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Fine Mapping and Haplotype Structure Analysis of a Major Flowering Time Quantitative Trait Locus on Maize Chromosome 10

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ABSTRACT

Flowering time is a major adaptive trait in plants and an important selection criterion for crop species. In maize, however, little is known about its molecular basis. In this study, we report the fine mapping and characterization of a major quantitative trait locus located on maize chromosome 10, which regulates flowering time through photoperiod sensitivity. This study was performed in near-isogenic material derived from a cross between the day-neutral European flint inbred line FV286 and the tropical short-day inbred line FV331. Recombinant individuals were identified among a large segregating population and their progenies were scored for flowering time. Combined genotypic characterization led to delimit the QTL to an interval of 170 kb and highlighted an unbalanced recombination pattern. Two bacterial artificial chromosomes (BACs) covering the region were analyzed to identify putative candidate genes, and synteny with rice, sorghum, and brachypodium was investigated. A gene encoding a CCT domain protein homologous to the rice *Ghd7* heading date regulator was identified, but its causative role was not demonstrated and deserves further analyses. Finally, an association study showed a strong level of linkage disequilibrium over the region and highlighted haplotypes that could provide useful information for the exploitation of genetic resources and marker-assisted selection in maize.

FLOWERING time is a critical issue for reproductive success of plants. To ensure that the timing of the reproductive switch is optimal, plants integrate both endogenous and environmental signals. In domesticated species, adaptation of flowering time has been a key factor for the spread of agriculture. This is particularly true in maize, domesticated in Mexico and now cultivated in a wide range of latitudes. The timing of flowering is also a critical selection criterion for plant breeders as it limits the cultivation area of varieties and also impacts on yield and harvest quality as recently reviewed by Jung and Muller (2009). Photoperiod sensitivity plays an important role in flowering time regulation in maize, at least in tropical germplasm in which it can considerably delay the reproductive switch under long-day conditions (Gouesnard *et al.* 2002), impeding the exploitation of this material in temperate breeding programs (Goodman 2004).

The photoperiod, autonomous, vernalization, and gibberellin pathways constitute the four major signaling pathways regulating flowering time in the model plant Arabidopsis (Boss *et al.* 2004; Ausin *et al.* 2005). The genetic control of the floral transition has also been studied in various cultivated cereals such as rice, barley, and wheat (for review, see Cockram *et al.* 2007; Colasanti and Coneva 2009; Greenup *et al.* 2009), but remains poorly understood in maize. Among the genes identified, *INDETERMINATE1* (*ID1*) encodes a zinc finger protein and has no Arabidopsis ortholog (Colasanti *et al.* 1998). It has been shown that *id1* mutants produce many more leaves and exhibit a severe delay of flowering time. Another locus, *delayed flowering1* (*dlf1*), has been identified on the basis of its loss of function (Muszynski *et al.* 2006). The *dlf1* gene encodes a bZIP protein homologous to the FD protein, which in Arabidopsis interacts with the floral integrator FT to promote the floral transition at the shoot apex (Abe *et al.* 2005). Muszynski *et al.* (2006) therefore hypothesized that *dlf1* should interact with an *FT* ortholog in

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maize. Gene homology across species indeed suggests a variation on a common theme, as exemplified by *zfl1* and *zfl2*, homologous to the Arabidopsis transcription factor *LEAFY* (Parcy *et al.* 1998; Bomblies *et al.* 2003) but only *zfl1* appears to regulate flowering time while *zfl2* may impact on morphological traits (Bomblies and Doebley 2006). Also, *conz1*, a *CONSTANS*-like gene has been characterized by Miller *et al.* (2008) who demonstrated that, similarly to the Arabidopsis gene *CONSTANS* (Suarez-Lopez *et al.* 2001; Valverde *et al.* 2004) and the rice *Heading Date 1* (*Hd1*) (Yano *et al.* 2000), *conz1* exhibits a day-length-dependent expression pattern; however, its implication in the photoperiod pathway in maize remains unclear. Finally, through positional cloning, Salvi *et al.* (2007) refined the major flowering time QTL *Vgt1* to an ~2-kb noncoding region acting as a *cis*-regulatory element that controls the *ZmRAP2.7* gene, which is homologous to the Arabidopsis *TARGET of EAT1* (*TOE1*).

These studies only offer limited insights into the molecular determinism of flowering time in maize, considering the tens of quantitative trait loci (QTL) detected for this trait (Chardon *et al.* 2004; Buckler *et al.* 2009). Among these QTL, a region of chromosome 10 appears of major importance and has been detected in multiple genetic backgrounds (Lubberstedt *et al.* 1997; Rebai *et al.* 1997; Bohn *et al.* 2000; Moutiq *et al.* 2002; Blanc *et al.* 2006; Wang *et al.* 2008) including a cross between maize and its wild ancestor teosinte (Briggs *et al.* 2007). Recently, K. Chenu, A. Bouchez and C. Giauffret (unpublished results; technical report available upon request from the corresponding author or C. Giauffret) conducted a QTL detection in a population developed from a cross between the day-neutral European Flint inbred line FV286 and the short-day highland tropical line FV331. They detected a major QTL ($R^2 = 41\%$) in this same region and, by developing isogenic material, showed its implication in photoperiod sensitivity.

In this study, we first report the fine mapping of this major flowering time QTL using a large segregating population derived from these near-isogenic lines. Second, we analyzed the collinearity between this region of maize chromosome 10 and corresponding regions of various monocotyledonous species and we tried to identify putative candidate genes. Finally, we aimed to characterize the QTL region by means of association mapping using a panel of diverse maize inbred lines.

MATERIALS AND METHODS

Plant material and phenotypic observation: Recombinant inbred lines (RILs) were developed from a cross between the day-neutral European Flint inbred line FV286 and the short-day tropical highland inbred line FV331. Near-isogenic lines (NILs) were derived by selfing F_5 RILs displaying residual heterozygosity for the QTL of interest (K. Chenu, A. Bouchez and C. Giauffret, unpublished results, technical report available upon request). Only RIL 146, which presented the highest homozygosity over the genome, was considered for the present study. Two late flowering NILs (146-4 and 146-9), with the FV331 allele at the QTL, were crossed with an early flowering NIL (146-5) with the FV286 allele, to generate the F_1 generation in Saint-Martin-de-Hinx (France) during the summer of 2003. F_1 plants were selfed in Chile during the winter of 2003/2004 and 425 F_2 plants per cross were grown in 2004 and evaluated for flowering time. Eighty-four heterozygous F_2 plants (considering marker *umc1246*) were selfed and their F_3 progeny (9304 plants) were sown during the summer of 2005 in Gif-sur-Yvette (France). Eight out of the selected F_2 individuals that proved recombinant in the region in further analyses were discarded so that 8473 F_3 plants were informative for the fine-mapping experiment. This population was phenotyped on a single-plant basis, selfed (successfully for 6538 plants), and genotyped to identify recombinant individuals. A first screening was performed on half of the F_3 individuals with markers *2am.acpcr* and *cl3584.idp* (supporting information, Table S1), which flanked the QTL region initially detected. As a result, 458 F_4 families from F_3 recombinant plants with enough seeds were evaluated in 2006 using a row design (two rows of 10 plants sown at a density of ~6 plants/m^2). Analysis of flowering times of those families allowed us to refine the QTL position between markers *cq.3pa* and *ch.idp* (Table S1). These two markers were consequently used to screen the second half of the F_3 population, leading to the identification of 28 additional recombinants. Their F_4 progenies were evaluated in 2007 using a similar design (with 25 plants per row and one repetition sown under plastic film) along with 32 F_4 families already evaluated in 2006. Finally, an ultimate reevaluation of 26 F_4 families was performed in 2008. All the plants, from the F_2 to F_4 generations, were individually phenotyped under natural long-day conditions in Gif-sur-Yvette (France). Male flowering time, corresponding to the date of appearance of the first visible anthers on a plant, was expressed in number of days from the first of July. Mean flowering time and the degree of segregation within the F_4 families were used to infer the genotype of the corresponding F_3 recombinants at the QTL.

DNA extraction and molecular analyses: DNA was extracted following a NaBisulfite method adapted from Tai and Tanksley (1991). Several markers were specifically designed for fine-mapping purposes. The QTL was first anchored to the B73 physical map (http://www.genome.arizona.edu/fpc/maize/ and http://www.maizesequence.org/index.html) using microsatellite markers, and then sequences generated from bacterial artificial chromosomes (BACs) covering the region were retrieved from the National Center for Biotechnology Information (NCBI) Web site (http://www.ncbi.nlm.nih.gov/). These sequences (either BAC ends or shotgun sequences) were repeatmasked (http://www.repeatmasker.org/), blasted against maize high-throughput genome sequences (HTGS) and genome sequence survey (GSS) sequences, and primers were designed on low-copy sequences using primer3 (Rozen and Skaletsky 2000). Amplification and sequencing of parental lines revealed polymorphisms used for marker development (Table S1). Last steps of the fine mapping, pinpointing an unsequenced region, BAC c0171E08, was ordered from the BACPAC Resource Center (BPRC) at the Children's Hospital Oakland Research Institute (Oakland, CA), sequenced at the French National Sequencing Center (Evry, France) and deposited in GenBank under accession no. GU142949.

PCR reactions were performed in 20-μl volumes containing 1× PCR buffer (QIAGEN, Valencia, CA), 1 unit of Taq polymerase (QIAGEN), 0.2 mm of each dNTP, 1.5 mm of $MgCl_2$, 8 pmol of each forward and reverse primer, and 15–30 ng of template DNA. Thermocycling, in particular annealing temperature and elongation duration, was adapted to each

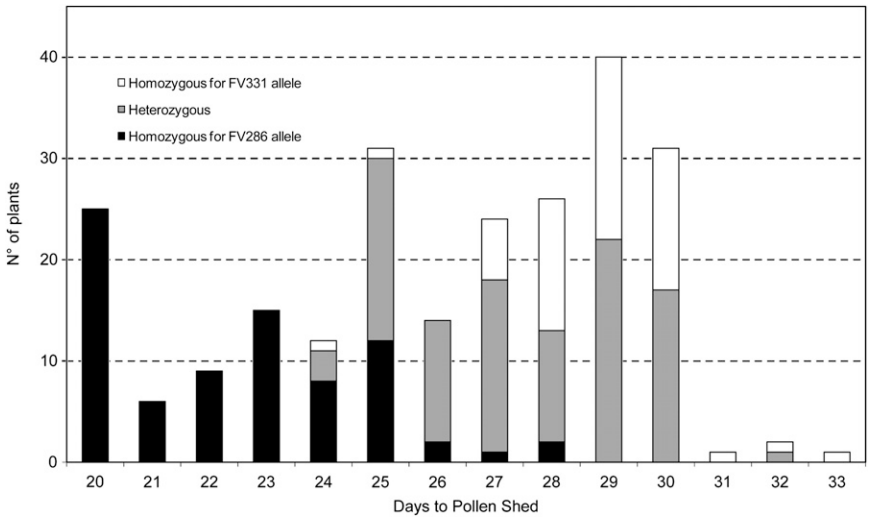

FIGURE 1.—Frequency distribution of days to pollen shed in 237 F_2 plants. Marker *umc1246* was used for genotyping (plants found to be recombinant in subsequent analyses were excluded from this analysis).

amplicon. Electrophoreses were carried out on either standard or high-resolution agarose, according to the length of the fragments. Gels were visualized under ultraviolet light after ethidium bromide staining. Sequencing of amplicons for association mapping was performed at the French National Center of Genotyping (Evry, France), following standard protocols.

Structural characterization of the region and analysis of synteny with monocotyledonous species: Five sequenced overlapping BACs (c0212J04, c0111P13, c0171E08, c0286M05, and c0111K09) were analyzed using TE-Nest (KRONMILLER and WISE 2008) and RepeatMasker (http://www.repeatmasker.org/) to localize transposable elements (TEs). Masked sequences were annotated using Fgenesh (http://linux1.softberry.com/berry.phtml, version 2.6). Using two nonambiguous genes to delimit the region, genomic sequences and corresponding gene annotations were retrieved from three monocotyledonous species: rice (*Oryza sativa*, http://rice.plantbiology.msu.edu), sorghum (*Sorghum bicolor*, http://www.phytozome.net/sorghum), and brachypodium (*Brachypodium distachyon*, http://www.brachypodium.org/). Local BLASTP analyses were then performed to compare the different sequences.

Association mapping and diversity analysis: A linkage disequilibrium (LD) mapping approach was conducted on 14 amplicons spanning the QTL interval in the final steps of the fine-mapping program (see Table S2). We considered either a panel of 375 inbred lines, described by CAMUS-KULANDAIVELU *et al.* (2006) or a subset of 96 inbred lines sampled in the different admixture groups on the basis of their phenotypes: lower and upper values for photoperiod sensitivity, estimated following the approach of GOUESNARD *et al.* (2002), were selected.

After sequence alignment and identification of polymorphisms, LD and associations with traits of interest were analyzed using TASSEL software (version 2.0.1, BRADBURY *et al.* 2007. Association tests were conducted using a general linear model accounting for population structure, as described in DUCROCQ *et al.* (2008). Finally, analysis of 256 landraces previously described by REBOURG *et al.* (2003) and DUBREUIL *et al.* (2006) was performed on a bulk of 15 plants per population and allele frequencies were estimated as previously described (DUBREUIL *et al.* 2006; DUCROCQ *et al.* 2008).

RESULTS

Validation of the QTL effect in a near-isogenic background: A total of 756 F_2 plants were successfully phenotyped for days to pollen shed (DPS) under natural field conditions. A clear segregation was observed with a difference of flowering time of >10 days between the earliest and the latest plants, *i.e.*, ~140 growing degree days (GDD). The relationship between this segregation and the genotype at the QTL was checked by assaying 250 plants with microsatellite *umc1246*, the closest linked marker known at this step of the study. Considering all individuals, the three genotypes (FV286/FV286, FV286/FV331, and FV331/FV331) differed significantly ($P < 0.001$) with the genotype at *umc1246* explaining 61% of DPS variation. The homozygous plants for the FV286 allele had an average flowering time of 22.45 days (±2.16 days) while the homozygous plants for the FV331 allele and the heterozygous plants flowered in 28.80 days (±1.46 days) and 27.53 days (±1.89 days), respectively. The effect of the heterozygous genotype appeared very close to that of the homozygous genotype for the FV331 allele, suggesting a possible dominant effect of the late allele (FV331) at this locus, as shown in Figure 1.

High-resolution mapping of the QTL: A population of 8473 informative F_3 plants was field planted in 2005. The screening of the first half of these plants with markers *2am.acpcr* and *cl3584.idp*, located 8000 kb apart, according to the maize physical map, resulted in the identification of ~800 recombinants within this region (Figure 2). Hence, 458 F_4 families with sufficient seed stock were observed in 2006. After sequential genotypic characterization of the region, the QTL interval was refined to a 700-kb interval between markers *ch.idp* and *cq.3pa*. The genotype of the nonrecombinant F_3 plants within this interval was highly consistent with the phenotype of their corresponding F_4 progeny, expressed as mean and standard deviation calculated from individual plant observations within each family. Therefore, these phenotypic values were compared to molecular data to refine the QTL position. Note that the progeny of strategic recombinant plants were systematically reevaluated at least in a second year, further

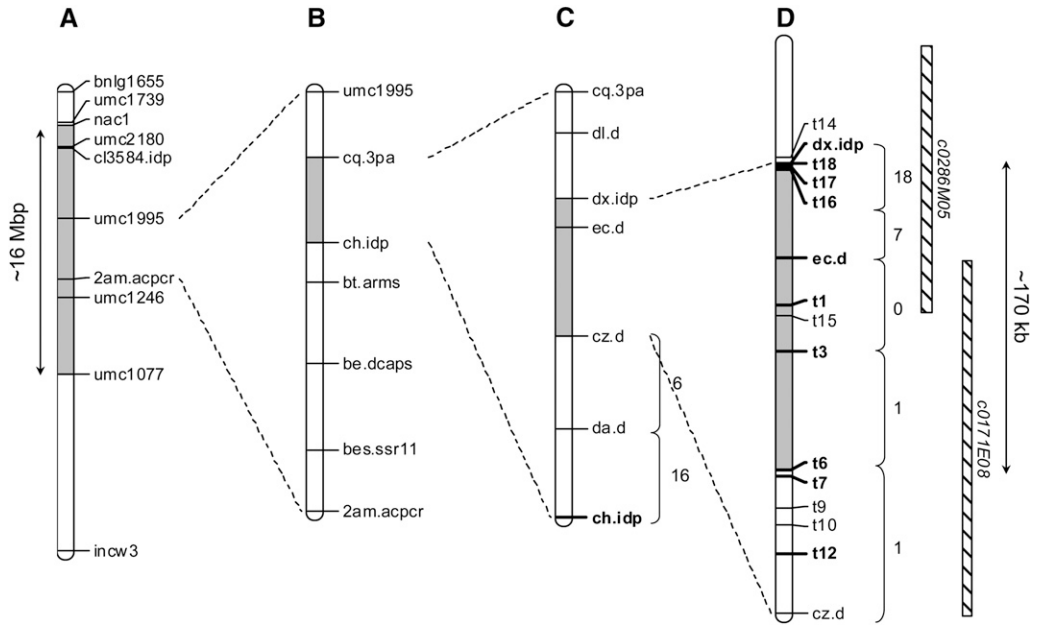

FIGURE 2.—Consecutive stages of the fine-mapping study. (A) Anchoring of the SSR marker used for genetic mapping of the QTL on the physical map. The shaded zone on the chromosome indicates the QTL confidence interval. This interval was estimated by genotyping the near-isogenic parents using SSR markers: these parents were polymorphic for markers *umc1995* and *umc1246* and monomorphic for markers *nacl* and *umc1077* (with FV286 and FV331 alleles fixed, respectively). (B) Physical map of the QTL region after the first screening of F_3 recombinants and phenotyping evaluation performed in 2006. Development of additional markers enabled the progressive refinement of the QTL position (C and D). Markers/amplicons used for association mapping appear in boldface type. BAC c0286M05 and c0171E08 are represented by hatched rectangles on the right of the figure. Numbers alongside brackets indicate the number of recombination events identified in our material between the corresponding markers.

reducing the risk of erroneous phenotype-based determination of the genotype at the QTL (Figure 3).

Screening of the second half of the F_3 population with markers *cq.3pa* and *ch.idp* led to the identification of 28 supplemental recombinants. Subsequent phenotypic characterization and marker development enabled us to progressively refine the QTL to an interval of 170 kb between markers *t16.idp* and *t6.idp* (Figure 3). This interval was covered by two overlapping BACs, namely c0286M05 (available under accession no. AC199625) and c0171E08, sequenced in the frame of this study (accession no. GU142949) to allow the development of additional markers. Some of the primer pairs revealed difficulties for the amplification of the FV331 allele. In this case, the genotype of the F_3 recombinants was determined by genotyping F_4 individual plants to distinguish between heterozygous and homozygous plants for the FV286 allele. However, a high transposable element content of the region (that limited marker density) and an unbalanced recombination pattern have prevented us from refining the QTL confidence interval. Indeed, as shown in Figure 3, 18 recombinants were found between *dx.idp* and *t16.idp*, which are 5 kb apart, whereas only 8 recombinants were found between *t16.idp* and *t6*, 7 of which were located in the 50-kb *t16.idp/ec* interval. As a consequence, seven recombinants support the left limit of the QTL interval, whereas a single recombinant (namely, "6763") supports its right limit but the phenotypic evaluation of its progeny is unambiguous (Figure 3).

Candidate genes and syntenic relationships: We aimed to identify putative candidate genes in the QTL region by analyzing the two BACs covering the interval. The draft sequence of BAC c0286M05 (AC199625; 170,132 bp) was made of seven contigs. Sequencing of BAC c0171E08 yielded a 174,044-bp contiguous sequence. We identified a large number of transposable elements (70 and 45% masked by RepeatMasker for c0286M05 and c0171E08, respectively). Nested TEs were identified using TE-nest, revealing a complex structure of transposable element insertions. Gene prediction, using Fgenesh, identified five and eight putative genes on BACs c0286M05 and c0171E08, respectively. On the basis of BLASTP results (Table S3), six of the predicted genes were considered as TE-related genes, three were considered as hypothetical genes, and two had no homologs found. Only two predicted genes had characterized homologs: a gene located on BAC c0171E08, encoding a pectin methylesterase inhibitor (PMEI) protein and a gene located on BAC c0286M05, encoding a CCT (CO, CO-LIKE, and TIMING OF CAB1) domain protein. However, the *PMEI* gene is located a few tens of kb outside the confidence interval of the QTL (Figure 3) and the *CCT* gene is located outside but very close to its left limit (the two exons of this gene lie between *dx* and *t16* amplicons).

To check the pertinence of the BAC annotation, we investigated the syntenic relationships between this region of maize chromosome 10 and other genome-sequenced monocotyledonous species, *i.e.*, rice, brachypodium, and sorghum. Two genes, encoding an adenylate kinase protein and an arginase protein, were chosen to delimit a 800-kb region including the QTL interval. A good collinearity was observed between this region

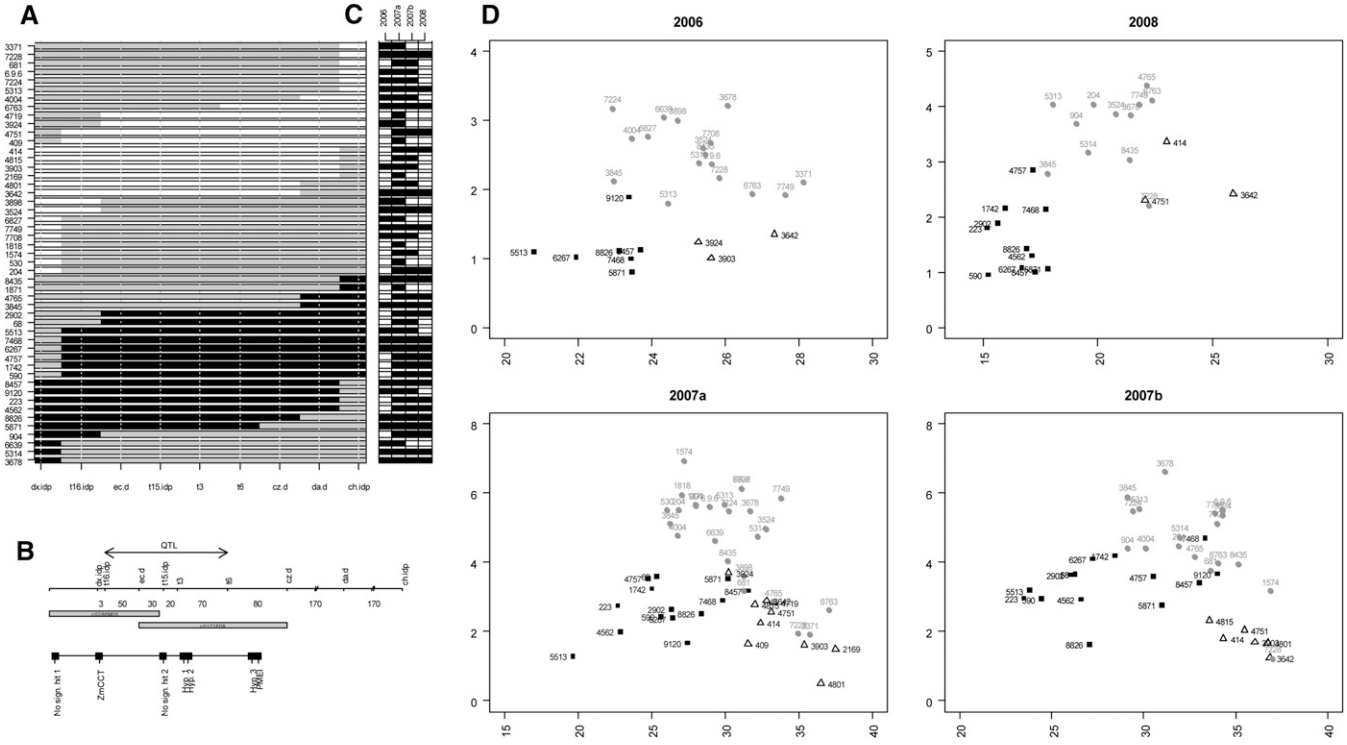

FIGURE 3.—(A) Graphical representation of the genotypes of recombinant F_3 plants in the QTL region (solid, FV286/F286; open, FV331/FV331; shaded, FV286/FV331). (B) Scaled representation of markers and putative genes in the vicinity of the QTL region (Hyp, gene considered as hypothetical on the basis of BLASTP results; no sign. hit, no significant BLASTP result; see Table S3 for details). For the sake of clarity, TE-related genes are not displayed on this figure. Numbers between markers indicate approximate distances (based either on the c0171E08 sequence or on the physical map). Genes from BAC c0286M05 were unambiguously located relative to the QTL by blasting primers of physically anchored markers against c0286M05 contigs. (C) Matrix representing the different years or conditions for phenotypic evaluation. Solid boxes indicate that the progeny of the corresponding individual was observed. Note that the summer of 2007 was particularly wet and cool, which had an impact on plant development. The years 2007a and 2007b refer to sowing conditions (under plastic film and normal sowing, respectively). (D) Phenotypic values expressed as mean (*x*-axis) and standard deviation (*y*-axis) obtained within the corresponding F_4 progenies during the different experiments. Colors and symbols correspond to the genotype at the QTL, as inferred from genotypic data, and all the available phenotypic information (solid squares, FV286/FV286; open triangles, FV331/FV331; shaded circles, FV286/FV331).

of maize chromosome 10 and a 600-kb region on rice chromosome 4, a 45-kb region on Brachypodium supercontig 3 and a 270-kb region on top of sorghum chromosome 6 (results are summarized in Figure S1). Four genes were conserved in the four different species with high homology: the adenylate kinase gene and the arginase gene (flanking the targeted region), as well as an AdoHcyase gene and a PMEI gene (thus supporting our annotation of BAC c0171E08). Most of the nonconserved genes were annotated as hypothetical or TE related. The *CCT* gene reported above on BAC c0286M05 was found in the targeted syntenic region of sorghum (68%, $E = 6.10^{-85}$) but not in Brachypodium and rice. However, a BLASTP analysis of the predicted amino acid sequence of this maize *CCT* gene (*ZmCCT*) against rice proteins showed a strong homology (41%, $E = 3.10^{-30}$) with GHD7 (ACA14489), a major regulator of yield potential and heading date in rice (XUE *et al.* 2008), located in the centromeric region of chromosome 7. Conversely, a TBLASTN of GHD7 protein against maize HTGS returned BAC c0286M05 as the first result (57%, $E = 5.10^{-20}$). The homology was mainly located in the CCT domain, as further supported by inserting this domain in the phylogenic tree previously constructed by GRIFFITHS *et al.* (2003) (results not shown). This homology appears particularly interesting, since *Ghd7* was proven to act in the photoperiod pathway of rice, and CCT domain proteins are known to be conserved across a wide range of species and play a major role in flowering responses to the environment and in the regulation of circadian rhythms (WENKEL *et al.* 2006). However, the two exons of the *ZmCCT* gene are located outside the confidence interval of the QTL. We therefore hypothesized that the causative factor might be located in the promoter of *ZmCCT* or in an upstream regulatory element. However, preliminary expression analysis of the *ZmCCT* gene did not reveal significant differences between the parental lines of our NIL population (results not shown).

Association mapping and analysis of genetic diversity: To characterize the genetic diversity in the region and possibly help in refining the position of the QTL, as

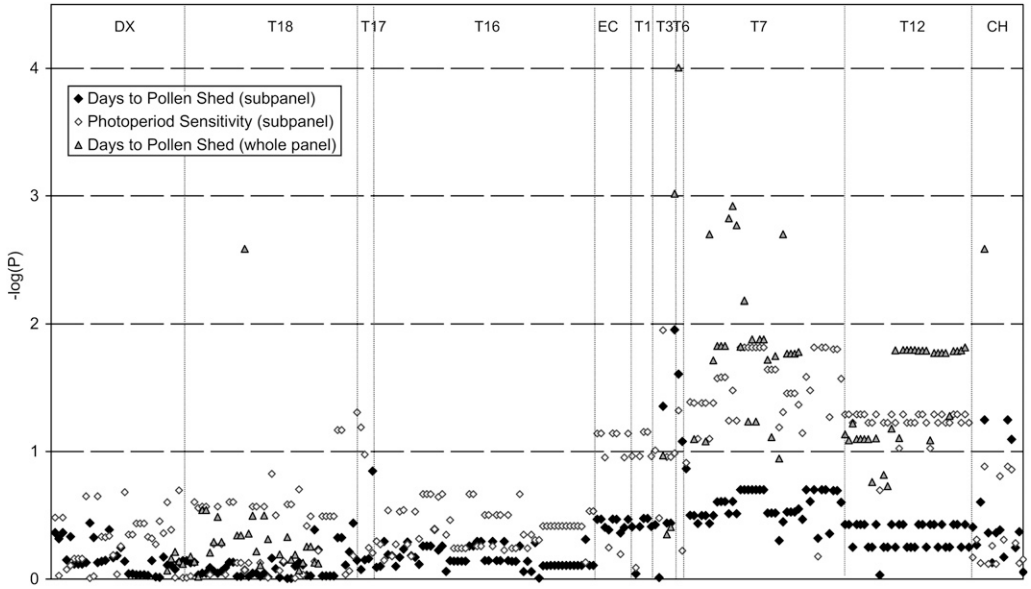

FIGURE 4.—Association of polymorphisms with flowering time and photoperiod sensitivity across the QTL region. Level of statistical association is expressed as $-\log(P)$. Solid and open diamonds indicate association with DPS and photoperiod sensitivity, respectively, across a subset of 96 lines. Shaded diamonds indicate association with DPS across the whole panel composed of 375 maize inbred lines. The vertical dotted lines separate the different sequenced amplicons.

shown by SALVI *et al.* (2007), we carried out an association study in this region of maize chromosome 10. Fourteen amplicons were sequenced on a panel of 96 maize inbred lines: 4 were located in the vicinity of the *ZmCCT* gene (*dx*, *t16*, *t17*, and *t18*), 9 were designed on the basis of the BAC c0171E08 sequence, and 1 amplicon (*ch*) was located ~300 kb downstream (Figure 2). Association tests were performed on 251 polymorphisms identified in 11 informative amplicons (Figure 4). Significance levels were generally weak (three *P*-values <0.05, considering DPS), maybe due to the relatively low power of the design including a limited number of individuals. The pattern of statistical association indicated lower *P*-values in *t3 and t6* amplicons (minimum *P*-value = 0.011 for *t3.544* considering DPS) and only one significant test (considering photoperiod sensitivity) for polymorphisms located around the *ZmCCT* gene. Also, a striking level of linkage disequilibrium was observed among amplicons derived from BAC c0171E08 (Figure S2), with 79% of the r^2 values higher than 0.8 and 97% of the D′ values equal to 1, over this region of ~170 kb. On the other hand, linkage disequilibrium was relatively lower between polymorphisms located around the *ZmCCT* gene. As a consequence, most of the polymorphisms observed between amplicon *ec* and amplicon *t12* distinguish two haplotypes, the less frequent one being exclusively composed of flint lines (European and Northern Flint groups) and the most frequent haplotype including lines from the different admixture groups, especially FV286, the early flowering parent of our NIL population. As detailed hereafter, FV331 belongs to none of these haplotypes. In the *ZmCCT* gene region (corresponding to amplicons *dx*, *t16*, *t17*, and *t18*), the most frequent haplotype was split in two with one haplotype being specific to dent and a few tropical lines. Consistently, the modeling of the ancestral structure (VEYRIERAS *et al.* 2007) highlighted three ancestral haplotypes in the entire region.

Remarkably, FV331, our tropical photosensitive parent, proved to be particularly singular. Indeed, only 7 out of the 14 targeted amplicons were successfully amplified (including the 4 located around the *ZmCCT* gene). Sequences obtained from these amplicons were clearly distinct from those of the 95 other inbred lines, with one exception for the *ch* amplicon located 300 kb downstream of the QTL interval. FV331 therefore exhibited a unique haplotype in this region. As a consequence, the effect of the FV331 allele could not be tested by association mapping, which requires higher allelic frequencies. Significant associations were therefore due to segregation of other alleles.

To confirm the above results, four amplicons showing limited redundancy were sequenced on a 375-inbred line panel that we also genotyped with two additional markers (*t6.idp* and *ch.idp* located in amplicons *t6* and *ch*, respectively). As expected due to a higher power, more significant effects were observed (Figure 4) with the lowest *P*-value of 1.10^{-4} obtained for *t6idp* (the right flanking marker of the QTL interval) considering DPS. Three alleles could be distinguished for this polymorphism: one of them was specific to the flint haplotype previously mentioned. Interestingly, although this allele was found in early materials, it appears to delay flowering time according to the association test (lsmean = 1149 GDD after adjustment of population structure effect). The second allele was found in lines of different admixture groups (including FV286) with a lsmean of 1101 GDD. The third allele was specific to dent lines and had an early flowering effect (lsmean = 1033 GDD). This last allele was also detected through polymorphism *t3.544* ($P = 1.10^{-3}$). Note that no amplification was observed for FV331 at the *t6* locus, which can be

considered a "fourth allele." When genotyping the maize populations with the *t6.idp* marker (Figure S3), we found that the late flint allele was exclusively observed in the Northern Flint and the European populations, especially in central Europe, and was absent from the tropical material. It was also absent from a set of 20 teosintes, suggesting a particular origin that remains to be determined. The early dent allele frequency was quite low across the different materials. Conversely, the intermediate common allele was found with a high frequency in a majority of landraces, excepting Northern European populations. Moreover, two additional rare alleles were also detected in our landrace panel.

DISCUSSION

Phenotypic observations conducted during our study confirmed the strong effect of this QTL of maize chromosome 10, the FV331/FV331 genotype delaying flowering time by 6.4 days (~100 GDD) with respect to the FV286/FV286 genotype. Moreover, the phenotype of heterozygous plants suggests a partial dominance of the FV331 allele over the FV286 allele.

The fine-mapping approach enabled us to refine the QTL to an interval of 170 kb. The low recombination within this region limited mapping resolution and discouraged further efforts for screening of additional recombinants in this genetic background. This absence or very limited occurrence of recombination events could be due to the singular genomic structure of FV331 in this region, highly different from FV286 (and other lines of the panel), as suggested by a specific sequence and the nonamplification of several amplicons. Highland maize, such as FV331, is known to have a smaller genome size than other sources due to a lower content of TEs and has been chosen for this reason for the Mexican maize genome sequencing project (Martinez de la Vega *et al.* 2008). Since numerous transposable elements were identified in the region, we could also hypothesize that they may influence recombination as illustrated by Dooner and He (2008). However, low recombination does not seem specific to the cross we studied since a very high LD was observed in the region among materials displaying closer genome structure (according to our association study). Interestingly, the rupture in haplotype structure observed within the *ZmCCT* gene coincides with a high recombination in the fine-mapping population. This suggests that other factors could affect recombination in the region.

Similarly, the presence of this major flowering time QTL is not specific to our population. The region encompassing bins 10.03 and 10.04 has repeatedly been detected in QTL mapping studies using diverse materials, as illustrated by the meta-analysis conducted by Chardon *et al.* (2004) and additional recent results obtained by Blanc *et al.* (2006), Briggs *et al.* (2007), Wang *et al.* (2008), and Buckler *et al.* (2009). Although we cannot exclude that more than one QTL could be involved, the confidence intervals reported in these studies are in accordance with the region we targeted. Moreover, parental inbreds of Blanc *et al.* (2006) were analyzed in the association study we conducted. We found that one of the parental lines used by Blanc *et al.* (2006), namely FV283, harbors the late flint haplotype in the region, whereas the other parents (D171, F810, and F9005) have the same common haplotype. This is in accordance with QTL mapping results that showed that FV283 had a late allele at the QTL (additive effect of 1.17 days for silking date). This effect has been recently confirmed by introgression of the FV283 allele for this region in a FV2 background (FV2 carrying the common allele). In 2008, phenotyping of 125 BC5S1 plants indicated a highly significant effect of the FV283 genotype (screened with markers *cq.3pa* and *ch.idp*), which delayed flowering time and also increased the anthesis–silking interval (A. Charcosset, personal communication). One can thus predict an allelic series at this major QTL with at least four classes of effect from the latest to the earliest one: FV331, FV283 (late flint allele), FV286 (common allele), and the dent allele. Considering the possible pitfalls of association genetics due to the relationship between population structure and adaptive traits, the effect of this last allele will require further validation (reproducibility on different panels, introgression, *etc.*), but it could be particularly interesting for the exploitation of genetic resources since this allele seems to have a low frequency in temperate material in which it could constitute a source of earliness. Markers developed in our study could therefore be beneficial for maize breeders. However, beyond the application in marker-assisted selection, the identification of the causative factor would offer a better understanding of the mechanisms implied in photoperiod sensitivity and regulation of flowering time in maize. Further efforts will thus be necessary for positional cloning of this QTL. The preliminary analyses we carried out focused on *ZmCCT* since its homology with *Ghd7* made it a good functional candidate. However, the two exons of this gene are located outside the QTL confidence interval and no effect was detected by association mapping. Considering that several positional clonings refined QTL positions to noncoding regions, as for *Tb1* (Clark *et al.* 2006) or *Vgt1* (Salvi *et al.* 2007), with *cis*-regulatory elements acting on genes located tens of kb downstream, we hypothesized that a similar mechanism could play a part in the QTL we focused on. Preliminary experiments conducted on leaf samples showed similar RNA levels of *ZmCCT* in FV286 and FV331, which does not support this hypothesis but calls for further investigations. Also, other putative genes present in the region should be reconsidered. Moreover, it is important to underline that the BAC sequences we used came from the B73 inbred line. Considering, the tropical origin of FV331 and the

singularity of the sequences we observed, we cannot exclude that the causative gene or factor is specific to this inbred line and was therefore not observed in our BAC sequences. Indeed, gene movement and structural rearrangement are frequent in maize (Ramakrishna *et al.* 2002; Messing and Dooner 2006). A finer structural analysis of the region will be required, either on the basis of the ongoing production of full genome sequence for a tropical highland maize (Martinez De La Vega *et al.* 2008) possibly related to FV331, or by developing a BAC specific to this region of FV331.

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Fine Mapping and Haplotype Structure Analysis of a Major Flowering Time Quantitative Trait Locus on Maize Chromosome 10

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TABLE S1

List of markers developed in the frame of the fine-mapping approach

Name	Forward Primer	Reverse Primer	Reference sequence	Marker Information
cl3584.idp*	GGGTGGTGAGCGGTAATAAG	AGGTACCGGATACGGTGTATG	AY110491	8 bp IDP
2am.acpcr#1	AGTACCTCTTCCCTTTGAGG	GGAGAACATTGCTGAGAACC	CG888792	Allele competitive PCR
2am.acpcr#2	GTCTTCTCAGAGATGATTGCAGT (F286 specific) CACGACGTTGTAAAACGACGTCTTCTCAGAGATGGTTGCAG C (F331 specific with M13 tail)	CACTGTGTCCCAGAACTACGA	CG888792	2 consecutive PCR
cq.3pa	ATTGGTTGGTGCTAATGTGC	GCCTACAACTTGTATCTGAGGAT (F286 specific)CTCCTCAATGGTTCTTAGCTA	CG886830	Three primer assay
ch.idp*	AACAGAGGGATGGTCACACC	CAACAATAGCATCGACATGG	CC750108	9 bp IDP
be.dcaps	ATCCAGGAGATGATCATTGG	GCCTTTATTACTTTAGGATAT	CC450044	dCAPS (EcoRV)
bes.ssr11	ATTAGGAGCATCCTCAACTTCG	CTGGTGAACTAAAGCCAAAAGG	CC761612	SSR
bt.arms	AGCTGCAGACCCTACTAGTGCGT (F286 specific)GTTTGACGATCTGAGCATGGAGT	GACTTGTTGCTTCGTGTAATCAATG (F331 specific) ATGGTACATGATGGAACCTGAGGT	CG997512	ARMS PCR
dl.d	TGTAGCTCCTCCTTCTCAGC	TGTGTCTCTTGTGGTTCTTGC	CL008777	Dominant (0/1)
ec.d	CAAACTTTCAGGAACGTGGT	GGAACCTCTGGCTGCATAAC	CG704448	Dominant (0/1)
cz.d	CACCTACAGCAGTCCAAACG	GTTGAGGCTTTAGGGTGAGC	CG702371	Dominant (0/1)
da.d	TGTTGTTGCTAGTACCTCACG	GGCCACAGGATTAAGAATCAAC	CG833450	Dominant (0/1)
dx.idp*	GGCCCATCAACAATCTTAAA	CAAATGCGGTAGATCAGTCG	AC199625	IDP
t6.idp[1]*	TAAGCTTCACTGCCCTATGC	ACTTCAGGGATCTTTTGAGC	AC199625	Dominant (0/1)
t15.idp	AAAGTAGGCCGAAGACAAGC	ACTTACGTGCGCCATTGAA	AC199625	12 bp IDP
t14.d	CTCTTCCTCGACCCATAACC	GGATTCATCCATCAACTAGCC	AC199625	Dominant (0/1)
t16.idp	TCGCTCGATCTCTCAGAAAC	CACAAGAGGAAGTAGGGTTGC	AC199625	IDP

Most of these markers were specifically developed for the FV286xFV331 population. * indicate markers that we successfully tested on more diverse material. [1] This marker is dominant in the NIL population and codominant (INDEL) in other material.

TABLE S2

List of sequenced amplicons used for association mapping

Name	Forward Primer	Reverse Primer	Designed on
DX	GGCCCATCAACAATCTTAAA	CAAATGCGGTAGATCAGTCG	c0286M05 (AC199625)
Target18 (t18)	GACCTAGCTTGTCGTCTGATTG	AGGCTTCGTCATTCGGTTAC	c0286M05 (AC199625)
Target17 (t17)	TTCTGCTGCTTCGTCTCTGG	ATTTCGTGCACCAGAGTGTC	c0286M05 (AC199625)
Target16 (t16)	ATCACTGGCACCACTTATCC	TGTCTCCTATAAGCGCAACC	c0286M05 (AC199625)
EC[1]	CAAACTTTCAGGAACGTGGT	GGAACCTCTGGCTGCATAAC	c0171E08
Target1 (t1) [1]	AAAGGTAGACTAGGAGGGGTAGC	GGGAAGAACAGATCGAGGAG	c0171E08
Target15 (t15)	CAGAGGACGTGACGTAAAGC	CGGCTCTTTCTATTCTGTTCG	c0171E08
Target3 (t3) [1]	AAAGCTATCCGTCCGATCC	TTTGGCCCACTACTCTTTCC	c0171E08
Target6 (t6) [1]	TAAGCTTCACTGCCCTATGC	ACTTCAGGGATCTTTTGAGC	c0171E08
Target7 (t7) [1]	TCCGCTCTTCTAGCCCAGAC	AGCATGTCATAGAGGCTAGGG	c0171E08
Target9 (t9) [1]	GCCATGCGATATACTTACGC	CCTCCTCCGTTCTTATGTCC	c0171E08
Target10 (t10) [1]	TCTACGACTACGCGAGGAAG	CATGCTACACGGGTGGATAG	c0171E08
Target12 (t12)	CAACTTGTGTTATCTAGCGTTGC	AAGCTTCCCCATGATCTCG	c0171E08
CH	GACCCTGACTCACTTTGTCC	TAACACGACAAGCTGTTTGG	b0130H12.r (CC750108)

[1] These amplicons are equivalent to dominant markers in the NIL population.

TABLE S3

BLASTP results for genes predicted on BAC c0286M05 and c0171E08

BAC	Predicted gene	First Hit Accession	Annotation (species)	E. value	Comment
c0286M05	1*	EEH41564	hypothetical protein (*Paracoccidioides brasiliensis*)	0.94	
c0286M05	2*	XP_002446018	hypothetical protein (*Sorghum bicolor*)	3.00E-74	Third hit = ACA14488, GHD7 (Oryza sativa, Evalue=3e-30)
c0286M05	3	AF391808_7	RIRE2 orf3 (*Zea mays*)	6.00E-92	
c0286M05	4	ABQ44355	polyprotein (*Zea mays*)	0	
c0286M05	5	AAN40028	putative RIRE2 orf3 (*Zea mays*)	2.00E-11	
c0171E08	1	ABQ44355	polyprotein (*Zea Mays*)	6.00E-51	
c0171E08	2*	YP_379909	putative ATPase involved in DNA repair (*Chlorobium chlorochromatii*)	5.6	
c0171E08	3*	XP_002453913	hypothetical protein (*Sorghum bicolor*)	8.00E-56	
c0171E08	4*	ACN36685	unknown (*Zea mays*)	3.00E-53	
c0171E08	5	ACN34364	unknown (*Zea mays*)	4.00E-109	TE-related
c0171E08	6*	NP_001142422	hypothetical protein (*Zea mays*)	0.008	
c0171E08	7*	XP_002447409	hypothetical protein (*Sorghum bicolor*)	8.00E-34	Other hits indicate similarity with Plant invertase/pectin methylesterase inhibitor
c0171E08	8	ABF67936	Milt putative polyprotein (*Zea mays*)	1.00E-08	

* These genes are reported in Figure 3 : c0286M05#1 = No Sign. Hit 1; c0286M05#2 = *ZmCCT*; c0171E08#2 = No Sign. Hit 2; c0171E08#3 = Hyp. 1; c0171E08#4 = Hyp. 2; c0171E08#6 = Hyp.3; c0171E08#7 = PMEI

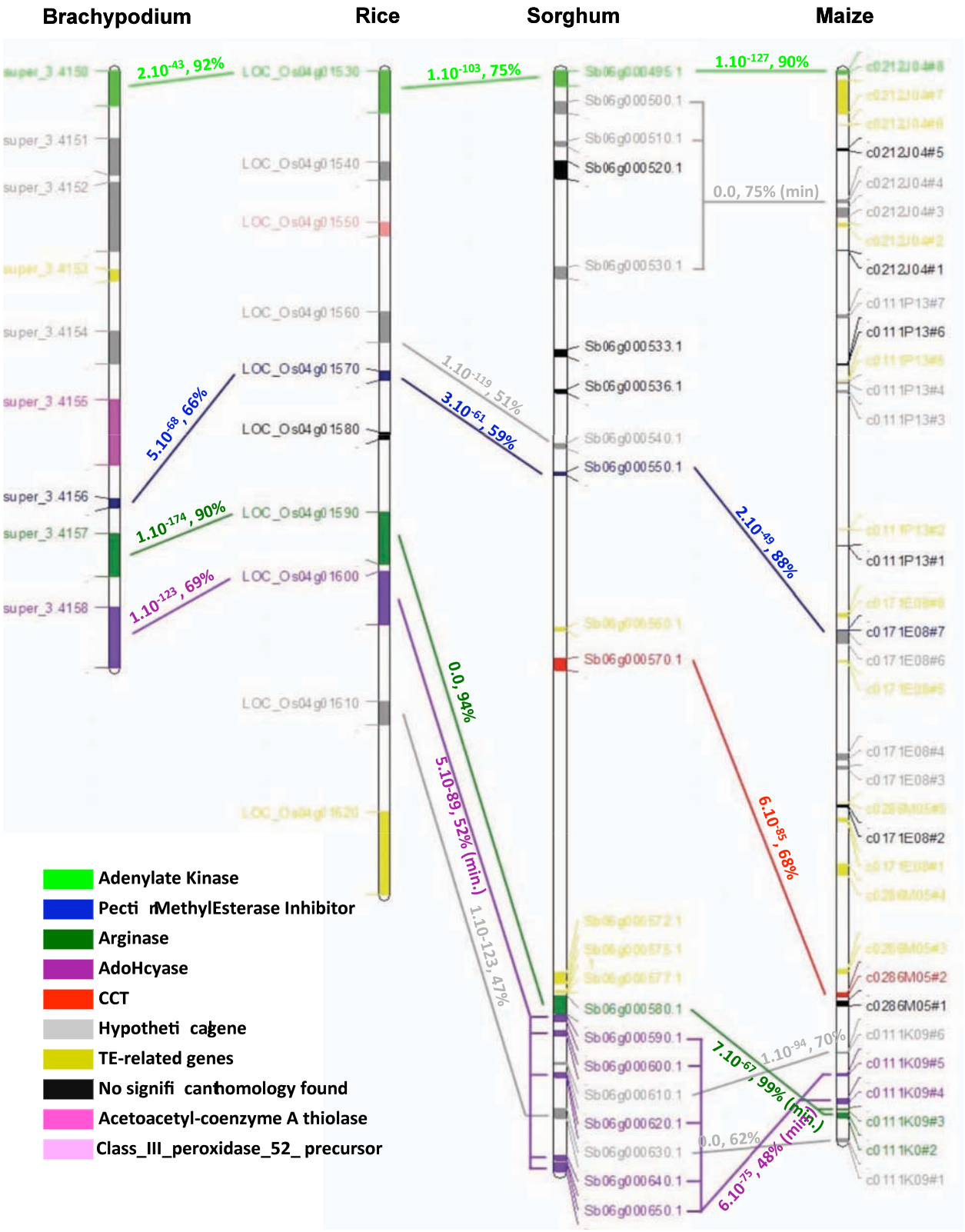


FIGURE S1.—Synteny between the targeted region of maize chromosome 10 and regions of 600 kb on rice chromosome 4, 45 kb on Brachypodium supercontig 3, and 270 kb on top of sorghum chromosome 6. For the sake of readability, the sorghum region has been enlarged and the maize region has been reversed upside-down.

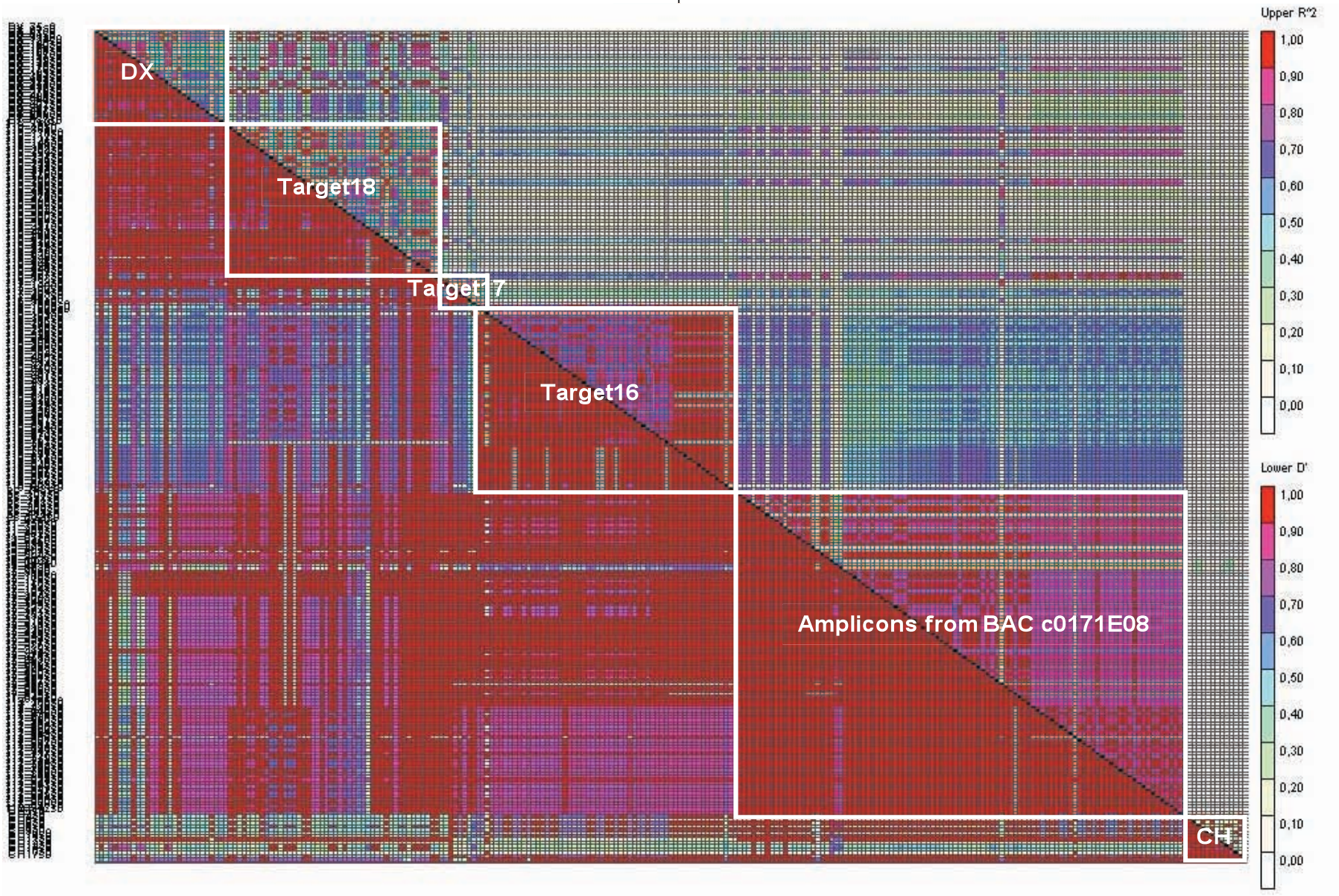
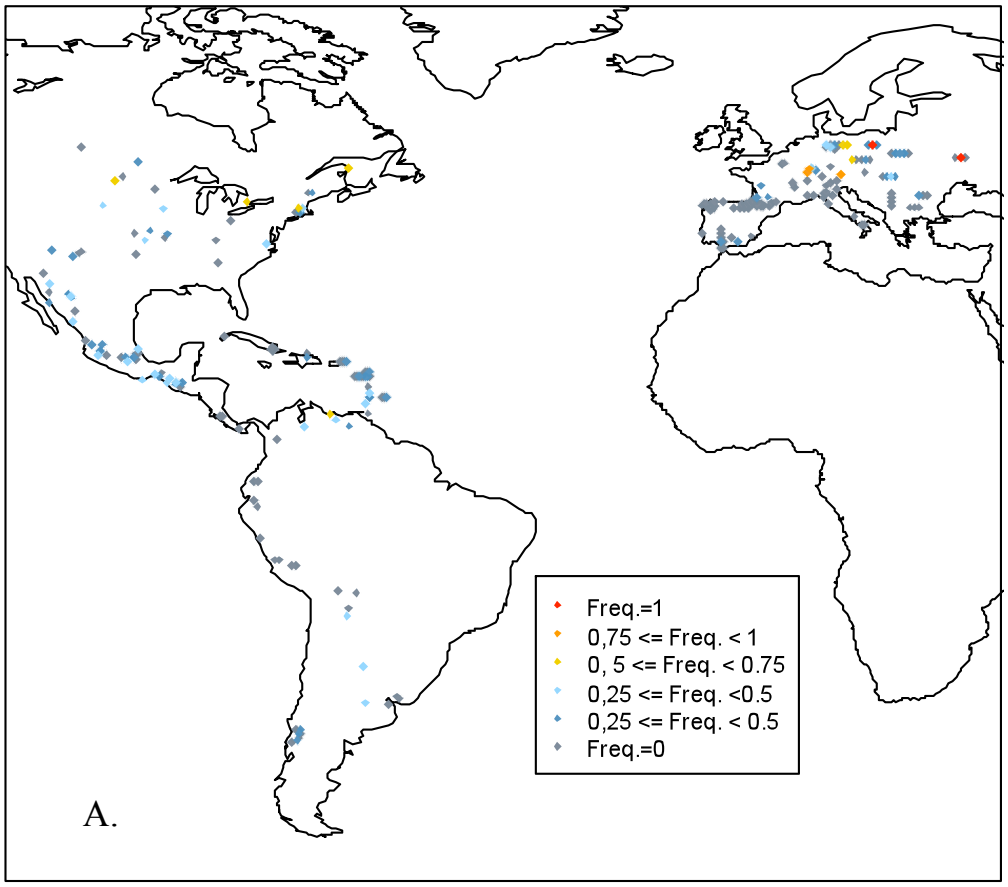
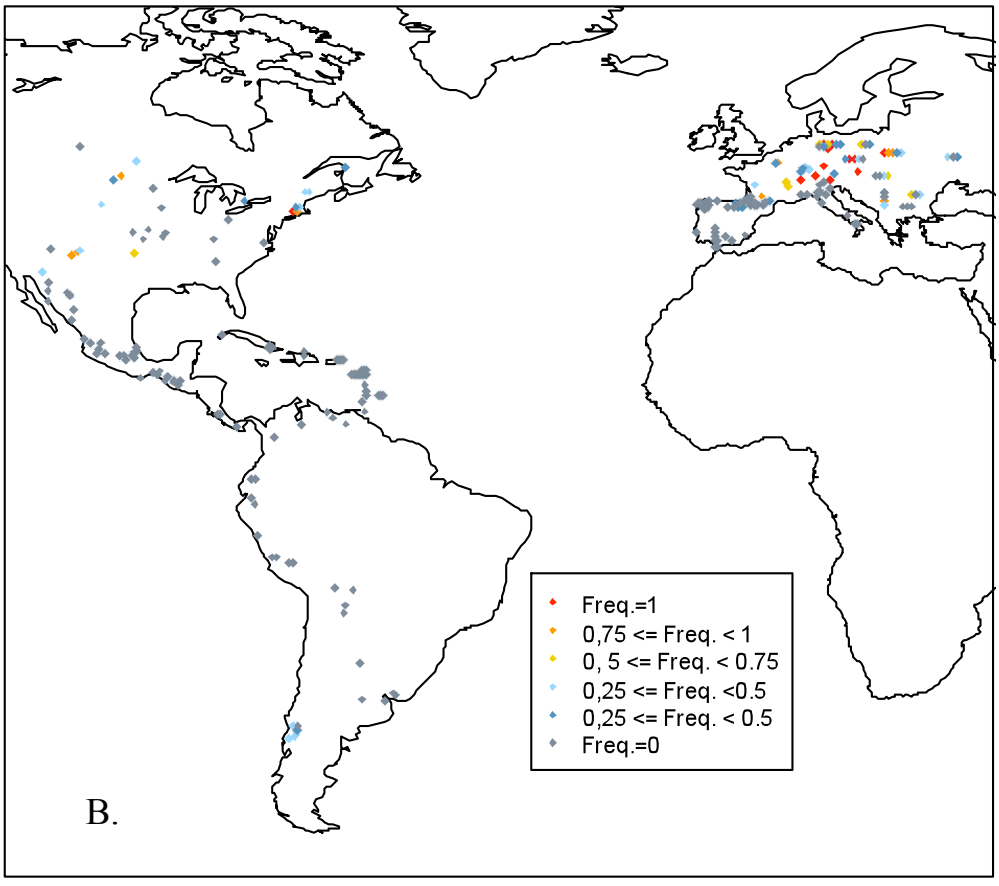


FIGURE S2.—Linkage disequilibrium over the QTL region. Note that *Target16* (*t16*) polymorphisms are in higher LD with those from BAC c0171E08 than with those from *Target17*, *Target18* and *DX*, although *Target16* is located ~50kb from BAC c0171E08 and *DX*/*Target16* interval is only 5 kb long.

Dent Allele Frequency



Flint Allele Frequency



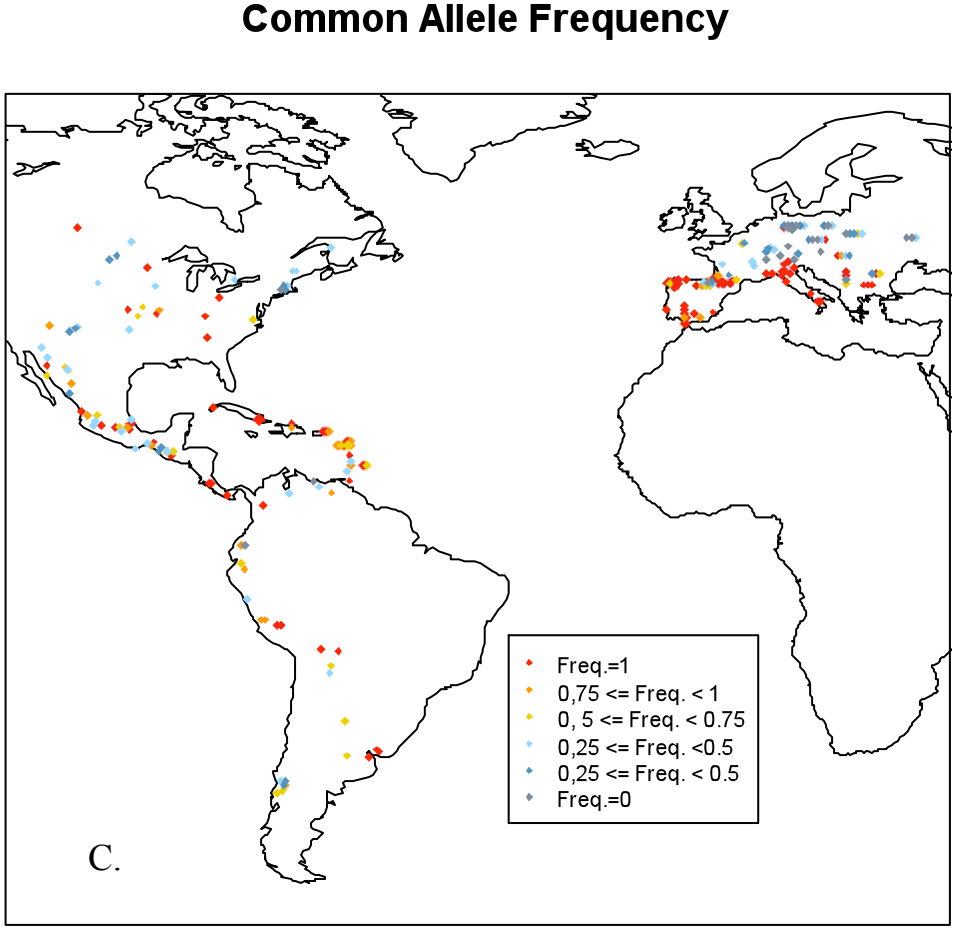


FIGURE S3.—Geographical distribution of (A) the "dent", (B) "flint" and (C) "common" alleles obtained through genotyping of marker *t6.idp* across a panel of 256 European and American landraces.