Published February 14, 2019

RESEARCH

Quantitative Trait Loci for Cold Tolerance in Chickpea

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ABSTRACT

Fall-sown chickpea (*Cicer arietinum* L.) yields are often double those of spring-sown chickpea in regions with Mediterranean climates that have mild winters. However, winter kill can limit the productivity of fall-sown chickpea. Developing cold-tolerant chickpea would allow the expansion of the current geographic range where chickpea is grown and also improve productivity. The objective of this study was to identify the quantitative trait loci (QTL) associated with cold tolerance in chickpea. An interspecific recombinant inbred line population of 129 lines derived from a cross between ICC 4958, a cold-sensitive *desi* type (*C. arietinum*), and PI 489777, a cold-tolerant wild relative (*C. reticulatum* Ladiz), was used in this study. The population was phenotyped for cold tolerance in the field over four field seasons (September 2011–March 2015) and under controlled conditions two times. The population was genotyped using genotyping-by-sequencing, and an interspecific genetic linkage map consisting of 747 single nucleotide polymorphism (SNP) markers, spanning a distance of 393.7 cM, was developed. Three significant QTL were found on linkage groups (LGs) 1B, 3, and 8. The QTL on LGs 3 and 8 were consistently detected in six environments with logarithm of odds score ranges of 5.16 to 15.11 and 5.68 to 23.96, respectively. The QTL *CT Ca-3.1* explained 7.15 to 34.6% of the phenotypic variance in all environments, whereas QTL *CT Ca-8.1* explained 11.5 to 48.4%. The QTL-associated SNP markers may become useful for breeding with further fine mapping for increasing cold tolerance in domestic chickpea.

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Abbreviations: CF, Central Ferry farm; CIM, composite interval mapping; FT1, Freezing Test 1; FT2, Freezing Test 2; GBS, genotyping-by-sequencing; LG, linkage group; LOD, logarithm of odds; QTL, quantitative trait locus/loci; RIL, recombinant inbred line; SNP, single nucleotide polymorphism.

Fall-sown legumes in temperate regions are emerging as a viable option for crop rotations in cereal-based cropping systems. Recently, winter pea (*Pisum sativum* L.) has been shown to be economically viable in traditionally winter wheat (*Triticum aestivum* L)–summer fallow rotations, conserving more stored soil water than winter wheat (Schillinger, 2017). Winter-hardy, food-quality pea and lentil (*Lens culinaris* Medik.) cultivars have been released (Muehlbauer and McPhee, 2007; McGee and McPhee, 2012; McGee et al., 2013). Yield gains for fall-sown vs. spring-sown pea and lentil are impressive, with increases of 480 to 590 kg ha^{-1} for lentil and as much as 1830 kg ha^{-1} for pea (Chen et al., 2006). Chickpea (*Cicer arietinum* L.) is an integral part of cereal-based cropping systems worldwide, as it significantly contributes to agricultural sustainability by fixing atmospheric N, breaking disease cycles, improving the cereal grain yields, and providing diet

Published in Crop Sci. 59:573–582 (2019).
doi: 10.2135/cropsci2018.08.0504

diversification. As a spring-sown crop, the short growing season of chickpea limits its grain yield, leaves very little crop residues to combat soil erosion, and hardly contributes to soil organic matter (Kumar and Abbo, 2001). The development of a fall-sown, winter-hardy chickpea could improve yields by extending the growing season, help the crop escape heat stress during flowering and late-season drought, and provide additional protection from cool temperatures during seed set in the early spring (Singh et al., 1997). Chickpea lacks winter hardiness because of genetic bottlenecks and subsequent loss of critical alleles during its evolution under domestication (Abbo et al., 2003). However, alleles for winter hardiness and a vernalization requirement still exist and are prevalent in chickpea's wild progenitor, *C. reticulatum* Ladiz (Abbo et al., 2002).

Chickpea wild relatives have been recommended as sources of cold tolerance for decades (van der Maesen and Pundir, 1984; Singh et al., 1998; Toker, 2005). In a study that compared wild with domesticated chickpea, it was reported that wild species had significantly higher levels of cold tolerance than the best cold-tolerant cultivars (Toker, 2005). Singh et al. (1998) concluded that *C. reticulatum* was one of the most cold-tolerant annual *Cicer* species. The use of crop wild relatives, such as *C. reticulatum*, carries significant agronomic challenges, such as reticulated seed, prostrate growth habit, and pod dehiscence (Ladizinsky, 1975; Ladizinsky and Adler, 1976). However, *C. reticulatum* holds the advantage of a vernalization requirement that contributes to winter hardiness by delaying flowering (Abbo et al., 2002). Using interspecific recombinant inbred line (RIL) populations, vernalization in chickpea was recently mapped to Linkage Group (LG) 3 (Samineni et al., 2016; van-Oss et al., 2018).

Several quantitative trait locus (QTL) studies for cold stress have been conducted in several related legumes, including pea, lentil, barrel medic (*Medicago truncatula* Gaertn.), and faba bean (*Vicia faba* L.) (Kahraman et al., 2004; Lejeune-Hénaut et al., 2008; Avia et al., 2013; Klein et al., 2014; Sallam et al., 2016). However, no QTL studies have been published to date regarding cold tolerance in chickpea.

Understanding the genetic basis of cold tolerance in chickpea is important to be able to introgress novel alleles efficiently into elite germplasm. Thus, the objectives of the current study were to phenotype and genotype a RIL population and identify QTL associated with cold tolerance in chickpea. The identified QTL-associated single nucleotide polymorphism (SNP) markers have potential for use in introgressing improved cold tolerance into domesticated chickpea.

MATERIALS AND METHODS

Plant Material

The chickpea population used in this study was 127 F_6–derived F_{10} RILs (CRIL2, C = chickpea, 2 = number of populations in the USDA breeding program) from the wide cross of *C. arietinum* ICC4958 × PI 489777 *C. reticulatum*. PI 489777 *C. reticulatum* is the wild progenitor of domestic chickpea that has a prostrate growth habit and small (15.4 g, 100-seed weight), dark brown seeds covered by tiny tubercules (Ladizinsky and Adler, 1976). Cultivar ICC4958 is a semi-spreading *desi* type with larger (34.0 g, 100 seed weight), brown seeds (Saxena et al., 1993). PI 489777 is the cold tolerance source and ICC4958 is the cold-susceptible parent. The cross was made by the USDA in 1992 (X92C031) to study Fusarium wilt resistance (caused by *Fusarium oxysporum* Schlechtend.), and RILs were created via single-seed descent in a greenhouse (Brim 1966; Snape et al., 1985; Ratnaparkhe et al., 1998; Tekeoglu et al., 2000).

Field Study

The RIL population was fall sown using a randomized complete block design with three replications in eastern Washington for 4 yr: 3 Oct. 2011 on the USDA Central Ferry (CF) farm; 28 Sept. 2012 on the CF farm and Washington State University Whitlow and Spillman farms; 25 Sept. 2013 on the CF farm and Spillman farm; and 1 Oct. 2014 on the CF farm. The CF farm is located in Central Ferry, WA (46°39′5.1″ N, 117°45′45.4″ W; 198 m asl). The Whitlow (46°44′3.2″ N, 117°7′25.8″ W) and Spillman (46°41′45.1″ N, 117°9′2.7″ W) farms are near Pullman, WA. However, Pullman was dropped from the experiment because of insufficient fall germination. The CF farm has a Chard silt loam soil (coarse-loamy, mixed, superactive, mesic Calcic Haploxerolls) and was irrigated with subsurface drip irrigation for 10 min d^{-1}. All seeds were treated with fungicides (mefenoxam [13.3 mL a.i. 45 kg^{-1}], fludioxonil [2.4 mL a.i. 45 kg^{-1}], and thiabendazole [82.9 mL a.i. 45 kg^{-1}]) insecticide (thiamethoxam [14.3 mL a.i. 45 kg^{-1}]), and sodium molybdate (16 g 45 kg^{-1}) prior to planting. Thirty seeds were planted per plot; each plot was 152 cm long, double rows, with 30-cm center spacing. The distance from plot to plot was 152 cm, and the distance between paired plots was 100 cm. Preemergence herbicide (α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine; Treflan, Dow Chemical) was applied. Hourly temperature data were collected from a weather station located on the CF farm (Davis Instruments) (Supplemental Fig. S1; Landry et al., 2016, 2017). Winter damage was assessed on 8 Mar. 2012 and 27 Feb. 2013 using a qualitative visual damage assessment scale (0 = no leaf damage, 1 = 25% leaf damage, 2 = 50% leaf damage, 3 = 75% leaf damage, and 4 = 100% leaf damage or dead) in 2012 and 2013. Percentage of surviving plants was determined (stand count) on 27 Mar. 2014 and 29 Mar. 2015 (Supplemental Table S1), and height was taken in 2015 only at the green pod stage in late June (Supplemental Table S2). Seed size was determined by scanning 20 seeds using the WinSEEDLE software (Regent Instruments, 2007), which calculated seed ellipsoid and seed circle volumes (Supplemental Table S3).

Controlled Conditions

Controlled environment experiments (Freezing Test 1 [FT1] and Freezing Test 2 [FT 2]) were conducted using 104 RILs because of lack of sufficient seed, and the two parents of CRIL2. The experimental design for both experiments was a randomized complete block design repeated twice, where each

replication entry had four plants. Each freezing test consisted of 104 RILs, eight pairs of the parents and check lines. Based on 2014 and 2015 field data, the five most cold-tolerant RILs (CRIL2-111, -96, -122, -53, and -5) and the five most susceptible RILs (CRIL2-1, -11, -85, -29, and -47) were selected as checks. The freezing protocol used in the freezing chamber was based on the procedure for winter wheat (Zhu et al., 2014).

Seeds were scarified before each experiment using a multitool (Dremel Multipro, Ball Bearing Model 5) and germinated on moist paper at 20°C for 4 d, then planted in a soilless mix Sunshine #5 (Sun Gro Horticulture) in 6.35-cm × 6.35-cm × 5.66-cm trays. Four plants per replication were grown for 3 wk in the greenhouse maintained at 22 to 25°C/16 to 19°C day/night temperatures. Slow-release fertilizer Osmocote (15–9–12 N–P–K) with micronutrients (The Scotts Company) was applied 2 wk after planting. Plants were then moved to a growth chamber for 5 wk. Temperatures in the growth chamber were recorded using a HOBO data logger (Onset Manufacturing Company). Acclimation started at 1 wk with 10°C and 16 h of light, 2 wk with 5°C and 12 h of light, and 2 wk with 3°C and 10 h of light. The last step was freezing that started at −3°C with no light for 16 h, then −5°C with no light for 2 h. Temperature was dropped further to −7°C with no light for 2 h during FT 1 because of a freezing chamber technical issue. From the freezing growth chamber, the plants were moved to the acclimation growth chamber at 4°C for 24 h, then returned to initial greenhouse conditions, and the plants were rated for cold damage after 7 d. Evaluation of cold damage was based on leaf damage that was scored using a 1-to-9 visual scale developed by Fiebelkorn (2013) (Table 1, Supplemental Tables S4a and S4b).

Linkage Map Construction

DNA was extracted from leaf samples collected from 124 3-wk-old, F_6–derived F_{10} RILs using the Qiagen DNeasy 96 Plant Kit. Genotyping-by-sequencing (GBS) libraries were prepared using the two-enzyme method described by Poland et al. (2012) that uses methylation-sensitive restriction enzymes (*Pst*I/*Msp*I). Libraries were sequenced using Illumina Hiseq 2500. The Illumina DNA-sequencing raw reads were demultiplexed and cleaned using the process-radtags program in Stacks (Version 1.47; Catchen et al., 2013), and high-quality reads were aligned on reference genome developed from the *kabuli* cultivar 'CDC Frontier' (Varshney et al., 2013), as per Thudi et al. (2016), using the Burrows Wheeler Alignment tool (Version 0.5.9) with default alignment parameters (Li and Durbin, 2009). Sequence variants were identified using the genotypes program in Stacks (Catchen et al., 2013). A total of 747 SNP markers were used to construct a linkage map (Supplemental Table S5). A maximum of 0.2 was set for the allowable missing portion of missing data for SNPs. A linkage map was constructed using Joinmap 4.0 (Van Ooijen, 2006), and minimum logarithm of odds (LOD) for group selection was five, with recombination frequency set between 0.25 and 0.05 cM. Distances were calculated using the Haldane's mapping function (Haldane, 1919). The Monte Carlo maximum likelihood algorithm (Geyer and Thompson, 1992) was used for ordering the markers. The segregation ratio at each marker locus was statistically analyzed against the expected Mendelian segregation ratios of 1:1 using χ^2 tests.

Table 1. Description of visual scores assigned to CRIL2 plants in the controlled environment experiments of Freeze Test 1 and 2 as developed by Fiebelkorn (2013).

Score	Visual identification
1	Plant completely green
2	Plant with minimal freezing damage
3	Plant at least 75% green
4	Plant between 50 and 75% green tissue
5	Plant 50% green
6	Plant between 25 and 50% green tissue
7	Plant 25% green
8	Plant almost dead but has minimal green
9	Plant completely dead

Data Analysis

The data were analyzed separately for each response variable (percentage leaf damage or percentage surviving stand count). Genotype and replication (blocking factor) were treated as random effects, and year was treated as a fixed effect. For freeze tests, genotype was treated as a random effect, and replication and experiment were treated as fixed effects, as each had only two levels.

The statistical model used was

$$Y_{ijk} = \beta_0 + \beta_1 x_i + u_1 z_k + u_2 z_j + \varepsilon_{ijk}$$

where Y_{ijk} is the response variable, β_0 is the overall mean, and β_1 is the effect of year. The random variables u_1, u_2, and ε_{ijk} (the effects from replication, the genotype effects, and the error term, respectively) were distributed as follows:

$$\begin{pmatrix} u_1 \\ u_2 \\ \varepsilon \end{pmatrix} \sim N\left[0, \begin{pmatrix} \sigma_b^2 & 0 & 0 \\ 0 & \sigma_g^2 & 0 \\ 0 & 0 & \sigma_e^2 \end{pmatrix}\right]$$

where σ_b^2, σ_g^2, and σ_e^2 are the variances for replication, genotype, and error.

The data (for leaf damage and stand count) being binomial traits, a generalized linear model was used for each variable using the binomial family and logit link function:

$$Y_{ijk} = \log\left(\frac{\pi}{1-\pi}\right)$$

The data were also analyzed with a general linear model using the square root transformation, log transformation, and arcsine of the square root transformation on the response data. The freeze test data were analyzed using a general linear model. Residuals from all models were examined for homoscedasticity and normality. The experiment was analyzed in R version 3.3.4 with Asreml (Butler et al., 2007; R Development Core Team, 2015). Heritability estimates were generated for each environment using the Sommer package in RStudio 2015 (Covarrubias-Pazaran, 2018). Broad-sense heritability was calculated as

$$H^2 = \frac{V_G}{V_G + V_{GE}/N_{env} + V_E/(2N_{env})}$$

where V_G is the genotype variance, V_{GE} is the genotype × environment variance, V_E is the environmental variance, and N_{env} is the number of environments.

QTL Detection with Composite Interval Mapping

A genetic linkage map made up of 747 SNPs and cold tolerance phenotypic data obtained from each of the six environments was used for QTL analysis using "R/qtl" in R software version 3.3.4 by employing the composite interval mapping (CIM) method (Broman et al., 2003, 2010; Arends et al., 2010). The statistical significance of the QTL was assessed by using Churchill and Doerge's permutation test with 1000 replications and a significance level of $P \leq 0.05$ (Churchill and Doerge, 1994). The additive effects and R^2 of the detected QTL were estimated using the "fitqtl" function of R version 3.3.4. The 95% Bayes interval was used to obtain interval estimates of QTL locations.

RESULTS

As expected, the two parents consistently differed in their reactions to the winter temperatures, and transgressive segregants with increased cold tolerance were identified in most environments (Fig. 1, Supplemental Tables S2 and S3). The 2011–2012 field study had the mildest low temperatures (Supplemental Fig. S1A), and no plant death was observed. The subsequent 3 yr reached lower temperatures (−12.5, −5.9, and −17°C, respectively; Supplemental Fig. S1B–S1D), with accompanying plant death of the most susceptible lines and the *C. arietinum* parent ICC 4958.

Given the residual pattern, field leaf damage (2011–2012, 2012–2013), and stand count were best modeled with the binomial distribution and the logit link function. Based on the residual pattern, stand count (2013–2014, 2014–2015) was modeled after an arcsine transformation using a standard linear model with normal distribution. The variance components indicated that genotypes explained a high percentage of the total variance in the 4 yr of field studies but a low percentage of the variance in the freezing tests (Table 2). Additionally, there was a relatively high error term in the freeze tests.

The predicted values for leaf damage and stand count were moderately correlated with each other ($r = 0.62$, $\rho = 0.64$ [Pearson and Spearman rank correlations, respectively]). Replication consistently had no effect, as either a fixed effect or a random effect. Correlations between leaf damage years and stand count years were higher ($r = 0.75$ and 0.85, respectively) using untransformed data (Supplemental Table S1). Correlations between the 4 yr of field cold tolerance studies were higher than between the two freeze tests (FT2, Table 3). Heritability estimates were as follows: CF 2012 and 2013 had an H^2 of 0.82 (SE = 0.03), and CF 2014 and 2015 had an H^2 of 0.96 (SE = 0.01) for the field environments, and controlled environments had an H^2 of 0.67 (SE = 0.07).

Linkage Map

An interspecific genetic linkage map containing 747 SNPs mapped on eight LGs (Supplemental Table S5, Supplemental Fig. S2) was constructed. The map spanned a total distance of 393.7 cM at an average density on 1.8 SNPs cM^{-1}. Nearly half of the loci (48%) exhibited segregation distortion from the expected ratio of 1:1 for RILs. The smallest LG in size was LG 7, which spanned 24.8 cM and contained 58 markers. Linkage Group 4 was the largest, with 203 markers spanning 90.3 cM. The densest LG, LG 5 (A), had an average marker density of 2.8 SNPs cM^{-1}.

QTL detection using Composite Interval Mapping

Using CIM QTL analysis for cold tolerance, three QTL were identified, of which two were large-effect QTL that were significant in all six environments, whereas one QTL was only detected in one environment (Table 4, Supplemental Table S6). The minimum LOD score set by Churchill and Doerge's permutation test (Churchill and Doerge, 1994) was 3.1. All the detected QTL for cold tolerance had *C. reticulatum* (PI 489777) as the contributing parental allele. The QTL were named following Hamon et al. (2011). The first QTL, *CT Ca-3.1*, was detected on LG 3 at 43.8 cM in all the six environments. *CT Ca-3.1* had a confidence interval between 0.5 and 13.4 cM. The minimum phenotypic variation for cold tolerance trait explained by this QTL was 7.15%, with a LOD score of 6.89 in the 2015 CF field environment, whereas the maximum was 34.57%, with a LOD score of 15.11 in the 2013 CF field environment. The second QTL, *CT Ca-8.1*, was detected on LG 8 in the four CF field sites and two controlled environments. The confidence interval for *CT Ca-8.1* was between 0 and 9.04 cM. The minimum phenotypic variation for cold tolerance trait explained by *CT Ca-8.1* was 11.47%, with a LOD score of 5.68 in FT2, whereas the maximum was 48.41%, with a LOD score of 23.96 in the 2014 field environment. The third QTL, *CT Ca-1.1*, was detected on LG 1(B) in the 2015 CF field environment. The phenotypic variation for cold tolerance trait explained by this QTL was low (2.6%), with a LOD score of 3.14, just above the threshold to declare a QTL.

The minimum LOD score was set at 3.4 for plant height and seed volume, and significant QTL were identified (Table 4). Two QTL were identified for plant height: *Ht Ca-4* was found on LG 4, with a LOD score of 6.5, whereas *Ht Ca-8* was found on LG 8, also with a LOD score of 6.5. *Ht Ca-4* explained 20.21% of the phenotypic variance, whereas *Ht Ca-8* explained 19.97% of the phenotypic variance. For seed ellipsoid volume, two QTL were identified. The first QTL, *SEV Ca-1(A)*, was found on LG 1(A), with a LOD score of 7.4, explaining 15.93% of phenotypic variance. The second QTL, *SEV Ca-4*, was found on LG 4, with a LOD score of 11.8, explaining 29.41% of phenotypic variance.

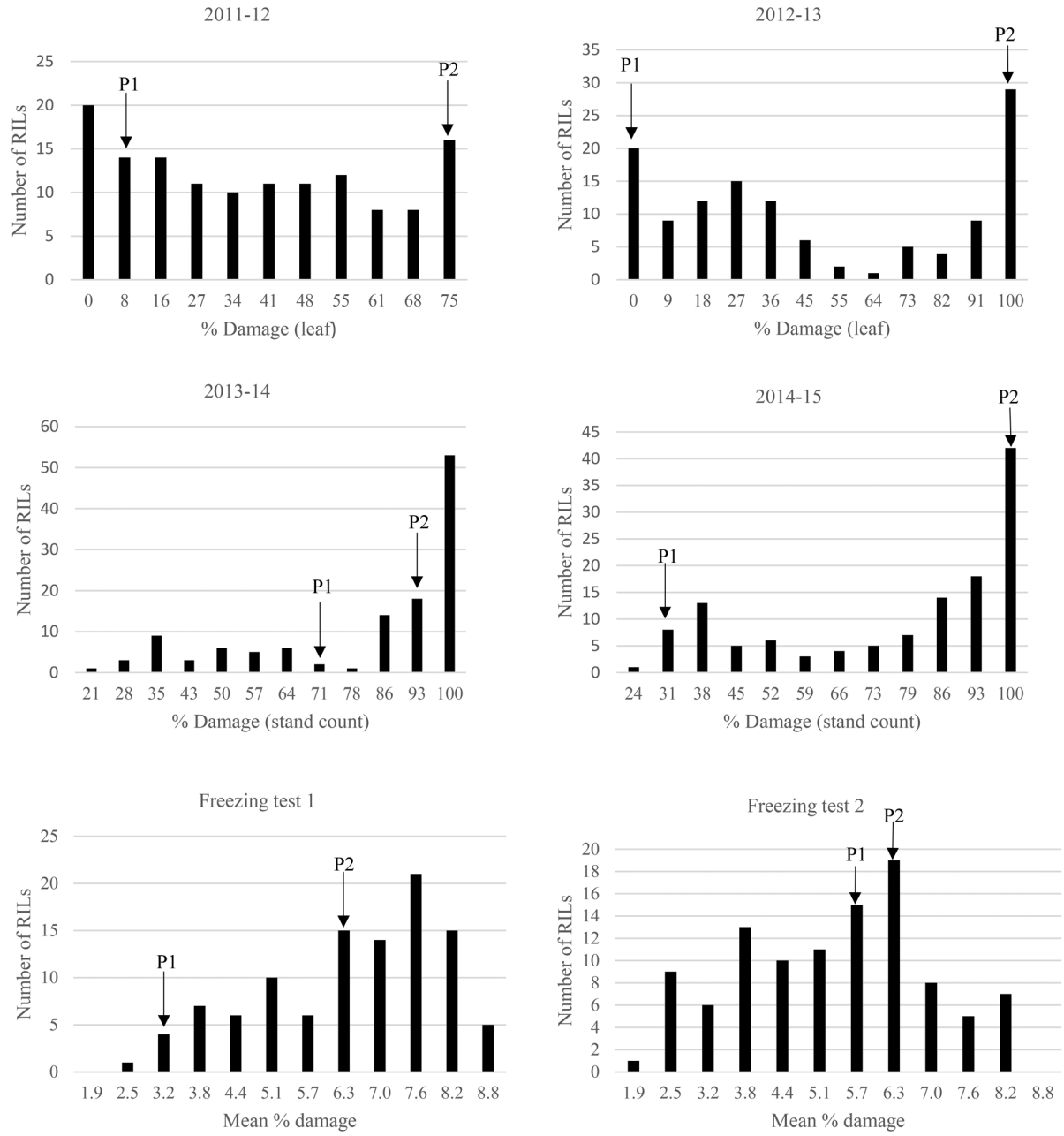

Fig. 1. Frequency histogram of the cold tolerance ratings of the CRIL2 recombinant inbred line (RIL) population across 4 yr in the field, measured by percentage leaf damage or percentage stand count, and the two controlled freeze tests measured with a 1-to-9 ordinal scale, with 9 being dead plants. P1 is PI 489777, the cold-tolerant parent, whereas P2 is ICC 4958, the cold-susceptible parent.

DISCUSSION

The two most significant QTL, identified using CIM, for cold tolerance in the CRIL2 chickpea population explained high percentages of the phenotypic variance that could be used in breeding winter-hardy chickpea. Up to 34 and 48% of the phenotypic variance for cold tolerance was explained by *CT Ca-3.1* and *CT Ca-8.1*, the QTL on LGs 3 and 8, respectively. As expected, all large-effect QTL that were associated with cold tolerance were derived from *C. reticulatum*. Similar results were reported in pea, where a QTL (gene region *Hr*) explained up to 43% of the variation for cold tolerance (Lejeune-Hénaut et al., 2008; Klein et al., 2014). It is also similar for barrel medic, lentil, and faba bean, where the one consistent QTL explained up to 39, 28, and 23% of the variation, respectively (Kahraman et al., 2004; Avia et al., 2013; Sallam et al., 2017). This is not surprising, given the moderate to very high broad-sense heritability (0.67–0.96) for cold tolerance in the CRIL2 population. Four consistent cold

Table 2. Variance components of the field study and the controlled freeze tests.

Trait	Genotype	Replication	Error
		%	
2012–2013 damage (leaf)	92.12	0.22	7.66
2014–2015 damage (stand count)	98.20	0.12	1.68
Freeze Tests 1–2	25.90	0.01	74.09

tolerance QTL in pea were identified in multiple environments (Lejeune-Hénaut et al., 2008; Klein et al., 2014), whereas only two cold tolerance QTL were identified in multiple environments in this chickpea study. This could be due to species difference, or the linkage map density in this chickpea QTL study may be an issue (Mallikarjuna et al., 2017; Supplemental Table S5; Supplemental Fig. S2).

Although the Mallikarjuna et al. (2017) chickpea consensus linkage map (363.8 cM) is similar to this report's linkage map (393.7 cM, Supplemental Table S5), there are gaps (Supplemental Fig. S2) compared with high-density chickpea maps (Deokar et al., 2018). We hypothesize that the two enzymes, *Pst*I/*Msp*I, of the Poland et al. (2012) GBS were not the best combination for chickpea, since only 747 SNPs were identified. Currently, we are testing the one enzyme *Ape*KI GBS of Elshire et al. (2011) for chickpea. The SNPs in CRIL2 were successfully identified using CDC Frontier as the reference genome, which has emerged as a very useful and highly cited chickpea reference genome (Thudi et al., 2016). Almost half of the markers exhibited segregation distortion and were not used in the linkage map. Hybrid progeny populations derived from interspecific crosses between domesticated cultivars and wild accessions of their immediate wild progenitors usually show higher polymorphism levels as compared with intraspecific crosses between domesticated parents. Such polymorphism is fundamental for our ability to produce accurate and dense genetic maps. However, a prominent weakness of such experimental populations is the distorted, non-Mendelian allelic segregation, which is an almost universal phenomenon of genetic analyses based on interspecific cross progeny, as evident from numerous

Table 3. Pearson phenotypic correlation coefficients between the cold tolerance ratings determined in the four field studies (percentage damage) and two freeze tests (FT, plant damage scores) for cold tolerance of the ICC4958 × PI 489777 CRIL2 population (Supplemental Tables S1, S4a, and S4b).

Environment	2012–2013	2013–2014	2014–2015	FT1	FT2
2011–2012	0.75***	0.62***	0.62***	0.57***	0.36*
2012–2013		0.64***	0.60***	0.59***	0.41**
2013–2014			0.85***	0.71***	0.53***
2014–2015				0.69***	0.50*
FT1					0.51***

*, **, *** Significant at the 0.05, 0.01, and 0.001 probability levels, respectively.

Table 4. Quantitative trait loci (QTL) detected for cold tolerance, plant height, and seed volume in the ICC 4958 × PI 489777 recombinant inbred line (RIL) population.

Trait	QTL name †	Environment‡	LG§	Position	Closest left marker from the position	LOD¶ peak	1.5-LOD support interval	R^2#	Additive effect (*a*)††
				cM			cM	%	
Cold tolerance	*CT Ca-3.1*	CF-2012	3	43.8	2574_3	10.8	0.5	24.0	–0.54
		CF-2013	3	43.8	2574_3	15.11	0.5	34.57	–0.92
		CF-2014	3	43.8	2574_3	5.21	13.4	7.79	–7.08
		CF-2015	3	43.8	2574_3	6.89	13.4	7.15	–7.16
		FT-1	3	43.8	2574_3	6.29	7.9	9.92	–0.54
		FT-2	3	43.8	2574_3	5.16	13.1	12.31	–0.58
	CT Ca-8.1	CF-2012	8	13.4	9648_8	7.89	9.0	13.5	–0.39
		CF-2013	8	10.8	9604_8	7.24	5.71	12.19	–0.53
		CF-2014	8	10.8	9604_8	23.96	0	48.41	–17.53
		CF-2015	8	10.8	9604_8	18.62	0.8	35.67	–10.36
		FT-1	8	10.8	9604_8	14.27	1.9	32.37	–0.94
		FT-2	8	10.8	9604_8	5.68	7.1	11.47	–0.55
	CT Ca-1.1(B)	CF-2015	1 (B)	0	1185_1	3.14	0	2.6	–1.45
Seed size	*SEV Ca-1 (A)*	CF-2015	1 (A)	29.7	999_1	7.36	1.1	15.93	–0.18
	SEV Ca-4	CF-2015	4	55.7	3594_4	11.75	7.7	29.41	–0.82
Height	*Ht Ca-4*	CF-2015	4	77.1	4740_4	6.51	11.3	20.21	–5.8
	Ht Ca-8	CF-2015	8	13.4	9648_8	6.53	5.1	19.97	5.9

† Ca, *Cicer arietinum*; CT, cold tolerance; Hgt, height; Wgt, weight; Vol, volume.

‡ CF, USDA Central Ferry farm; FT, freeze test.

§ LG, chickpea linkage group as assigned in the draft genome sequence by Varshney et al. (2013).

¶ LOD, logarithm of odds.

R^2, percentage of phenotypic variance explained by an individual QTL ($P < 0.005$).

†† Additive allelic values of ICC 4958

reports that need no reiteration here. Indeed, for many decades, geneticists have been lamenting the occurrence of unequal segregation of alleles in such crosses (Zamir and Tadmor, 1986) and likewise the differential survival of zygotes after interspecific crosses (Gadish and Zamir, 1987). For this reason, the high frequency of distorted segregation observed in the present work is no exception.

Although *CT Ca-3.1* and *CT Ca-8.1* cold tolerance QTL were detected with the freeze test (FT1 and FT2) data, the high experimental errors and low correlation values indicated that the testing procedure for chickpea would need to be improved. Assessing leaf damage at different temperature drop points and rates is important for improving the procedure in future studies. Assessment of additional traits such as plant roots, tissues, turgidity, and discoloration after each freeze test may capture the full severity of plant cold damage not detected using just the visual scores of Table 1 (Landry and Hu, 2018). The ability to complete a cold tolerance test every 8 wk throughout the year as opposed to once per year in a field study will allow breeders to select more frequently and cycle generations faster, thus decreasing the amount of time required for variety development.

The QTL *CT Ca-3.1* is a good candidate region for the vernalization gene previously mapped in the CRIL2 population and in one other population where a *C. reticulatum* accession was one of the parents (Samineni et al., 2016; van-Oss et al., 2018). The same QTL was mapped in this study. Vernalization is the acceleration of flowering after a plant's exposure to cold temperature (Sung and Amasino, 2004). Flowers are known to be delicate structures on a plant that are sensitive to various stresses (Kim et al., 2009), and vernalization protects a plant from cold stress by stopping it from flowering before winter and allowing it to flower when temperatures are optimal (Kim et al., 2009). The vernalization requirement has been well described in *C. reticulatum* (Abbo et al., 2002; Sharma and Upadhyaya, 2015; van-Oss et al., 2015, 2018). We mapped the vernalization gene (*MtVRN2*), a repressor of the *flowering locus T* (*FT*) gene homolog from *Medicago truncatula*, to the *CTCa-3.1* confidence interval using the CDC Frontier chickpea reference genome (Jaudal et al., 2016). This is a start at candidate gene identification; fine mapping will be necessary to elevate its status. A draft genome was published for both CRIL2 parents, which should facilitate this endeavor (Jain et al., 2013; Gupta et al., 2016). In a more distantly related legume, *Lupinus angustifolius* L., a gene (*LanFTc1*) also interacts with *flowering locus T* (*FT*), controlling its vernalization requirement in lupin (Nelson et al., 2017) and thus rendering the *M. truncatula* gene especially interesting in chickpea.

The frost tolerance gene *Hr* in pea (*early flowering 3*, *ELF3* ortholog) is also a flowering control gene of interest (Lejeune-Hénaut et al., 2008; Weller et al., 2012). Recently, *Efl1*, an *ELF3* ortholog in chickpea, was mapped to the early flowering QTL on chickpea LG5, so it likely is not the vernalization gene in chickpea (Ridge et al., 2017). Other genes of interest are the *CBF/DREB1* genes, which are key to cold acclimation in numerous plants, with evidence published for their role in pea and barrel medic cold tolerance (Thomashow, 2010; Tayeh et al., 2013). A Basic Local Alignment Search Tool (BLAST) search revealed a homolog on LG 4 of chickpea, but it was not found in this QTL study, possibly due to linkage map density of LG 4 (Supplemental Table S5, Supplemental Fig. S2).

Several QTL for chickpea plant height have been identified in other studies on chickpea (Gowda et al., 2011; Kujur et al., 2016). Although two studies reported QTL for plant height on the same LGs as in this study (*Hgt Ca-4* and *Hgt Ca-8*), they were not in the same regions, ranging from 31.3 to 37.3 cM distal to *Hgt Ca-4* and *Hgt Ca-8*, respectively (Gowda et al., 2011; Kujur et al., 2016). These two studies used *C. arietinum* intraspecific RIL populations that did not segregate for the prostrate growth habit of *C. reticulatum*. Therefore, this was not unexpected. However, two studies, using a similar wide cross, mapped a gene–gene region for prostrate growth habit to LG 3 (Cobos et al., 2009; Aryamanesh et al., 2010) that was not detected in this study. Further, Upadhyaya et al. (2017) identified eight SNPs on LGs 1, 3, 4 (3), 6, and 7 of Scaffold682 associated with plant growth habit using categories rather than height per se. The QTL on LG 4 does co-localize with *Ht Ca-4*.

For chickpea seed size, many studies have reported QTL by using 100-seed weight (Cobos et al., 2007; Gowda et al., 2011; Karami et al., 2015). While none of these studies reported QTL for seed size using seed volume, all three reported QTL for seed size on LG 4, where the *SEV Ca-4* seed volume QTL resides. The closest published QTL of Gowda et al. (2011) was 3 cM from the *SEV Ca-4* marker, well within the confidence interval to be considered potentially the same QTL.

The identification of these QTL is a start on efficiently moving positive alleles for cold tolerance into cultivated chickpea. The main QTL identified in this study have large confidence intervals (13.4 and 9 cM). Creating a higher density map and performing fine mapping may help reduce the confidence interval and make SNP discovery more precise (Gao and Zhu., 2013; Jaganathan et al., 2015). This can increase the efficiency of moving the cold tolerance trait from *C. reticulatum* into domesticated chickpea.

Conflict of Interest

The authors declare that there is no conflict of interest.

Supplemental Material Available

Supplemental material is available online for this article. The dataset for this article can be found at http://dx.doi.org/10.5061/dryad.jt052vq.

Acknowledgments

The authors gratefully acknowledge the funding sources for this project: a Fulbright Fellowship awarded to D. Mugabe, Grant no. IS-4427-11C from the United States–Israel Binaional Agricultural Research and Development Fund (BARD) (C.J. Coyne, G. Vandemark, H. Zhang, and S. Abbo), and the USDA-ARS Current Research Information System (CRIS) Projects 5348-21000-026-00D (C.J. Coyne, E. Landry, and J. Piakowski) and 5348 21000-024-00D (R. McGee and G. Vandemark). The authors acknowledge technical assistance provided by Britton Bourland, Landon Charlo, Kurt Tetrick, and Jarod Pfaff.

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