the founder lines of the v-set. Therefore, it may be possible that the genotypes with the same band size had inherited it from the same parent. On the other hand, the visible band of E20_1556 in BPP was inherited from BPL4628 (not from the founder lines). This indicates that BPL4268 may share genomic regions with other founder lines. To address this challenge, the founder lines and BPL4628 were genotyped using 189 SNPs to study their genetic relatedness using PCoA. As a result, BPL4628 was grouped with four founder lines, namely Arrisot, Banner, Bulldog, and Bourdon (Figure 3). For further confirmation, we analyzed the 12 parents with the same RAPD primer (E20) to figure out whether any founder parents of the v-set had the same band or not. The analysis of gel electrophoresis by the same primer is presented in Figure 5. The target band was labeled with a white arrow. The results indicate that nine founder parents were found to have the same band size. As such, this could be a possible reason for the presence of this band in the v-set.

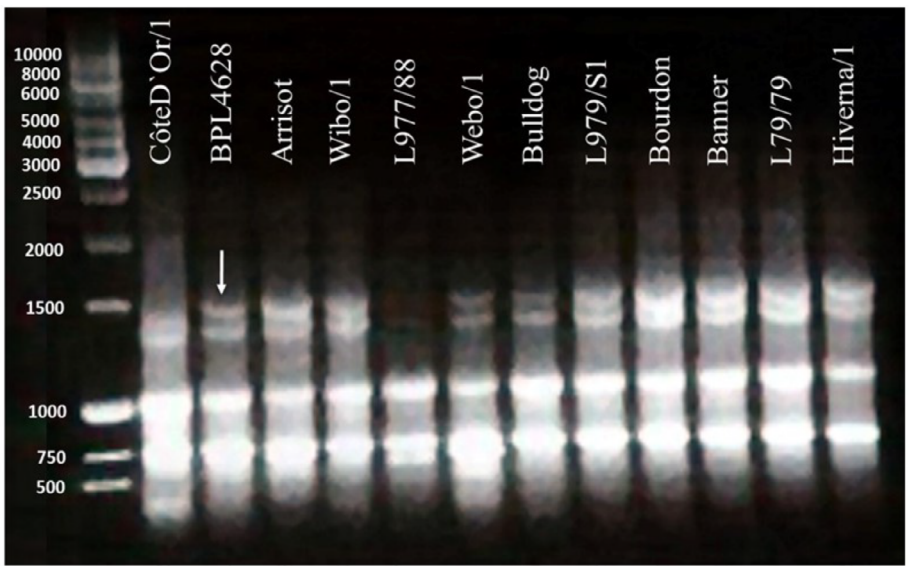

Figure 5. Gel electrophoresis for all founder lines (parents of v-set) + PBL4628 using E20 primer.

In conclusion, the validation of RAPD markers associated with frost tolerance in a different genetic background of the winter faba bean can be exploited to improve the winter faba bean through MAS. This will help to accelerate the breeding program by producing cultivars showing high resistance to frost stress in combination with other desirable traits, e.g., high yielding and high winter hardiness.

Acknowledgments

The authors would like to thank Prof Dr Wolfgang Link, Department of Crop Sciences, Georg-August-Universität, Göttingen, for funding this study. The authors thank Dr Mustapha Arbaoui, Centre Hospitalier Universitaire Hassan II, Fez, Fès-Boulemane, Morocco, for his valuable discussion of this work. This work was conducted in the Molecular Genetic Lab, Division of Plant Breeding, Department of Crop Sciences, Georg-August-Universität, Göttingen.

References

Amar S, Ecke W, Becker HC, Möllers C (2008). QTL for phytosterol and sinapate ester content in *Brassica napus* L. collocate with the two erucic acid genes. Theor Appl Genet 116: 1051-1061.

Arbaoui M (2007). Detailed genetic analysis of faba bean (*Vicia faba* L.) winter-hardiness and related traits. PhD, University of Göttingen, Göttingen, Germany.

Arbaoui M, Link W (2007). Effect of hardening on frost tolerance and fatty acid composition of leaves and stems of a set of faba bean (*Vicia faba* L.) genotypes. Euphytica 162: 211-219.

Arbaoui M, Link W, Satovic Z, Torres AM (2008). Quantitative trait loci of frost tolerance and physiologically related trait in faba bean (*Vicia faba* L.). Euphytica 164: 93-104.

Basunanda P, Radoev M, Ecke W, Friedt W, Becker HC, Snowdon RJ (2010). Comparative mapping of quantitative trait loci involved in heterosis for seedling and yield traits in oilseed rape (*Brassica napus* L.). Theor Appl Genet 120: 271-281.

Cheng L, Yang A, Jiang C, Ren M, Zhang Y, Feng Q, Wang S, Guan Y, Luo C (2015). Quantitative trait loci mapping for plant height in tobacco using linkage and association mapping methods. Crop Sci 55: 641.

Chessel D, Dufour A, Thioulouse J (2004). The ade4 package - I: One-table methods. R News 4: 5-10.

Collard BCY, Jahufer MZZ, Brouwer JB, Pang ECK (2005). An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: the basic concepts. Euphytica 142: 169-196.

Díaz-Ruiz R, Torres AM, Satovic Z, Gutierrez M V, Cubero JI, Román B (2010). Validation of QTLs for *Orobanche crenata* resistance in faba bean (*Vicia faba* L.) across environments and generations. Theor Appl Genet 120: 909-919.

Gupta M, Verma B, Kumar N, Chahota RK, Rathour R, Sharma SK, Bhatia S, Sharma TR (2012). Construction of intersubspecific molecular genetic map of lentil based on ISSR, RAPD and SSR markers. J Genet 91: 279-287.

Hassan M (2013). QTL Mapping: Methodology and Applications in Wheat Breeding. Saarbrücken, Germany: LAP LAMBERT Academic Publishing.

Herzog H (1988). Winter hardiness in faba beans: varietal differences and interrelations among selection criteria. Plant Breed 101: 269-276.

Kolb FL, Bai G-H, Muehlbauer GJ, Anderson JA, Smith KP, Fedak G (2001). Host plant resistance genes for Fusarium head blight. Crop Sci 41: 611.

Li H, Hearne S, Bänziger M, Li Z, Wang J (2010). Statistical properties of QTL linkage mapping in biparental genetic populations. Heredity 105: 257-267.

Novalina N, Sagala AD (2013). Construction of hevea brasiliensis geneticlinkage map and identification of quantitative trait loci using RAPD markers. Int J Adv Sci Eng Inf Technol 3: 71-75.

Palta JP, Whitaker BD, Weiss LS (1993). Plasma membrane lipids associated with genetic variability in freezing tolerance and cold acclimation of solanum species. Plant Physiol 103: 793-803.

Sallam A, Dhanapal A, Liu S (2015a). Association mapping of winter hardiness and yield traits in winter faba bean (*Vicia faba* L.). Crop Pasture Sci 67: 55-68.

Sallam A, Martsch R (2015). Association mapping for frost tolerance using multi-parent advanced generation inter-cross (MAGIC) population in faba bean (*Vicia faba* L.). Genetica 143: 501-514.

Sallam A, Martsch R, Moursi YS (2015b). Genetic variation in morpho-physiological traits associated with frost tolerance in faba bean (*Vicia faba* L.). Euphytica 205: 395-408.

Singh AKSBD (2015). Marker-Assisted Plant Breeding: Principles and Practices. 1st ed. New Delhi, India: Springer.

Torres AM, Weeden NF, Martín A (1993). Linkage among isozyme, RFLP and RAPD markers in *Vicia faba*. Theor Appl Genet 85: 937-945.

Williams AF, Tse AG, Gagnon J (1988). Squid glycoproteins with structural similarities to Thy-1 and Ly-6 antigens. Immunogenetics 27: 265-272.

Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18: 6531-6535.

Xu L, Wang L, Gong Y, Dai W, Wang Y, Zhu X, Wen T, Liu L (2012). Genetic linkage map construction and QTL mapping of cadmium accumulation in radish (*Raphanus sativus* L.). Theor Appl Genet 125: 659-670.

Zhao J, Dimov Z, Becker HC, Ecke W, Möllers C (2007). Mapping QTL controlling fatty acid composition in a doubled haploid rapeseed population segregating for oil content. Mol Breed 21: 115-125.