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QUANTITATIVE TRAIT LOCI FOR COLD TOLERANCE IN THE MAIZE IBM POPULATION

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B73 and Mo17 represent the main families of elite maize (*Zea mays* L.) inbred lines. B73 and Mo17 significantly differed in their proportion of germination under low-temperature conditions, and the IBM population derived from the cross B73 × Mo17 provides breeders a great opportunity for locating quantitative trait loci (QTLs) for cold tolerance in elite maize germplasm, the objective of this study. Under low-temperature conditions, the recombinant inbred lines significantly differed for all traits except vigor. No QTLs were detected at optimal conditions, but two QTLs involved in leaf color at low temperature were located in the short arm of chromosome 3 and the long arm of chromosome 6. The final fit for QTLs detected in our study explained 14.2% of phenotypic variance and 28.2% of genetic variance. However, in cross-validation analysis, QTLs detected in chromosomes 3 and 6 were detected in only 12.3% and 25.7% of all cross-validation runs, respectively, and explained 3.7% of the genetic variance. Although Mo17 could bring some favorable alleles, marker-assisted selection is not advisable because differences between allelic variants are small and explain a low proportion of genotypic variance. The locus *luteus11* is proposed as a candidate gene for the QTL located in chromosome 6, while the QTL located in chromosome 3 probably corresponds to an unknown gene.

Keywords: cold tolerance, quantitative trait loci, marker-assisted selection, maize.

Introduction

Today's cold-tolerant maize (*Zea mays* L.) varieties produce low yields (Revilla et al. 2005), and high-yielding germplasm is generally low temperature sensitive. However, there is variability for cold tolerance among high-yielding genotypes; actually, recombinant inbred lines (RILs) derived from lines B73 × Mo17 (the IBM population) significantly diverged for germination at low temperature (Kollipara et al. 2002). In order to check the possibility of improving cold tolerance in the widely used Corn Belt Dent germplasm, the IBM population was chosen because B73 and Mo17 represent the main families of elite maize inbred lines and an extensive genetic map is available. The IBM population offers high possibilities for quantitative trait locus (QTL) resolution and detection power.

Thus far, breeding for cold tolerance in maize has had very limited success, producing a few inbred lines with low yield. Low heritability and complexity of resistance to low temperature seem to be the main handicaps. Cold tolerance is a complex trait with polygenic inheritance that involves additive as well as dominance and maternal effects (Mahajan et al. 1993; Revilla et al. 2000, 2005). Cold-tolerance-related traits usually show low heritability, mostly because of an important genotype × environment interaction, for which a main factor is seed origin (Revilla et al. 2005).

Marker-assisted selection (MAS) offers an interesting possibility for improving cold tolerance (Collard et al. 2005), as long as an appropriate base population with high yield and variability for cold tolerance is used, a fine genetic map is avail-

able, the genotype × environment interaction of seed origin is appropriately managed, and the QTLs identified are reliable. A precise identification of markers tightly linked to QTLs is required for MAS. At present, little is known about maize QTLs involved in cold tolerance. Different genome regions that were involved in the expression of physiological traits related to tolerance to low temperature have been identified in previous studies (Fracheboud et al. 2002, 2004; Jompuk et al. 2005). Also, QTLs associated with the development of root and shoot at low temperatures were identified (Hund et al. 2004). Hund et al. (2005) reported QTLs for specific leaf area and for photosynthesis-related traits at low temperatures in the same chromosome regions. The objective of this study was to check the possibilities of detecting QTLs for cold tolerance in the Corn Belt maize cross B73 × Mo17.

Material and Methods

Plant Material

A population of 302 intermated RILs derived from B73 and Mo17 (the IBM population) was employed for QTL analysis. RILs were obtained from the Maize Genetic Cooperation Stock Center (Urbana, IL). The RIL scores provided by the Maize Mapping Project (<http://www.maizemap.org/>) were used to make a high-density genetic linkage map (<http://www.maizegdb.org/ibm302scores.html>). Information about marker and amplified sequences is available at <http://www.maizegdb.org/>. Seeds from RILs were multiplied in northwestern Spain in two consecutive years (2003 and 2004) in order to estimate the genetic variance explained, adjusted for QTL × seed origin interaction (Utz and Melchinger 2003).

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Phenotypic Analysis

The parental inbred lines and RILs were evaluated in a growth chamber under low-temperature and optimal conditions. The cold chamber (20 m³) was equipped with three shelves separated at 0.5-m intervals. For evaluation at low temperature, conditions were set at 14 h light (provided by seven very high-output fluorescent lamps with a photosynthetic photon flux of 228 $\mu\text{mol m}^{-2} \text{s}^{-1}$) at 14°C and 10 h without light at 8°C. The two different seed origins (2003 and 2004) were evaluated separately in two consecutive experiments. Under optimum conditions, the temperatures were 25° and 18°C in light and dark, respectively. Evaluations were arranged following a randomized complete block design with three replications (one replication on each shelf). Each trial was carried out for a period of 30 d under low-temperature conditions and 15 d at optimum conditions. Genotypes were planted in 21-L trays filled with 12 L of sterilized and watered peat (Gramoflor, Vechta, Germany). Sowing depth was 2 cm, and seeds were planted in rows spaced 5 cm apart, with 2 cm between seeds. All trays were watered again with 2.5 L of water 20 d after planting, which was enough to keep the plants turgid. Cold tolerance was assessed using agronomic traits, useful for breeders, instead of physiological traits. Six related cold-tolerance traits were considered: leaf color (from 1 = albino to 9 = dark green) and vigor (from 1 = weak to 9 = vigorous) as visual ratings, proportion of emergence, proportion of survival (proportion of live plants over emerged plants), days to emergence (days from planting to 50% plants emerged), and emergence rating ($100 \times [\Sigma(\text{no. plants emerged at time } i / \text{time from planting}) / \text{time from planting to end of emergence}]$, where time was recorded in days). Emergence was recorded every 2 d, up to a maximum of 30 d. Under each condition (low and optimal temperature), comparisons among the LSMEANS of the parental inbreds and the RIL population and simple correlation coefficients between traits were computed with SAS, version 9.1. (SAS Institute 2000). An ANOVA of RILs was performed for each trait by using the procedure PROC MIXED of SAS, version 9.1. RILs were considered random effects, and best linear unbiased prediction was employed in order to obtain the estimate of each RIL score for each seed origin.

QTL Analysis

The original linkage map has more than 2000 markers (simple sequence repeats, single nucleotide polymorphisms, RFLPs, microarrays, and INDEL); however, following the recommendations of the software PLABQTL (<http://www.uni-hohenheim.de/~ipspwww/soft.html>), QTL analyses were performed separately for RILs at low- and optimal-temperature conditions, using a linkage map with an average distance between loci of about 10 cM, yielding a final set of 587 markers. A likelihood odds (LOD) threshold of 4.9 was chosen for declaring the putative QTL significant. The LOD score was obtained by the permutation test method (Churchill and Doerge 1994), yielding an individual Type I error rate of 0.31% and an experiment-wise error rate of 33%. The analysis and cofactor election were carried out following PLABQTL's recommendations, using an "F-to-enter" and an "F-to-delete" value of 9. Significance thresholds used for QTL identification were high because we wanted to reduce the possibility of false positive QTLs as much as possi-

ble. All putative QTLs were examined for QTL \times environment interaction. The proportion of phenotypic variance explained by all QTLs was determined by the adjusted coefficient of determination of regression (R_{adj}^2), fitting a model including all detected QTLs. The proportion of genotypic variance explained by all QTLs for one trait (p) was calculated as $p = R_{\text{adj}}^2 / h^2$, where h^2 is the heritability of the trait.

Fivefold cross validation (CV/G) was performed for the RILs following the procedures described by Utz et al. (2000). The whole data set was randomly split into $k = 5$ data subsets. Four of these subsets were combined to form the estimation set (ES), and the remaining subset formed the test set (TS), in which predictions derived from the ES were tested for their validity by correlating predicted and observed data. We used 1000 replicated CV/G runs. Estimates of medians, percentiles, and frequency of QTL detection in the ES and TS were calculated over all replicated CV/G. The PLABQTL (Utz and Melchinger 2003) software package was used for all calculations.

Results

Under optimum conditions, there were no significant differences between the parental inbreds and the RIL population for any trait (table 1), but significant differences among RILs were detected for all traits (data not shown). At low-temperature conditions, differences between the parental inbreds and the RIL population were significant ($P < 0.05$) for proportion of emergence, and large intervals of variation were found for all traits (table 1); variation among RILs was significant for all traits except vigor (table 2). Under low-temperature conditions, the year \times RIL interaction was significant for vigor, proportion of emergence, and emergence rating, thus showing the importance of seed origin for evaluations of cold tolerance. All traits showed a normal distribution, except the percentage of survival (data not shown). Logarithmic transformation, recommended for percentage, did not improve data distribution for this trait, and so data were not transformed for analysis.

Under optimum conditions, no QTLs were detected. In low-temperature conditions, two QTLs were detected in this study, both of them associated with leaf color at low temperatures (table 3). The heritability was 37.5% for this trait. For the QTLs detected, the Mo17 alleles had a positive additive effect. These QTLs were located in the short arm of chromosome 3 and in the long arm of chromosome 6. The QTL identified in chromosome 3 explained 13% of the phenotypic variance, while that located in chromosome 6 explained 16.9%. The final fit in this study explained 14.2% of phenotypic variance and 28.2% of the genetic variance for leaf color (table 3). When QTL analyses were carried out independently for each seed source, only one QTL in chromosome 6 was detected for each seed source, presenting confidence intervals of 162–174 and 116–126 cM.

In cross-validation analysis, the median QTL effects calculated from CV/G were similar to the values calculated from the full data set. The tenth and ninetieth percentile values indicate low variances associated with the effects. The QTLs located in chromosomes 3 and 6 were detected in 12.3% and 25.7%, respectively, of all cross-validation runs and explained only 3.7% of the genetic variance (table 3).

Table 1
Means and Range of Best Linear Unbiased Prediction (BLUP) Values for Six Cold-Tolerant-Related Traits in the Evaluation of Maize IBM Recombinant Inbred Line (RIL) Population and Parents Evaluated at Low-Temperature and Optimum Conditions in a Chamber

Genotype	Score ^a		Emergence			Survival (%)
	Leaf color	Vigor	Days	Percentage	Rating	
Low-temperature conditions:						
B73	5.3 ^A	5.5 ^A	20 ^A	35 ^B	3.7 ^A	100 ^A
Mo17	4.3 ^A	4.8 ^A	27 ^A	75 ^A	4.8 ^A	96 ^A
RILs	4.0 ^A	4.4 ^A	23 ^A	47 ^B	3.2 ^A	95 ^A
Minimum ^b	3.2	4.2	17	6	2.8	84
Maximum ^c	4.8	4.8	23	82	7.3	96
Optimum conditions:						
B73	8.0 ^A	7.5 ^A	4 ^A	81 ^A	12.9 ^A	100 ^A
Mo17	8.0 ^A	6.5 ^A	4 ^A	100 ^A	16.7 ^A	100 ^A
RILs	7.9 ^A	6.7 ^A	4 ^A	79 ^A	13.0 ^A	99 ^A
Minimum ^b	6.7	6.4	4	32	5.1	88
Maximum ^c	8.3	8.8	7	99	16.9	100

Note. Means with the same uppercase superscript letter, within the same column, are not significantly different at $P = 0.05$.

^a Scores for color and vigor: from 1 = albino leaves to 9 = dark green leaves; score for vigor: from 1 = weak plants to 9 = vigorous plants.

^b Minimum of RIL means. Means were computed with the BLUP of each RIL for each seed origin.

^c Maximum of RIL means. Means were computed with the BLUP of each RIL for each seed origin.

In general, simple correlation coefficients between traits were low, although they were higher under optimum than under low-temperature conditions (table 4). Leaf color did not show high correlations with any trait. In general, the coefficients of correlation between vigor or emergence rating and the remaining traits were similar at optimum and low-temperature conditions, while the coefficients of correlation between the proportion of emergence and other traits at optimum conditions differed from those at low-temperature conditions.

Discussion

Because no QTLs were detected at optimum conditions, QTLs found at low-temperature conditions would be considered especially involved in the response to low temperatures and related to cold tolerance. Therefore, all discussion will be focused on the evaluation under low-temperature condi-

tions. The inbred B73 had significantly lower germination than did Mo17, but no QTLs were found for proportion of emergence, likely because of the significant year \times RIL interaction. In addition, some RILs showed low seed emergence rates at optimal conditions, likely as a result of seed infection by *Fusarium verticillioides*, because, in a previous study, we had found that this fungus affected more than 50% of seeds of different hybrids (Butrón et al. 2006). An alternative approach would be to analyze QTLs for percentage reduction in emergence rate due to low-temperature conditions rather than analyze QTLs for emergence rate at low-temperature conditions. However, the percentage of reduction in emergence rate ($[\text{emergence at } 25^{\circ}\text{C} - \text{emergence at } 14^{\circ}\text{C}]/\text{emergence at } 25^{\circ}\text{C}$) cannot be computed properly because of the lack of homogeneity in the cold chamber and the different ages of the seeds evaluated at low- and optimal-temperature conditions. Evaluations under low-temperature conditions were made in 2005, while evaluations at optimal conditions were made in 2007.

Table 2
Mean Squares of the ANOVA of Recombinant Inbred Lines (RILs) for Six Cold-Tolerant-Related Traits Evaluated in Cold Chamber under Low-Temperature Conditions

Sources of variation	df	Leaf color	Vigor	Days to emergence	Proportion of emergence	Emergence rating	Proportion of survival
RIL	251 ^a	2.17 ^{**}	1.75	27.95 [*]	1700 [*]	10.19 ^{**}	249.44 ^{**}
Year \times RIL	184 ^b	1.21	1.78 ^{**}	20.44	1281 ^{**}	6.79 ^{**}	152.11
Error	644 ^c	1.14	1.34	19.57	351	4.75	153.10

^a 256 for proportion of emergence and 252 for proportion of survival.

^b 181 for color, 183 for vigor, and 192 for proportion of emergence.

^c 536 for color, 554 for vigor, 896 for proportion of emergence, and 689 for proportion of survival.

* $P = 0.05$.

** $P = 0.01$.

Table 3
Summary of Quantitative Trait Loci (QTLs) Affecting Leaf Color Evaluated in a Cold Chamber in the Maize IBM Recombinant Inbred Line (RIL) Population

QTL bin ^a	Confidence interval (cM)	LOD score	Flanking markers	R^2_{adj}	p^b	\hat{a}^c	Cross-validation $\hat{a}_{TS,ES}^d$			
							Median	Percentile (tenth, ninetieth)	Frequency (%)	p^e
3.01	28–43	5.07	<i>mmp158a, mmp38</i>	13.0		.097	.099	.082, .119	12.3	
6.03	163–172	6.75	<i>umc2316, umc65a</i>	16.9		.101	.108	.088, .123	25.7	
Final fit				14.2	28.2					3.7

Note. LOD = likelihood odds; TS = test set; ES = estimation set.

^a Bin locations are designated by an X.Y code, where X is the linkage group containing the bin and Y is the location of the bin within the linkage group (Gardiner et al. 1993).

^b Proportion of genotypic variance explained by detected QTL, calculated as R^2_{adj} /heritability in the whole data set.

^c Median of QTL detection calculated based on whole data set.

^d Median, percentiles, and frequency of QTL detection were calculated based on 200 fivefold CV/G runs.

^e Proportion of genotypic variance explained by detected QTL, calculated as R^2_{adj} /heritability in 200 cross-validation runs.

The highly significant differences ($P < 0.01$) among RILs and the lack of significant year \times RIL interactions for leaf color and proportion of survival should allow the detection of QTLs for those traits if there were some genes with significant contribution to cold tolerance in the IBM population. Significant QTLs have been found for color, which is the trait with the simplest genetic control among the traits evaluated. Under low growth temperature, the variation of the chlorophyll content was large and therefore detectable by the visual rating used. However, under optimum temperature, a visual rating was probably not precise enough to detect the smaller differences in leaf color. Therefore, we cannot exclude that the QTLs detected for color play a role under optimal conditions, but they seemed to become especially important under low-temperature conditions. For the QTLs detected, the Mo17 alleles had a positive additive effect, although the inbred B73 did not significantly differ from Mo17 for leaf color. Therefore, QTLs for which B73 presents favorable alleles would probably be undetected because of their minor effects. Finally, because the recorded traits were poorly correlated, the QTLs detected for leaf color would not have any important effect on emergence-related traits, vigor, and proportion of survival.

Departure from a normal distribution of proportion of survival could compromise the statistical power for QTL detection (Mao and Xu 2004). The complexity of cold-tolerance-related traits results from the involvement of many genes with large environmental effects and genotype \times environment interactions.

Small variations of temperature around a critical value usually generate large experimental errors, raising the coefficients of variation. However, such constraints did not prevent us from finding significant QTLs in other studies, as long as differences among RILs were significant and even though the parents were not significantly different and the coefficients of variation were above 40% (Hund et al. 2004). Under those circumstances, the possible QTLs we could detect would be too weak to be of any value for MAS.

Lack of significant QTLs for complex traits could just mean that the parents do not contribute significantly different genes for cold tolerance or that the effects of the segregating genes are small and of similar magnitude. QTLs related to maintenance of color at low temperature have been detected by other authors. Fracheboud et al. (2002) found QTLs related to photosystem II activity and pigment composition in leaves, located in chromosome 3, that are expressed only in leaves developed at suboptimal temperatures. Jompuk et al. (2005) detected a QTL for leaf greenness close to the centromere of chromosome 3. However, in this study, the QTL detected for leaf color in chromosome 3 was located far away from the centromere, where QTLs previously reported for photosystem II activity and pigment composition in leaves were located. Some genes related to leaf color have been located in bin 3.00, namely, *golden plant2* (this mutant phenotype has yellow or golden leaves) and *defective crownN1053A* (green striped leaves), but they also mapped far away from the left-flanking

Table 4
Simple Correlation Coefficients among Traits Recorded on Recombinant Inbred Lines under Optimum (above Diagonal) and Low-Temperature (below Diagonal) Conditions

	Leaf color	Vigor	Days to emergence	Proportion of emergence	Emergence rating	Proportion of survival
Leaf color		.37**	-.12	.17*	.16*	.27**
Vigor	.39**		-.42**	.48**	.49**	.16*
Days to emergence	-.33**	-.27**		-.37**	-.45**	-.02
Proportion of emergence	-.19**	.15*	.25**		.97**	.09
Emergence rating	.28**	.52**	-.77**	.24**		.10
Proportion of survival	-.10	-.01	.45**	.23**	-.26**	

* $P = 0.05$.

** $P = 0.01$.

marker of the QTL in the IBM2 2005 Neighbors Frame 3 map (<http://www.maizegdb.org/cgi-bin/displaymaprecord.cgi?id=978379>).

Fracheboud et al. (2004) and Jompuk et al. (2005) identified a QTL located in chromosome 6 related to the efficiency of photosystem II activity under cool conditions. The nearest marker for the QTL detected on chromosome 6 by Fracheboud et al. (2004) was *bnlg1617*, located in bin 6.04 in the IBM2 2005 Neighbors 6 map (<http://www.maizegdb.org/cgi-bin/displaymaprecord.cgi?id=978382>). The right-flanking marker for our QTL, *umc65a*, is considered a core marker (a locus that defines a bin boundary) and defines bin 6.04. So, our QTL is likely situated at the end of bin 6.03. This indicates that our QTL is located near the QTL defined by Fracheboud et al. (2004). Furthermore, if we consider that the interval for the QTL detected is only a lower boundary for the true support interval (Utz and Melchinger 2003), both QTLs detected might be the same, and they could correspond to the locus *luteus11* that was located in the interval *umc2316-umc65a* in the IBM2 2005 Neighbors Frame 6 map (<http://www.maizegdb.org/cgi-bin/displaymaprecord.cgi?id=978382>). Mutants for the locus *luteus11* show altered leaf and/or seedling color.

QTLs identified by the authors mentioned above explained a similar proportion of phenotypic variance as that showed by QTLs reported in this study. QTLs found by Fracheboud et al. (2002), involved in the pigment composition of the third leaf of maize seedling and in different photosynthetic parameters at low temperatures, explained between 0.1% and 20.4% of phenotypic variance. Although three QTLs identified by Fracheboud et al. (2004) explained between 37.2% and 54.4% of the phenotypic variance, most QTLs reported in the literature explained less than 15%.

Van Berloo and Stam (1999) pointed out that for a heritability range of ca. 10%–30%, MAS is usually more effective than conventional breeding methods, but, according to Utz et al. (2000), for MAS to be superior to classical phenotypic selection, the QTL positions must be estimated with high precision to choose markers showing a minimum of recombination with the QTL, the QTL effects must be estimated on the basis of their true genetic effects, and, finally, the QTL detected must explain a sufficient proportion of genotypic variance. For this reason, an analysis of cross validation was carried out. Previous studies developed for identifying QTLs related to cold tolerance did not carry out cross-validation analyses, so the effect of the QTLs reported by these authors might have been overestimated, increasing the risk of failure in future programs of MAS.

The cross validation and the use of two environments for seed production reduce considerably the apparent contribution of the QTLs originally identified and increase the consistency of the results. Because the percentage of genotypic variance explained in the CV/G analysis is much lower than that in the full data set, there was a strong upward bias in the calculation of the genetic variance explained by the model. Therefore, the QTLs detected in our study have a very small effect and could not be detected with enough precision to be used in MAS programs. The probability of obtaining these results rises as the number of genes involved in a trait increases, and the results show that there are no real possibilities of applying a MAS program for improving cold tolerance of these Corn Belt inbred lines without introducing favorable alleles from other maize sources. Considering that B73 and Mo17 are involved in the pedigree of many elite inbred lines, this negative conclusion can be generalized to an important number of the inbred lines used nowadays. In order to improve cold tolerance of these elite Corn Belt inbred lines, a most promising approach would be to introduce genes for cold tolerance from the already available sources (Revilla et al. 2000, 2005; Rodríguez et al. 2006).

Our results suggest that genes involved in the maintenance of the photosynthetic activity and leaf color at low temperatures are located in the short arm of chromosome 3 and the long arm of chromosome 6. Nevertheless, the genetic variance explained by these QTLs is too low, and MAS selection would not be effective for improving the cold tolerance of this germplasm. In order to improve the cold tolerance of this germplasm, introducing new favorable alleles from cold-tolerant germplasm could be a more suitable option for further breeding programs. The locus *luteus11* is proposed as a candidate gene for the QTL located in chromosome 6, while the QTL located in chromosome 3 probably corresponds to an unknown gene.

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