RESEARCH

Association Analyses to Genetically Improve Drought and Freezing Tolerance of Faba Bean (*Vicia faba* L.)

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ABSTRACT

In parts of Central Europe, such as Germany, climate change will lead to increasing area utilization for winter types of faba bean (*Vicia faba* L.) with improved tolerance to drought and freezing. Here, we present the first genome-wide association analysis focusing on drought and freezing stress in a set of 189 German winter faba bean lines. We assessed proline, glycine betaine, soluble sugars, water content, membrane stability, and chlorophyll content in leaves of juvenile plants, with and without drought stress. To describe freezing tolerance under growth chamber conditions, we monitored the freezing symptoms of juvenile plants, such as loss of color and turgidity, freezing survival, and regrowth after freezing—achieving medium to high repeatabilities ($0.43 < h^2 < 0.93$). With 175 single-nucleotide polymorphism (SNP) assays and 1147 amplified fragment length polymorphism (AFLP) assays, a total of 1322 (mostly mapped) DNA markers were utilized. We detected a total of 21 putative quantitative trait loci (QTL) for six of the traits, each explaining 6 to 15% of the phenotypic variance. Several phenotypically promising inbred lines were identified. The present results will greatly improve the prospects for including winter faba bean into German crop rotations in the near future.

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Abbreviations: AFLP, amplified fragment length polymorphism; GWBP, Göttingen winter bean population; GS, combined genetic similarities; LD, linkage disequilibrium; QTL, quantitative trait loci; RIL, recombinant inbred lines; SPAD, dimensionless unit of plant tissue chlorophyll content; SNP, single-nucleotide polymorphism.

Faba bean (*Vicia faba* L.) is a partially allogamous diploid annual grain legume (pulse crop) with 2n = 2x = 12 chromosomes and a genome size of about 13,000 Mb (Satovic et al., 2013). Its mature seed comprises about 45% starch and about 30% protein and is used for human consumption and animal nutrition. According to FAOSTAT (2015), the global faba bean area was about 2×10^6 ha in 2013, ranking between the global areas of lentil and lupine. China is the country with the highest faba bean production and home to about 45% of the global area. Another 14% of faba bean hectarage is in the Maghreb, and a similar proportion lies near the river Nile in Egypt, Sudan, and Ethiopia. Australia has about 5% of the global area, and the largest American grower is Peru with 3% (nearly 57,000 ha). In 2013, about two thirds of the total EU area (~230,000 ha) existed north of the Alps and Pyrenees. Germany's faba bean hectarage was 16,500 ha in 2013 and increased to 20,500 ha in

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2014, with organic field comprising about half of this area (EUROSTAT, 2015; FAOSTAT, 2015).

The current Intergovernmental Panel on Climate Change report (IPCC, 2014) considered it a virtual certainty that we will experience more frequent high temperatures and corresponding dry spells, with the continued occurrence of occasional cold winter extremes. Thus, the genetic improvement of abiotic stress tolerance is an important objective. In the face of a greater threat of drought, many temperate crops benefit from autumn sowing and the correspondingly faster development in spring and summer. Given the state of climate change, winter crops offer promise for higher and more reliable yields. The predicted milder winters will further allow autumn sowing even for crops with limited winter hardiness such as winter faba bean. Genetic improvements of their winter hardiness will open even more opportunities for autumn sowing.

Over the past decade, a number of studies have reviewed the abiotic stress tolerance of faba bean. Stoddard et al. (2006) described field-based and controlled-condition experiments assessing faba bean tolerance to drought and freezing, with consideration of physiological issues such as ^{13}C discrimination and proline accumulation. They further mentioned tolerant genotypes, such as ILB938 (drought tolerant) and Côte d'Or (freezing tolerant). Khan et al. (2007) presented experimental evidence pointing to stomatal conductance, leaf temperature, and ^{13}C discrimination as promising tools for breeding drought tolerant faba bean. In their 2010 report focusing on drought avoidance, Khan et al. (2010) proposed ^{13}C discrimination as the "gold standard" for assessing transpiration efficiency. They cited Amede et al. (1999) with regard to the lack of osmotic drought adjustment of known faba bean types, and described a need for screening of a genetically more diverse set of germplasm. They underscored that the realization of genetic progress related to drought tolerance will require not a single trait approach but the combination of different aspects of abiotic and biotic stress tolerance.

Araújo et al. (2015) reported details of the abiotic stress responses of faba bean, mentioning the relatively large amount of water that a faba bean seed must imbibe to germinate. They discussed the potential osmotic drought adjustment of faba bean, as well as the promise of interspecific crosses with *V. narbonensis* and *V. johannis* to improve freezing tolerance. The latter cannot yet be realized as such interspecific crosses with faba bean have, to date, been unsuccessful.

Across large parts of the UK and many coastal regions of France, the winter conditions are mild enough to grow current winter faba bean cultivars, such as 'Wizard' (Smith, 2015). However, winters may occasionally be too harsh for these crops to be grown in Germany and the more continental parts of Europe, north of the Pyrenees and Alps. Link et al. (2010) reported the current knowledge of breeding and the research status regarding winter hardiness and freezing tolerance, concluding that a genetically wider set of accessions must be studied to uncover genotypes with higher tolerance to both drought and freezing. Such work has been performed by Olszewski (1996), who found new freezing-tolerant "genitors" (ILB3187, ILB2999, ILB3245, and ILB14), and by Khazaei et al. (2013) who identified new drought-tolerant types along with their typical physiological profile.

Faba bean was long considered a "genomic orphan" crop, but, since 2008, increasing genetic information has become available (Ellwood et al., 2008; Alghamdi et al., 2012). Recently, an appreciable number of genetic markers have become available for faba bean (Kaur et al., 2014; Webb et al., 2015). Substantial additional progress is expected based on the high level of synteny between *V. faba* (genome not yet sequenced) and *Medicago truncatula* (genome sequenced; Khazaei et al., 2014; Smýkal et al., 2015; Webb et al., 2015). Findings from the sequenced genomes of *Arabidopsis thaliana* and *M. truncatula* will help to further progress in faba bean research (Link et al., 2010).

Arbaoui et al. (2008b) identified the first putative quantitative trait loci (QTL) for aspects of freezing tolerance in faba bean. However, we still lack the tools required for marker-assisted breeding for abiotic stress tolerance of faba bean. The current research aimed to develop data and expertise required for a simultaneous improvement of drought tolerance and freezing tolerance in winter faba bean for growth in Germany with the ultimate goal of increasing and stabilizing its grain yield and thus promoting this highly valuable protein crop in our field rotations. A genome-wide association analysis was undertaken for this investigation.

MATERIALS AND METHODS

Genetic Materials

The genetic material used in this study included the so-called A-set (inbred lines for association analysis) and the M-set (inbred lines for linkage mapping). For the A-set, 189 inbred lines were available, and a number of check entries (including 11 founder lines, see below) were added. These A-set lines have been bred via single-seed descent from the so-called Göttingen Winter Bean Population (GWBP), and were inbred to generations beyond F9. The GWBP was created in 1989 through the combination of 11 founder lines: Hiverna/1, Webo/1, Wibo/1, L79/79/1, L977/88/S1wn, L979/S1/1/1sn (German lines), Côte d'Or/1, Arrissot (French lines), Banner/1, Bourdon/1, and Bulldog/1 (UK lines). After the initial mixing, eight generations of natural reproduction were allowed. At Göttingen, such German winter faba beans reproduce with roughly 50 to 60% outcrossing (Gasim et al., 2004). Thereafter, the single-seed descent (i.e., inbreeding) of the A-set lines was begun (Gasim, 2003).

For the M-set, the biparental RIL population of Arbaoui et al. (2008b) was used. The parents of this M-set were the freezing-tolerant pure line Côte d'Or/1 (a founder line of the GWBP) and the Chinese pure line BPL4328 (Duc and Petitjean, 1995). From this cross, a total of 101 pure lines (i.e., RIL) were derived via single-seed descent and were, in the present study, used to place the markers on a linkage map; thus, the M-set lines were not phenotyped.

DNA Marker Assessment

Amplified fragment length polymorphism (AFLP) and single-nucleotide polymorphism (SNP) marker assays were conducted to genotype these entries. For each inbred line, one individual was used for AFLP detection and one different individual for SNP detection. For AFLP analyses, DNA was extracted from 100 mg leaf material per juvenile plant using the Illustra Nucleon Phytopure Genomic DNA Extraction Kit (GE Healthcare, Great Britain), applying mercaptoethanol and following the manufacturer's protocol with small modifications. The AFLP analyses were performed following the protocol reported by Vos et al. (1995) using multiplex PCR (Ecke et al., 2010). In contrast to previous studies, we ran 19 cycles for pre-amplification, and the final AFLP amplification involved 11 cycles with an annealing temperature of 64.2°C and 24 cycles with an annealing temperature of 56.0°C. A total of 96 primer combinations were used, as described in detail by Welna (2014).

DNA fragments were detected using the ABI 3130xl Genetic Analyzer System (Applied Biosystems, Foster City, CA), with GeneScan 500LIZ used to check fragment size. Actual scoring was supported by GeneMapper software (Applied Biosystems, 2003). The resulting data was transformed into a 0/1 matrix (Ecke et al., 2010). We performed marker loci definition and recording of marker peak absence vs. presence for markers that were polymorphic between the M-set parents, or that were polymorphic between three of the A-set lines (S_048, S_122, S_253). These three A-set lines were chosen as additional guides for marker loci definition motivated by a study of the segregation of a subset of our AFLP markers in backcross families among them (Ali, 2015a). The GeneMapper-supported analysis was hence conducted jointly across the M-set and A-set. Scored yet unmapped markers occurred for two reasons: they were polymorphic in the M-set but could not reliably be mapped, or they were monomorphic in the M-set but polymorphic among the three A-set lines.

For SNP analyses, a sample of 50 mg freeze-dried leaf tissue from each tested plant was sent to LGC Genomics (Cambridge, UK) for DNA extraction and SNP analysis. Several hundred assays for Kompetitive Allele Specific PCR analysis were available from the work of Cottage et al. (2012a, 2012b). SNPs were selected if they were simultaneously polymorphic in the M-set and the A-set.

Phenotyping

We assessed phenotypic data related to (1) drought tolerance-related traits and to (2) freezing tolerance of the 189 A-set lines. For the first topic, these lines were analyzed for several physiological traits presumed to be generally associated with drought tolerance (Balko, 2005b; Stoddard et al., 2006), including contents of free proline, glycine betaine, and soluble sugars (μmol g^{-1} in dry matter), leaf water content and membrane stability index (%), and chlorophyll content (SPAD). Two replicates were realized, one from the 2011–2012 season and another from the 2012–2013 season. For each entry and replicate, ten potted juvenile plants were available. The plants were grown in a climate chamber, without stress, with 16-h light per day at 20°C (day) and 17°C (night). Testing was performed using samples of the youngest unfolded (i.e., third and fourth) leaves at about 25 d after sowing. Traits were assessed from detached leaflets or from punched-out leaflet disks, without stress application (control), and after stress application (details follow; Supplemental Table S1).

Leaflet discs were used to assess proline, sugars, and membrane stability, whereas detached leaflets were used to examine chlorophyll, leaf water, and glycine betaine. Control proline and control sugar contents from unstressed leaflet discs were analyzed in a sample of 25 discs, from ten leaflets per entry and replicate. Stress proline content was analyzed in 25 stressed leaflet discs, and another 25 stress-treated leaflet discs were used for sugar content analysis (Supplemental Table S1). An additional 25 stressed and 25 unstressed leaflet discs were analyzed for control and stress membrane stability, respectively. Chlorophyll was measured in ten detached leaflets, directly after cutting (control) and once again after stress treatment. The same leaflets were used to gravimetrically determine leaf water content. Control glycine betaine was analyzed in ten independent detached leaflets. Glycine betaine after stress was measured in the same leaflets already used for measurements of chlorophyll and water content after stress (Supplemental Table S1).

For proline analysis, drought stress was applied to the leaflet discs using PEG (420 g PEG6000 per kg water) in HEPES buffer (25 mM KCl, 3.7 mM $CaCl_2$, and 5 mM HEPES). Leaflet disks were placed on this medium at 20°C, 80% relative air humidity for 48 h under continuous light and incubated on a rotary shaker at 60 rpm. For the other traits, drought stress was induced without medium. These leaflets and leaflet discs were placed on a gossamer layer in Petri dishes and for 48 h exposed to under continuous light, at 20°C and with 80% relative air humidity, thus provoking wilting (Balko and Seddig, 1998; Balko, 2004, 2005b; Stoddard et al., 2006).

Free proline content was assessed photometrically using freeze-dried material according to the method described by Bates et al. (1973). Soluble sugars contents were also analyzed photometrically from freeze-dried material, following the method of Yemm and Willies (1954). For membrane stability assessment, leaflet discs were shaken in 50-mL test tubes with 20 mL deionized water for 24 h at 7°C. Electric conductivity (EC1) was measured. The samples were then boiled for 30 min followed by a second conductivity measurement (EC2). The Membrane Stability Index was calculated as [1– (EC1/EC2)] × 100, according to Chandrasekar et al. (2000). Chlorophyll content was determined indirectly using a SPAD502Plus tool (Konica Minolta, Langenhagen, Germany).

Glycine betaine content was analyzed in two steps. First, ultra-performance liquid chromatography was conducted on 80 leaflet samples (UPLC-ESI-MS with a SeQuant ZIC-HILIC column), using dried, milled, water-extracted, and 9:1

Acetonitrile-diluted material, after centrifugation (Spielmeyer and Pohnert, 2012; Gebser and Pohnert, 2013). In the second step, a near-infrared spectroscopy calibration was developed based on these results and on near-infrared spectra from the same samples. The ratio performance deviation was 2.29 in the calibration and 1.81 in the cross-validation. The calibration equation was used to predict the glycine betaine content of the remnant dried and milled leaflet samples.

During both seasons and for each trait, ten inbred lines were analyzed per calendar week. This established an incomplete block of a corresponding α-lattice design, with 20 incomplete blocks representing one replicate; thus 11 checks were added to the entries. The software PLABSTAT (Utz, 2004) was used throughout the analyses.

Phenotypic analysis of freezing tolerance was conducted between September and April in 2011–2012 and 2012–2013 in a plant growth chamber with juvenile, hardened, potted plants (Sallam et al., 2015). The chamber was an accessible 4-m^2 Vötsch VB4018 that provided 200 μmol s^{-1} m^{-1} light, and could produce temperatures down to −20°C. Air humidity fluctuated freely. The 4-m^2 area could contain 54 pots (size 17 × 17 cm^2), which represented one replicate. With two plants per entry and four entries per pot, we established an a-lattice design with 12 entries per incomplete block (i.e., 3 pots) and 18 incomplete blocks. There were $r = 2$ per lattice experiment, and ten such experiments (five per each of two seasons) yielding a total of 20 replicates.

The utilized soil was a 3:1 mixture of local compost soil and sand, with the moisture gravimetrically maintained at approximately 70% of field capacity throughout the experimental period. Germination occurred under mild conditions. At the two-leaf stage, plants were transferred into the growth chamber for a 10-d hardening at 4 to 5°C and with a 10-h photoperiod. To prepare for the frost test, each pot was plugged into an open polystyrene box. The actual test involved a sequence of three successive nights of freezing down to −16, −18, and −19°C. The linear approach towards each freezing level took 4 h, the freezing temperature was maintained for 4 h, and the linear return to mild recovery conditions (to ~5°C) took 6 h (Arbaoui et al., 2008a; Arbaoui and Link, 2008; Roth and Link, 2010; Sallam et al., 2015). After these three freezing nights, the pots were transferred back to the greenhouse under mild conditions, maintaining their randomization. A recovery phase of 4 d and a regrowth phase of 4 wk followed.

The following freezing traits are reported here (Sallam et al., 2015): plant height (cm) at end of hardening, loss of leaf turgidity due to freezing (scale 1–9, with 9 representing the greatest loss), loss of leaf color due to freezing (scale 1–9, with 1 representing no color change and 9 representing black), ultimate survival at end of the regrowth phase or number of days between frost test and death, and shoot regrowth (g $plant^{-1}$) at the end of the regrowth phase. Loss of turgidity and loss of color were each determined after the first, second, and third nights of freezing and again after 4 d of the recovery phase. These eight scores were added to obtain an accumulated "loss of turgidity and color" score [Loss(T+C); scale 8–72]. After the 4-d recovery phase, the main stems and tillers of these juvenile plants were chopped off at the second node. The remaining small trunk allowed us to make daily assessments of whether the plant was alive or dead, and any regrowth verified that the plant was alive. After 4 wk, all shoot biomass was harvested from living plants and weighed as fresh matter, and thus regrowth was assessed. For those plants that died, the number of days between the frost test and their death was transformed into a disposition to survive (0° to 90°; Roth and Link, 2010; Sallam et al., 2015). Disposition to survive was calculated as $\arctan(x_i/\mu_x)$, with x_i = number of days until death and μ_x = average number of days until death for those plants that died within the 4-wk regrowth phase. The surviving plants were scored as 90°.

The drought-related results were analyzed according to the α-lattice design of the experiments, rwesulting in lattice-adjusted means across the two replicates. These data were employed to calculate Spearman rank correlations. Repeatabilites (h^2) were calculated as implemented in PLABSTAT, with $r = 2$ of the drought experiments, in which

$$h^2 = \frac{\text{variance component of genotypes}}{\text{variance component of genotypes} + \frac{1}{2}\text{effective error MS}}.$$

The freezing-related results were first analyzed according to the α-lattice design of the ten experiments. The resulting lattice-adjusted means were utilized to analyze the series of experiments with the main effects "genotypes" and "experiments". Spearman rank correlations and h^2 were calculated accordingly. Here, with $r = 10$, we calculated

$$h^2 = \frac{\text{variance component of genotypes}}{\text{variance component of genotypes} + \frac{1}{10}\text{MS}}$$

for the combination of Genotypes × Experiments. The drought and freezing results were then further subjected to association analyses.

Association Analysis

To study the potential associations between DNA markers and phenotypic expressions of traits, we analyzed 2018 polymorphic markers and all traits of the 189 inbred lines of the A-set, employing TASSEL 3.0 (Bradbury et al., 2007). Initially, 189 SNP loci and 1829 AFLP loci were available. After filtering for a minor allele frequency of 5% with 175 SNP and 1147 AFLP, a total of 1322 markers remained. The Mixed Linear Model mode was applied with an optimum level of compression and a re-estimation of the variance component estimates after each marker. A kinship matrix was developed and employed. Again, the SNP and AFLP markers were used after filtering for a 5% minor allele frequency. SNP-based genetic similarities among the A-set lines were calculated using the simple-matching coefficient, and AFLP-based genetic similarities using the Jaccard coefficient (Piepho and Laidig, 1997).

Next, combined genetic similarities (GS) were calculated, weighing the SNP-derived and the AFLP-derived coefficients according to their numbers (175/1322 and 1147/1322, respectively). With these combined similarities, a principal coordinate analysis was conducted (NTSYSpc; Rohlf, 2000) to visualize potential structure among the A-set lines (Pearson and Manolio, 2008; D'hoop et al., 2010; Kollers et al., 2013; Vilhjálmsson

and Nordborg, 2013). In the same way, combined similarities were determined among the 11 founder lines of the GWBP. The average GS coefficient among the founders was utilized as a threshold to separate between kinship-relevant similarity and alike-in-state similarity among the 189 A-set lines. All similarities below that threshold were set as zero, while all above the threshold were linearly transformed to a scale from zero to one. Following TASSEL, kinship values were constructed by adding a constant value of 1.0 to each resulting number. The resulting kinship matrix was utilized for association analyses.

For each trait, we tested the significance of associations between marker alleles and trait expression using the P-values of the markers as calculated as by TASSEL, and according to the methods of Benjamini and Hochberg (1995) and Benjamini and Yekutieli (2005). A false discovery rate of 20% was applied (Honsdorf et al., 2010). Phenotypic effects as calculated by TASSEL corresponded to the difference between the means of the two marker classes. The variance explained simultaneously by all significant markers of a given trait was obtained from multiple regression analysis employing the BASIC mode of PLABSTAT.

Analysis of overall gamete phase disequilibrium (also called linkage disequilibrium; LD) between marker pairs was performed for the mapped markers (n = 805; 118 SNP and 687 AFLP) and repeated for all markers of the association analyses. Additionally, all markers found to be significantly associated with a trait were studied for mutual LD, whether mapped or not. Employing TASSEL, the selected LD type was "full matrix," and thus no sliding window was applied.

Mapping of Markers

A total of 130 SNP and 1415 AFLP were found to be polymorphic in the M-set and were used for mapping with Mapmaker 3.0. The Kosambi mapping function was applied, with a maximum distance between markers of 35 cM and a LOD of 3.0 (Ecke et al., 2010). After re-analysis of the resulting linkage groups with the 'order' command, further markers which were not yet mapped were manually placed with the 'try' command (Ecke et al., 2010; Welna, 2014).

RESULTS

The genetic linkage map was used to study linkage disequilibrium among pairs of mapped markers and to compare the present map with previously published maps. This map spans 1633.2 cM and shows 1159 marker loci, including seven major linkage groups embracing 1140 loci, including 9 to 22 SNP per major linkage group. These groups can be associated with the six *V. faba* chromosomes via n = 111 SNP which are in common with the consensus map of Cottage et al. (2012b) (see also Supplemental Fig. S1, S2; Welna, 2014; Webb et al., 2015).

The average GS among the 189 A-set lines was 0.329, whereas the average GS among the 11 founder lines was 0.320. In the A-set, 9% of the pairwise marker similarities fell below the threshold of 0.320 set by the founders—hence, 9% of the kinship matrix data was deemed "unrelated"—while eight line pairs showed GS of >0.750.

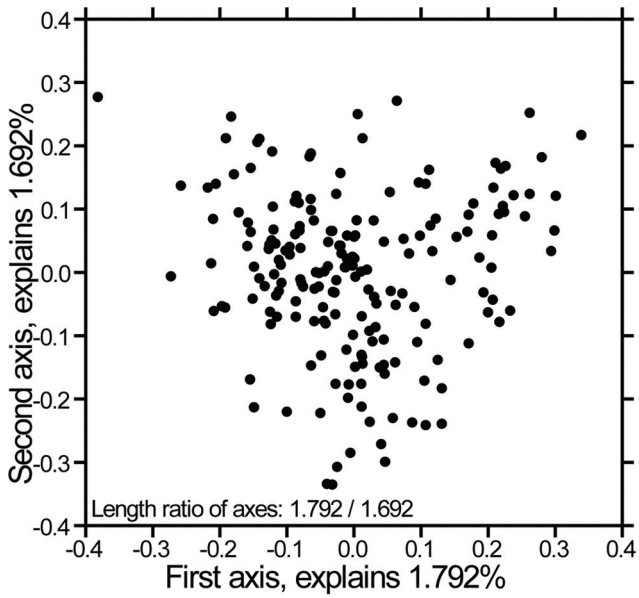

Fig. 1. First two axes of a principal coordinate analysis, based on genetic similarity estimates from 1322 genome-wide markers.

With its first two axes, principal coordinate analysis (Fig. 1) showed only 1.79 and 1.69% of the similarity variance in the data. Additional axes explained 1.44, 1.31, and 1.22%, and down to 0.61% of the similarity variance. The very even spread of lines across the area in Fig. 1 indicate a lack of marked subgrouping among these lines.

Among the 11 founder lines, the overall mean LD (expressed as r^2) was 0.1135. In contrast, the average r^2 in the A-set lines was very low: r^2 = 0.0075 for all pairs of the 118 mapped SNP markers, and r^2 = 0.0074 for the 687 mapped AFLP markers. The average r^2 was 0.0077 among all 1322 markers included in the genome-wide analysis. Among the 21 markers that were significantly associated with a trait (see below), the average r^2 value was 0.0076, with a range of 0.000 to 0.043.

Analysis of variance showed that all drought-related traits exhibited highly significant variation (F value, $p < 0.01$) due to genotypes, due to the wilt-inducing treatment, and due to genotype × treatment interactions (details not given). Response to drought stress was analyzed both as relative performance (stress in percent of control) and as absolute change due to stress, with the version yielding higher repeatability (h^2) reported herein. For analyses of membrane stability, sugars, and water, relative performance yielded higher h^2, while the "stress minus control" analysis yielded higher h^2 values for glycine betaine, SPAD values, and proline (details follow). The average of the latter was negative for SPAD values (Table 1). The induction of wilting reduced membrane stability to 36% of control, increased the sugar content by 2.3-fold, and reduced water content to 43% of control. It increased glycine betaine content by 5.2 μmol g^{-1} and reduced the arbitrary SPAD values by 4.7 units, from 37.3

Table 1. Phenotypic results of drought-related (2 replicates) and freezing-related traits (10 replicates).

Trait	Ave.	Min.	Max.	Coefficient of variation	$LSD_{0.05}$	h^2
DROUGHT STRESS: Control treatment						
Membrane stability, %	82.87	78.73	85.08	0.013	2.38	0.400
Glycine betaine, μmol g^{-1} in DM	10.07	8.56	11.55	0.058	1.02	0.614
Sugars, μmol g^{-1} in DM	437.95	169.32	865.70	0.286	198.24	0.678
Chlorophyll, SPAD units	37.31	26.73	47.15	0.098	3.12	0.906
Water, %	89.94	86.90	91.37	0.008	0.85	0.826
DROUGHT STRESS: Stress treatment						
Membrane stability, %	29.72	9.65	46.78	0.253	15.91	0.426
Glycine betaine, μmol g^{-1} in DM	15.25	12.37	18.69	0.078	2.39	0.477
Sugars, μmol g^{-1} in DM	959.10	532.0	1395.5	0.163	219.36	0.745
Chlorophyll, SPAD units	32.63	21.86	42.66	0.107	4.15	0.818
Proline, μmol g^{-1} in DM	425.60	203.3	609.10	0.144	66.77	0.846
Water, %	38.06	15.20	53.49	0.210	7.59	0.884
DROUGHT STRESS: Relative performance (stress in % of control) or absolute difference between treatments (Δ)						
%Membrane stability	36.18	11.73	57.32	0.258	18.73	0.481
ΔGlycine betaine	5.18	2.42	7.97	0.206	2.26	0.425
%Sugars	233.08	148.4	441.00	0.205	94.75	0.492
ΔChlorophyll	−4.68	1.29	−10.00	0.421	3.46	0.602
%Water	42.63	17.09	59.56	0.208	8.58	0.880
FREEZING STRESS: Stress treatment						
Regrowth, g $plant^{-1}$	0.66	0.08	1.66	0.523	0.60	0.614
Disposition to survive, °C	66.01	27.35	83.81	0.150	11.03	0.838
Plant height, cm	5.76	4.14	8.06	0.145	0.70	0.907
Loss(T+C), scale 8–72	35.48	22.57	58.73	0.165	4.34	0.929

to 32.6 units. The average control proline content was nearly zero, at 3.5 μmol g^{-1}; thus, the average proline content under stress of 425.6 μmol g^{-1} (Table 1) could also be reported as "stress minus control."

All freezing-related traits showed marked and highly significant variation ($p < 0.01$) among the A-set lines. Regrowth showed an exceptionally high coefficient of variation of 52.3%.

Repeatabilities based on the two replicates varied between $h^2 = 0.40$ for unstressed membrane stability up to $h^2 = 0.91$ for SPAD values under unstressed conditions, as well as between $h^2 = 0.61$ for regrowth after frost test and $h^2 = 0.93$ for loss of turgidity and color.

We detected very few significant ($p < 0.05$) correlations among the drought-related traits. Proline content after wilting was positively correlated ($r = 0.34$) with glycine betaine content after wilting and with gain of glycine betaine due to wilting ($r = 0.41$). Freezing-related traits were all correlated among each other. Greater plant height of the lines during the hardening phase was associated with heavier loss of turgidity and color ($r = 0.60$) as well as with decreased disposition to survive ($r = -0.48$) and decreased regrowth ($r = -0.40$). Regrowth and loss of turgidity and color were only correlated with an r value of −0.50 ($p < 0.01$ for all of these correlations). No meaningful correlations were observed between freezing and drought traits ($-0.22 < r < 0.31$ for all coefficients).

Out of 20 reported traits, 15 were subjected to association analyses (excluding the drought-related traits in their control treatments). Only 6 of these 15 traits allowed the identification of significant QTL. For these six traits, between 1 and 8 putative QTL were identified (Tables 1 and 2).

For drought, there were 12 markers with significant trait associations. One marker (E36M48-279) showed a signal in two traits: stress-induced gain of glycine betaine and of proline (Table 2), with absence of the AFLP peak ("allele 0") being associated with higher increases of both solutes. Multiple regression analysis indicated a multiple R^2 of 55.6% for sugar percentage and of 36.4% for proline.

Visual inspection of Q–Q plots (i.e., observed p-values of markers vs. expected p-values of markers; Pearson and Manolio, 2008) for drought-related traits supported the validity of data and results (Supplemental Fig. S3). Linear regression analysis of the proline phenotypes against the number of marker loci per A-set line with an "increase" marker allele yielded a correlation of $r = 0.57$ ($p < 0.01$).

Table 2. Association analyses results for drought- and freezing-related traits (minor allele frequency 5%; *n* = 189 inbred lines; mixed linear model, Kinship-matrix, FDR 20%).

	DNA marker	Linkage group and position		*p*-value	Effect†	Increase allele‡	R^2
		——————cM ——————					%
DROUGHT							
ΔGlycine betaine							
1	E36M48-279	7	94.4	4.72×10^{-5}	1.017	"0"	9.31
2	E41M58-139	–	–	1.81×10^{-4}	0.726	"0"	7.55
%Sugars							
3	E40M48-432	–	–	9.59×10^{-8}	24.23	"1"	14.66
4	E40M62-295	–	–	1.36×10^{-7}	23.41	"0"	14.49
5	E32M51-178	–	–	4.02×10^{-6}	22.47	"0"	11.17
6	E42M48-357	4	154.0	2.25×10^{-4}	21.58	"1"	8.43
7	E40M48-310	–	–	3.57×10^{-4}	15.97	"0"	6.86
Chlorophyll (Stress)							
8	E41M60-310	7	27.3	8.99×10^{-5}	2.37	"0"	8.19
Proline (Stress)							
9	E42M51-135	3	125.9	3.80×10^{-6}	44.28	"0"	12.32
10	E40M55-194	1	33.0	3.83×10^{-5}	42.04	"1"	8.71
1	E36M48-279	7	94.4	8.51×10^{-5}	57.14	"0"	9.06
11	E41M62-459	2	211.9	3.38×10^{-4}	44.03	"0"	6.94
12	E36M48-304	7	112.3	7.46×10^{-4}	76.67	"1"	6.60
FREEZING							
Disposition to survive							
1	E40M58-369	3	128.8	1.02×10^{-4}	9.742	"0"	8.11
2	Vf_Mt3 g086600	2	97.0	2.77×10^{-4}	8.859	"G"	7.14
Loss(T+C)							
2	Vf_Mt3 g086600	2	97.0	2.52×10^{-5}	5.964	"T"	9.47
3	Vf_Mt5 g046030	4	170.3	2.90×10^{-4}	3.635	"C"	9.00
4	E41M48-064	–	–	4.86×10^{-4}	2.867	"1"	6.56
5	E42M59-217	3	142.3	5.81×10^{-4}	4.816	"0"	6.42
6	E40M55-221	2	62.9	7.62×10^{-4}	3.381	"1"	6.15
7	E42M58-219	3	103.1	8.24×10^{-4}	2.946	"1"	6.31
8	E36M56-320	6	74.0	9.06×10^{-4}	3.452	"0"	7.39
9	E41M51-174	–	–	9.71×10^{-4}	2.989	"1"	5.98

† Difference between the means of the two marker classes as calculated by TASSEL 3.0 (Bradbury et al., 2007).

‡ To specify which homozygous marker class showed the higher average trait expression.

This value increased slightly ($r = 0.58$) if the phenotypic values of the entries were correlated with values predicted based on the actual marker effects. Two lines were homozygous at all five detected QTL for the "increase" marker allele—including the line S150 (Supplemental Fig. S4). Three drought-related markers were located on the same linkage group, at positions 27.3, 94.4, and 112.3 cM (Table 2). With $0.000 < r^2 < 0.015$ among them, each of these three markers most likely marks a unique QTL rather than several of them marking the same QTL.

Disposition to survive the frost test was associated with one AFLP and one SNP marker (Table 2), with about 7 and 8% of phenotypic variance explained per marker. Loss of turgidity and color was associated with eight marker loci, one of which was also shared with the other freezing trait (Vf_Mt086600, 9% variance explained, $p = 2.52 \times 10^{-5}$). The "G" allele of this SNP was associated with a higher disposition to survive, and the "T" allele with higher loss of turgidity and color. The second marker for disposition to survive, E40M458-369, did not show significant association with Loss(T+C), even when applying a false discovery rate as large as 30%.

Multiple regression analysis indicated a multiple R^2 value of 36.1% for the eight Loss(T+C) QTL jointly. The Q–Q plots for freezing-related traits again supported the data and results (Supplemental Fig. S5). Linear regression

of Loss(T+C) against the number of marker loci per A-set line with an "increase" allele yielded a correlation of $r = 0.52$ ($p < 0.01$). This value became $r = 0.53$ if phenotypic values were correlated with values predicted from the marker effects. Two lines were homozygous at seven or eight of the eight QTL for "decrease" of this trait, that is, higher freezing tolerance (again including the line S150; Supplemental Fig. S6). Three freezing-related markers were located on a common linkage group (Table 2), at positions 103.1, 128.8, and 142.3 cM. With $0.000 < r^2 < 0.030$ among them, each of these three markers most likely marks a unique QTL. Again, two markers for Loss(T+C) at positions 62.9 and 97.0 cM, on a common linkage group, showed $r^2 = 0.0001$.

DISCUSSION

Winter faba bean is uncommon in Germany, as areas like Göttingen experience harsh enough winters to kill such crops about once per decade. The inclusion of winter faba bean in German rotations would be a novelty—and would probably attract more attention from farmers and policymakers than any gradual yield improvement in spring faba bean. Climate change promises milder winters on average, and thus climate change and genetic improvement of winter hardiness may meet halfway to promote winter faba bean in Germany. Compared to spring faba bean, winter faba bean can better escape summer droughts (dehydration avoidance). Therefore, it is logical for breeders to work towards improving the dehydration tolerance (Blum, 2014) of winter faba bean, to make their defense against abiotic stress more complete (Flores et al., 2012).

The present study is the first genome-wide association analysis of faba bean focused on abiotic stress in winter faba bean. We evaluated the phenotypes of 189 inbred winter faba bean lines based on physiological aspects that are mainly connected with dehydration tolerance (membrane stability, chlorophyll and water content after drought stress, and solute accumulation). With the exception of glycine betaine accumulation, experience in assessing and interpreting these physiological parameters was available from previous experiments with spring faba bean (e.g., Venekamp and Koot, 1988; Stelling et al., 1994; Balko et al., 1995; Balko, 2005a, 2005b). Cromwell and Rennie (1953) previously detected relatively high amounts (1.1–1.5% in dry matter) of glycine betaine in *V. faba* shoots and roots. Glycine betaine accumulation is considered an adaptive response to drought stress (e.g., Jones and Storey, 1981; Gill et al., 2014, Lai et al., 2014), prompting our present assessment of glycine betaine. Stoddard et al. (2006) also proposed the investigation of glycine betaine as marker for osmotic stress response in faba bean.

We additionally examined freezing tolerance using the same set of inbred lines. The focus of these experiments was on freezing symptoms after controlled freezing stress in a plant growth chamber, based on prior experience with similar experiments (Arbaoui and Link, 2008; Roth and Link, 2010). Compared with the physiological experiments, there was greater input for the freezing phenotyping, with 20 instead of 2 replicates.

There exist a variety of alternative approaches for both experiments, including drought-stress application to of whole plants instead of detached leaflets or leaflet discs, and a wide variety of different protocols for examining freezing tolerance (Gehriger and Vullioud, 1982; Duc and Petitjean, 1995; Avia et al., 2013). Future studies will show how the current approach can be improved, and other or further measurements of physiological traits should be included. A subset of 40 lines of our plant material was studied for field-based ^{13}C discrimination as reported by Welna 2014 (Ali et al., 2013). Although ^{13}C discrimination is a self-evident trait, this parameter did not yield promising correlations with field-based performance under drought. Leaf water potential and stomata resistance are common analyses (for faba bean, see Ricciardi et al., 2001). Ali (2015b) performed an indirect yet rapid assessment of stomata conductance, using an infrared camera to measure the temperature depression of juvenile potted faba bean plants experiencing heavy drought, again utilizing a subset of our present faba bean lines. These results were significantly correlated ($r = 0.42$) with gain of dry matter after a life-threatening period of drought (about 3 wk). Similar to glycine betaine, trehalose could be an interesting compound to assess as an indicator of abiotic stress response (López et al., 2006).

Several previous studies have presented promising correlations of both proline accumulation and chlorophyll fluorescence with field-based reactions of spring faba bean yield to drought, as well as among proline, membrane stability index, changes in leaf water content, and chlorophyll fluorescence (Stelling et al., 1994; Balko et al., 1995; Gadallah, 1999; Balko, 2004, 2005a, 2005b). Link et al. (2010) reported a correlation of $r = -0.41$ between severity of freezing injury symptoms (using growth chamber tests similar to those in the present study) and field-based winter hardiness. In contrast to in the study of Arbaoui et al. (2008a), the pots in our present study were insulated with polystyrene such that the plant roots were kept at conditions milder than −5°C throughout. While both studies ensured that the shoots experienced full freezing as programmed, the conditions in our present study should be more similar to the field situation. The inbred line S_048 was ranked eighth according to disposition to survive and is proven to be a very winter-hardy line (Link and Martsch, unpublished data, 2015). However, further validation is still necessary.

At the start of the present experiments, about 778 SNPs were known in faba bean from the work of

Cottage et al. (2012b). Our initial 189 SNPs comprised the polymorphic subset of them for the given set of genotypes. These SNPs were detected based on BLAST correspondence to mRNA sequences of *M. truncatula* (Mt) and were developed as tools for exploitation of the Mt genome sequence for investigations of *V. faba* (Webb et al., 2015). We further added 1147 AFLP markers to the SNPs. Upon finding the unexpectedly low average LD (r^2 = 0.0075) and examining the low average LD of marker pairs with a distance of 1 cM or less (r^2 = 0.0522), the total number of utilized markers (n = 1322) was a limiting factor for this genome-wide association analysis. Obviously, several QTL were not detected and should be searched for using additional markers. Meanwhile, an additional 622 SNPs have now been identified by Kaur et al. (2014) in a different set of genotypes, and more are in the pipeline (e.g., Höfer et al., 2014; Webb et al., 2015).

LD among the 11 founder lines was r^2 = 0.1135. We simulated 364 samples of 11 haplotypes with two bi-allelic loci each, drawn from a basic pool with zero LD and allele frequencies of 20% and 80% throughout, which resulted in an average LD of r^2 = 0.1031. With allele frequencies of 50% throughout, the average LD became r^2 = 0.0972. This is similar to the measured LD among the 11 founder lines. Thus, it seems likely that the 11 founder lines represent a basic germplasm pool with a very low LD and are probably similar to the very low LD in our A-set of lines (r^2 = 0.0075). Seed exchange between different geographic locations over the past millennia, along with the partial allogamy of faba bean, may have caused this seemingly very low gamete phase disequilibrium. This is a disadvantage given our limited numbers of available markers, as QTL may remain undetected. However, when more markers are identified, this high resolution will increase the probability that informative markers will remain useful for several generations and across different germplasm pools.

Of the analyzed markers, 88% (n = 1159) represented an independent map position. The map size of 1633.2 cM (Supplemental Fig. S1, S2) is in reasonable agreement with the map sizes of 1216.8, 1403.8, and 1685.8 cM presented by Kaur et al. (2014), Webb et al. (2015), and Ellwood et al. (2008), respectively. The first version of the 1403.8-cM map, as presented at Hyderabad in 2012 (Cottage et al., 2012b), was based on 643 SNPs; a subset of which (n = 111) was included in the current work. Perfect colinearity was detected between the positions of the common subset of SNPs in our map and that of Cottage et al. (2012b) (Supplemental Fig. S3), with the exception of one SNP.

We found a rather low repeatability of glycine betaine values and of glycine betaine accumulation ($0.425 < h^2 < 0.614$, Table 1). This was likely because a portion of the data was predicted via the near-infrared spectroscopy calibration. Hence, the true relationship between proline and glycine betaine might be stronger than r = 0.34. The highest h^2 values after stress were found for water content (h^2 = 0.884) and %water (h^2 = 0.880); yet, no QTL was discovered. Similarly, no QTL was detected for membrane stability.

The weakest correlation between two freezing traits was found between regrowth and plant height (r = −0.40), and the strictest correlation was between regrowth and disposition to survive (r = 0.76; $p < 0.01$). The very high repeatabilities, e.g., of Loss(T+C) in which h^2 = 0.929, reflected the high total number of replications employed. Still, no QTL were detected for regrowth and plant height.

Surprisingly, the positive weak connection (r = 0.31, $p < 0.01$) between the soluble sugars under drought stress and plant height at the start of hardening was the only notable correlation between drought and freezing traits. In particular, drought-provoked proline accumulation and freezing traits were uncorrelated ($-0.06 < r < 0.08$), despite the fact that proline is also a physiological aspect of freezing tolerance (Arbaoui et al., 2008a).

The AFLP locus E36M48-279 was, with its allele "0," associated with glycine betaine increase of 1.02 μmol g^{-1} and a proline increase of 57.1 μmol g^{-1}. This may corroborate the correlation between these two traits. The major putative QTL for freezing tolerance (SNP Vf_Mt3 g086600) was shared with disposition to survive and Loss(T+)C, and seemingly explained 7 to 9% of the phenotypic variance. Three of the 189 inbred lines were homozygous at seven or eight of the Loss(T+C) markers for the allele associated with low freezing symptoms. One of these favorable lines (S_150) was also one of two lines that showed homozygosity at all five proline marker for the favorable allele, thus connecting the two types of abiotic stress.

To examine the plausibility of the present findings, association analyses were repeated with the general linear model (GLM) mode of TASSEL 3.0 (instead of a mixed linear model, MLM). Most of the results were very similar to those presented in Table 2; the same marker allele was associated with trait increase. When comparing the effect sizes estimated with MLM with their GLM-estimated counterparts, the differences ranged from between 0.7 and 16% of the MLM effect size, except for the putative QTL of chlorophyll (stress treatment) for which the MLM effect was 45% larger than the GLM estimate. For the relative content of soluble sugars (stress in percent of control, sugar percentage), different results were obtained when analyzed via GLM instead of MLM. All five significant MLM effect-size estimates were larger than their GLM counterparts, with differences ranging from 35 to 377% (average of 2.3-fold larger). Moreover, none of the MLM-detected markers showed significance under GLM. Further investigations are needed to determine whether a MLM–GLM comparison is worthwhile in cases like the

present, where population structure does not seem to be a major issue.

The model legume *M. truncatula* shows genetic variation for freezing tolerance (Pennycooke et al., 2008; Avia et al., 2013), which may be of interest for winter faba bean research. Avia et al. (2013) studied a biparental cross with 178 RIL, and found a number of convincing QTL and the most important one being on Mt chromosome 6. Tayeh et al. (2013a) revealed colinearity of this QTL with a *Pisum sativum* freezing tolerance QTL previously detected by Dumont et al. (2009). With considerable input, Tayeh et al. (2013b) narrowed down the set of candidate genes for this QTL to a few C-repeat binding factors/dehydration-responsive element binding protein1 that are well known for involvement in freezing tolerance (Thomashow, 2001). For freezing tolerance, the QTL identified in our experiments (Table 2) showed syntenic locations on the Mt genome on Mt chromosomes 3, 4, 5, 7, and 8 (with none on Mt chromosome 6). This could be deduced via the tight connection between the current map and the map of Cottage et al. (2012b) (Webb et al., 2015; Supplemental Fig. S2), as well as by the macro-synteny of both to *M. truncatula* (Satovic et al., 2013; Webb et al., 2015). Two of our markers for freezing tolerance could not be mapped reliably; thus, no syntenic location is known. To date, none of our putative QTL appear to be identical to that identified by Tayeh et al. (2013a) in Mt chromosome 6. With regards to drought tolerance, little information is available about QTL in *M. truncatula* (Arraouadi et al., 2012), or about candidate genes in faba bean (Abid et al., 2015) and in *M. truncatula* (Wang et al., 2013).

We propose a first glimpse candidate gene search for one of our putative QTL: SNP locus Vf_Mt3 g086600 (associated with freezing tolerance). The locus of this SNP can directly be found on Mt chromosome 3 at position 28,532,000 Bp (annotated as "unknown protein"; Mt3.5, Hapmap, 2015). Nearby, at 28,570,300 Bp a gene for glycine aldehyde dehydrogenase is displayed, an enzyme belonging to the glycine betaine pathway. This osmotically important solute may play a role in freezing tolerance. Yet, this is speculative and needs further investigation. Obviously the genome sequences of *M. truncatula*, as well as of *Lotus japonicas*, *Glycine max*, *Lens culinaris*, and *Cicer arietinum* (Satovic et al., 2013; Varshney et al., 2014; Webb et al., 2015) have become very important tools for future faba bean research.

To summarize the main conclusions: (i) marked genetic variation for aspects of drought and freezing tolerance traits occurred, (ii) little genetic connection between these two topics was displayed, (iii) LD in the current winter faba bean germplasm was very low, thus high numbers of markers and probably candidate gene search should be applied, and (iv) several putative QTL for drought tolerance related traits and for freezing tolerance were detected. We reported findings from the first association analysis in faba bean focused on abiotic stress. The present results markedly improve the prospects for introducing adapted winter faba bean in German field rotations.

Supplemental Material Available

Supplemental material is available with the online version of this article.

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