Mapping of DNA Markers Linked to a Thermosensitivity Gene in Sorghum

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Abstract

In sorghum (*Sorghum bicolor* Moench), flower initiation is reported to be controlled by the thermosensitivity locus T. Flower initiation by T-(TT or Tt) genotypes is delayed by exposure to temperatures over 20℃ under long-day conditions. DNA markers associated with this trait were isolated by bulk segregant analysis with amplified fragment length polymorphism (AFLP) markers. A total of 13 dominant and 1 co-dominant markers were identified from among the 1024 AFLP markers tested, and 7 of them could be assigned to a linkage map. Linkage analysis using 33 individuals from a BC_1F_2 population, which were classified by phenotype as either tt or T-, showed that the T locus for thermosensitivity was distal to the marker AFLP16. Quantitative trait locus (QTL) analysis also showed that the sorghum thermosensitivity trait is monogenic. Restriction fragment length polymorphism (RFLP) analysis was carried out to estimate a more precise location of AFLP16 by comparing it with 145 RFLP markers already mapped in the authentic sorghum genetic map. In the RFLP analysis, the T locus was mapped to sorghum chromosome 6, 4.0 cM from AFLP16.

Introduction

Flowering is the developmental turning point between the vegetative and reproductive phases in plants. The timing of flower initiation is critical for reproductive success, and the relationships between developmental stage and environmental factors such as photoperiod and temperature have been studied extensively. In sorghum (*Sorghum bicolor* Moench), which is a facultative short-day plant, the loci Ma_1 to Ma_4 [13], Ma_5 and Ma_6 [14], T [17], and D_1 and D_2 [18] control the relationships between flower initiation (FI) and environmental factors such as photoperiod and temperature. Among these, the T locus controls the thermosensitivity of FI. The FI of T-(TT and Tt) genotypes is accelerated by exposure to temperatures lower than 20℃ under long-day conditions (over 12.5 h), whereas the opposite phenomenon occurs at temperatures over 20℃ (i.e., FI of T-genotypes is delayed)[17]. The characteristics controlled by the T locus are similar to those of genes controlling vernalization in winter-annual plants such as wheat, barley, and *Arabidopsis thaliana*, although exposure to temperatures below 20℃ is not essential to induce FI in sorghum.

High-density restriction fragment length

polymorphism (RFLP) linkage maps have been constructed for gramineous species such as maize, wheat, and rice [1,3,6]. In sorghum, an RFLP linkage map was constructed using the mapped rice RFLP markers [9]. In addition, high-density genetic maps based on amplified fragment length polymorphism (AFLP), RFLP, and simple sequence repeat (SSR) markers have been constructed in sorghum [8]. To provide the information necessary to use the T gene to improve FI in crops, we conducted a detailed mapping study. We performed bulk segregant analysis to identify AFLP markers linked to the thermosensitivity gene (T) controlling FI in sorghum and mapped the chromosomal locations of these AFLP markers in greater detail by using RFLP analysis. We mapped the T locus in two ways: as a single gene and as a QTL. The mapping population was a BC_1F_1 population constructed by making crosses of ($TT \times tt$) × tt.

Materials and Methods

Plant material

Sorghum has at least nine loci for determining FI relative to photoperiod and temperature, including Ma_1 to Ma_4 [13], Ma_5 and Ma_6 [14], T [17], and D_1 and D_2 [18]. To construct a mapping population for T in a genetic background that was simplified with respect to the other FI loci, a BC_1F_2 population was established by backcrossing Daikoukaku ($ttd_1d_1D_2D_2$) to an F_1 hybrid (Natuibuki, $Ttd_1d_1D_2D_2$) produced by crossing MS175 ($TTd_1d_1D_2D_2$) and Daikoukaku [17]. The progeny of this backcross (BC_1F_1) were then selfed to generate the BC_1F_2 population. The BC_1F_2 plants were grown at 15-h daylength at 25℃ in a natural-light greenhouse to express the effect of T on the delay of FI. The days-to-emergence of the flag leaf (DEFL) in the BC_1F_2 population (90 plants) exhibited a segregation ratio that was not significantly different from 3 late: 1 early using chi-square, as would be expected for a single dominant gene controlling late flowering [17].

AFLP analysis

To identify AFLPs linked to the thermosensitivity trait, two bulk samples were prepared, each consisting of 12 individuals from the BC_1F_2 population having either late or early DEFL. To map the thermosensitivity gene as a Mendelian single gene, 15 individuals with tt and 18 with T- were used. DNA was isolated from 8 g of fresh leaf tissue per individuals by the CTAB method. AFLP analysis for the bulk DNA was performed following the procedure of Vos *et al.* [19] with some modifications. DNA digestion and ligation was performed using the fluorescent dye-based AFLP Plant Mapping Kit from Perkin Elmer Applied Biosystems (Foster City, CA, USA). Two pre-selective amplification steps were performed. First, amplification with an *Eco*RI (E) primer with the sequence 5′-GACTGCGTACCAATTC-3′ (E-000) and an *Mse*I (M) primer with sequence 5′-GATGAGTCCTGAGTAA-3′ (M-000) was performed to reduce nonspecific background on the polyacrylamide gels. This was followed by amplification with a second set of *Eco*RI (E-000 + A) and *Mse*I (M-000 + C) primers, each containing one selective nucleotide. Selective amplification was then conducted using the pre-amplified products and selective *Eco*RI and *Mse*I primers, each of which had three selective nucleotides. All of the *Eco*RI selective primers were 5′-end-labeled with the fluorescent dye FAM (Amersham Pharmacia Biotech, Tokyo, Japan). A total of 32 primer combinations of 4 E-primers with the 3′ ends AAC (e02), AAG (e03), ACA (e05), ACC (e06), and 8 M-primers with the 3′ ends CAA (m17), CAC (m18), CAG (m19), CAT (m20), CTA (m29), CTC (m30), CTG (m31) and CTT (m32) were used to construct the linkage map. Each AFLP marker was given a suffix according to its position from the top of the gel (i.e., e02m17-1 was above e02m17-2 on the gel). To obtain good separation of the amplified DNA fragments, buffer gradient electrophoresis was conducted with $1 \times$ TBE (100 mM Tris, 100 mM boric acid, 2 mM EDTA, pH 8.0) as the cathode buffer (–) and $1 \times$ TBE plus 0.5 M sodium acetate as the anode (+) buffer [12]. The amplification products were run on a 40-cm, 5% denaturing polyacrylamide gel at 1100 V for 2 h 45 min. After electrophoresis, the gels were scanned in a Molecular Imager (Bio Rad, Hercules, CA, USA).

RFLP analysis

To more accurately map the AFLP markers and the T locus, the same BC_1F_2 population and Daikoukaku parent as in the AFLP analysis were used for RFLP analysis.

DNA was isolated from 8 g of fresh leaf tissue by the CTAB method, and bulk DNA samples were made from pools of 10 individuals predicted to carry either *tt* or *T*- based on the position of the AFLP16 marker band. (The AFLP16 marker was found to be the closest to the locus controlling days-to-heading and thermosensitivity; see Results).

Restriction enzyme treatment, electrophoresis, and Southern hybridization analysis were carried out according to the method of Kurata *et al.* [6]. DNA probes created by the Rice Genome Program (RGP) of MAFF were used as hybridization probes (http://rgp.dna.affrc.go.jp/ publicdata/geneticmap2000/index.html). Linkage analysis was performed by using the F_2 model in MAPMAKER/ EXP 3.0 [7] and MAPMAKER/QTL ver. 1.1 [10]

Results and Discussion

The BC_1F_2 population, which was raised at 15-h daylength and constant 25℃, segregated for early and late flowering, as assessed based on DEFL (Fig. 1). The values of the BC_1F_2 individuals flanked 60 days, which was the mean of the parental DEFLs.

AFLP analysis

AFLP markers associated with the thermosensitivity gene were identified by screening 4 bulk DNA samples (E-1, E-2, L-1, and L-2) each made from 6 individuals predicted to carry *tt* (E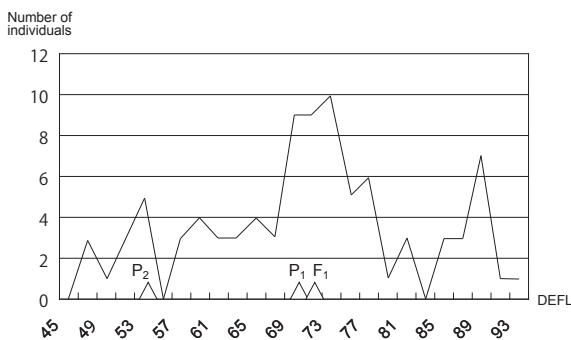) or *T*-(L) based on DEFL. From 1024 primer combinations, we identified 9 markers in a coupling phase with *T* (i.e., not observed in bulks E-1 and E-2 but present in bulks L-1 and L-2; Fig. 2A), 4 markers in repulsion phase with *T* (i.e., not observed in L-1 and L-2 but present in E-1 and E-2; Fig. 2B), and 1 co-dominant marker showing size polymorphism between the L and E groups (Fig. 2C). Using these 14markers, AFLP analysis was performed on 90 individuals of the BC_1F_2 population, and a genetic linkage map was constructed with 7 of the markers.
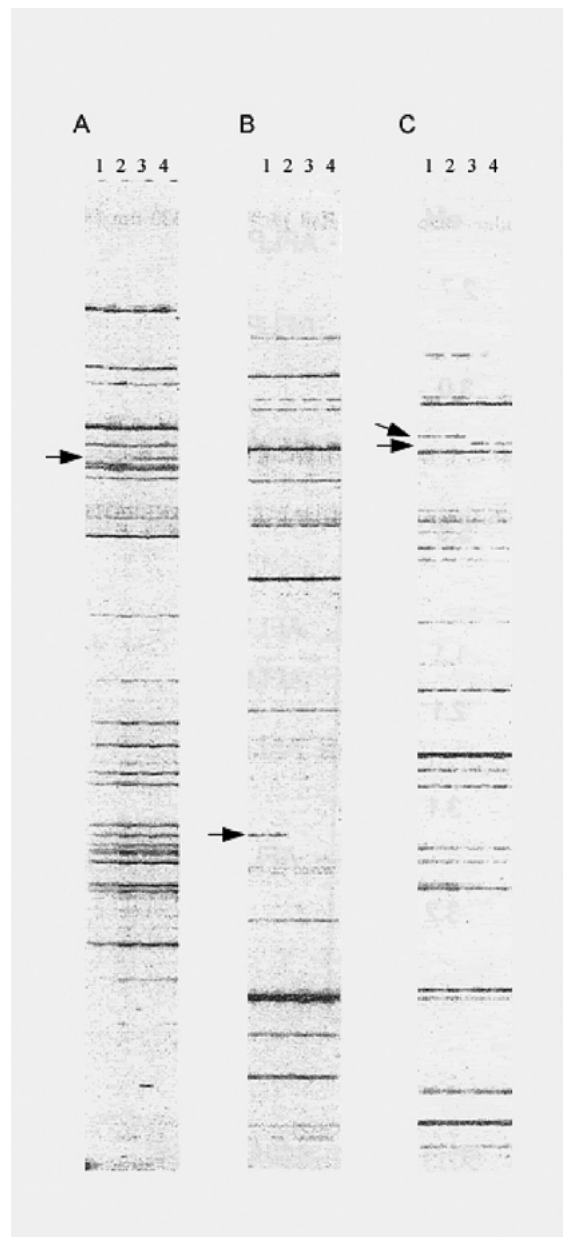

Fig.2. Examples of band types observed in AFLP analysis. A= coupling-phase marker (arrowhead=AFLP), B=repulsion-phase marker (arrowhead=AFLP11), C=co-dominant marker (arrowhead=AFLP16) Lane 1=bulk E-1 (*tt*), Lane 2=bulk E-2 (*tt*), Lane 3=bulk L-3 (*T*-), Lane 4=bulk L-4 (*T*-).

Fig.1. Segregation of days-to-emergence of flag leaf (DEFL) in a BC_1F_2 population derived from backcrossing of Daikoukaku ($ttd_1d_1D_2D_2$) to Natuibuki ($Ttd_1d_1D_2D_2$), an F_1 between MS175 ($TTd_1d_1D_2D_2$) and Daikoukaku. P_1=MS175, P_2=Daikoukaku, F_1=Natuibuki.

To map the thermosensitivity locus as a Mendelian single gene, a genetic linkage map was constructed using 15 individuals with tt and 18 with T-, after omitting 57 ambiguous individuals. The result showed that the thermosensitivity locus was located 3.2 cM from the AFLP16 marker (Fig. 3A). The very low frequency of polymorphic AFLP markers (15 out of 1024=1.5%) seemed to be due to the lack of polymorphism in the BC_1F_2 population.

Since traits associated with heading are generally considered to be quantitative [20], we used QTL interval mapping to investigate the precise position of the thermosensitivity locus by treating it as a quantitative trait. A QTL with LOD=14.2 was found at the location of AFLP16, providing further evidence that the thermosensitivity gene controls DEFL under long day length at temperatures over 20℃ behaves as a single gene and is located distal to AFLP16 (Fig. 3B). Although AFLP16 was located at the end of the linkage group and the thermosensitivity gene was not flanked on both sides by AFLP markers, the result of the QTL analysis confirmed the location of this gene.

RFLP analysis

RFLP markers were screened for their ability to detect polymorphism in MS175, Daikoukaku, and the four bulk DNA samples (E-1, E-2, L-1, and L-2) after digestion by eight common restriction enzymes. A set of 145 DNA markers out of approximately 607 RFLP markers in a recently constructed sorghum genetic map [9] were tested, and 79 of them were polymorphic (54.5%). Then, we compared the RFLP alleles in the parental varieties with those in the bulk populations to estimate the map location of the thermosensitivity gene. The markers were classified into three categories. The first category contained markers for which the Daikoukaku allele was found within the early-flowering bulk samples and the MS175 allele was found in the late-flowering bulk samples. The second category had both parental alleles in both bulk samples, and the third category contained markers for which the allele in both bulk samples was the same as that in Daikoukaku, showing that each of these regions was fixed for the genetic segment from Daikoukaku. Since markers of the first type were located on the chromosome 6 [9], the thermosensitivity gene was tentatively placed on the chromosome 6.
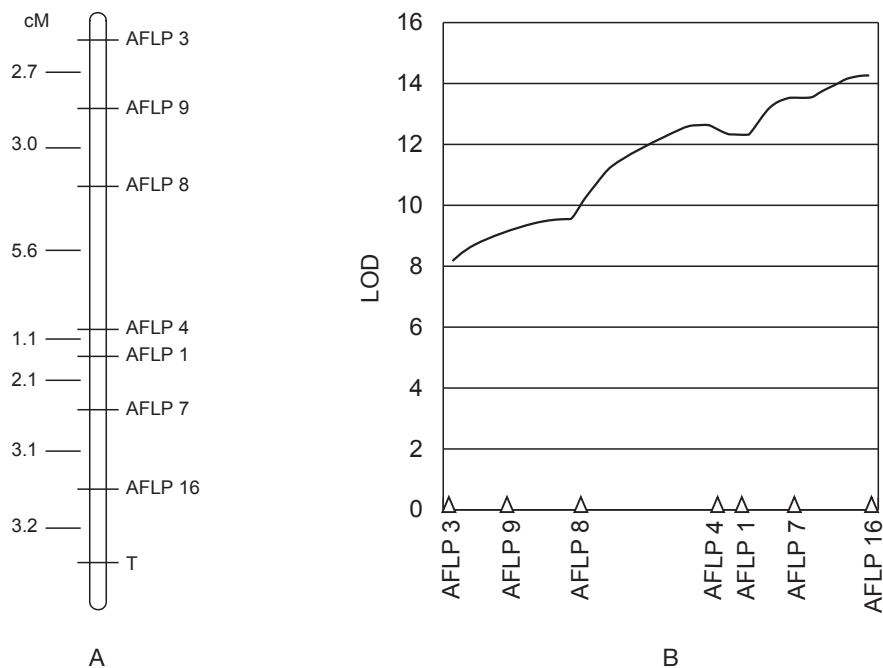

Fig.3. AFLP linkage map (A) and LOD scores from QTL analysis (B) of a sorghum BC_1F_2 generated from a backcross of Daikoukaku ($ttd_1d_1D_2D_2$) to (Natuibuki, $Ttd_1d_1D_2D_2$), an F_1 between MS175 ($TTd_1d_1D_2D_2$) and Daikoukaku [18].

A genetic linkage map consisting of the 8 RFLP markers in the first category and AFLP16 was constructed by using DNAs from 15 individuals with *tt* and 18 individuals with *T-*, which were the same individuals as in the AFLP analysis. The result showed that the thermosensitivity locus *T* was located 4.0 cM from AFLP16 (Fig. 4A). Interval mapping of the locus was also carried out using DNA of all 90 individuals to investigate the position of the thermosensitivity by treating it as a quantitative trait. A QTL showing LOD of 15.7 was found at AFLP16, providing further evidence that the thermosensitivity trait is monogenic (Fig. 4B).

The integrated relationship between the QTL for DEFL, the linkage map of the thermosensitivity gene, and a previously constructed sorghum RFLP linkage map [9] are shown in Fig. 4C. As shown in Fig. 4C, neither the thermosensitivity locus nor the QTL of DEFL could capture the locus, because it was beyond the last marker on either map. In this study, both bulk samples in BC_1F_2 showed the Daikoukaku pattern in the region from S10644 to the end of chromosome, indicating that it was fixed for the genomic region from Daikoukaku. Since the mapping population segregated for thermosensitivity, the *T* locus could not reside in this fixed region (Fig. 4C),
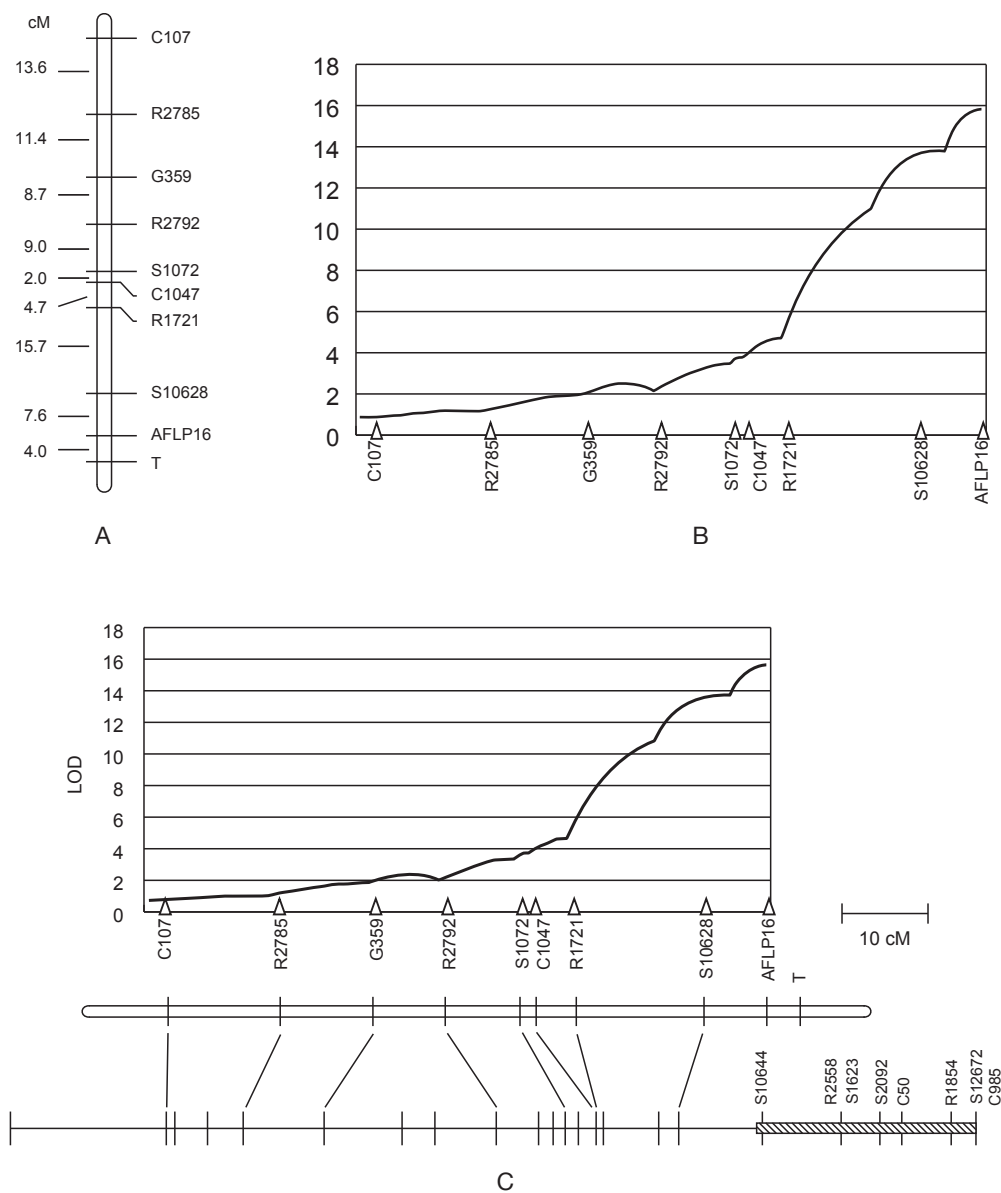

Fig.4. RFLP linkage map (A), LOD scores from QTL analysis (B), and their relationships to a published RFLP linkage map [9] (C) based on mapping in a sorghum BC_1F_2 population generated from backcrossing Daikoukaku ($ttd_1d_1D_2D_2$) to (Natuibuki, $Ttd_1d_1D_2D_2$), an F_1 between MS175 ($TTd_1d_1D_2D_2$) and Daikoukaku [18].

and because the region did not segregate for any of the markers we tested, we could not construct a complete genetic linkage map. However, in a previously reported sorghum linkage map [9], 6 RFLP markers were distal to marker S10628, which we mapped in this study and is the closest RFLP marker to the thermosensitivity locus; based on this information, the T locus appears to be located between RFLP markers S10644 and R2558.

RFLP marker is too laborious to use in conventional breeding and other more convenient DNA markers should be created after further narrowing down the flanking region. Then the thermosensitivity gene itself would be isolated and its introduction into flowering crops by genetic transformation would be very meaningful in various aspects. Because of its unique function, flower initiation of transgenic plant carrying the thermosensitivity gene is delayed by exposure to over 20℃ under long-day condition. Recently, Japanese rice culture is endangered by the occurrence of white-back kernel induced by high temperature damage in summer. Retardation of heading in high temperature sensitive rice excellent cultivars by the thermosensitivity gene would be one of possibilities to circumvent the damage. In many horticultural plants, the control of flowering time is very essential and the thermosensitivity gene could be also used practically.

By the way, Sorghum is considered to be a good candidate for biomass production, and transgenic approaches to improvement of this trait are expected. However, gene dispersal by pollen into Johnsongrass [11,15,16], which is a notorious perennial rhizomatous weed closely related to sorghum, is likely to raise concerns about the environmental safety of transgenic sorghum. If the thermosensitivity gene (T) is cloned and modified to enable control of flowering of sorghum, this might be a method to prevent hybridization with unwanted species. A high-density genetic recombination map of sequence-tagged sites for sorghum is available [2] so it seems feasible to determine the position of this thermosensitivity gene in the near future with the aid of BAC-based fluorescent *in situ* hybridization [4,5].

Acknowledgments

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ソルガム温度感応遺伝子に連鎖する DNA マーカーのマッピング

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摘　　要

　　ソルガムの花芽分化は，長日条件下で 20 度以上の温度により遅延する温度感応遺伝子により制御されていると言われる。AFLP を用いたバルク解析法によりソルガム温度感応遺伝子の DNA マーカーを単離した。1024 個の AFLP マーカーから 13 個の優性マーカーと 1 個の共優性マーカーが得られ，それらのうち 7 個が連鎖地図に割り当てられた。tt または T の表現型を示す 33 個体の BC_1F_2 集団による連鎖解析の結果，温度感応を司る T 座位は第 16AFLP マーカーの末端にあった。QTL 解析の結果もまた，温度感応性遺伝子が単一遺伝子であることを示した。第 16AFLP マーカーのより詳細な位置を特定するため，すでにマッピングされている 145 個の RFLP マーカーとの比較を行った結果，温度感応遺伝子の座位は第 6 染色体上にあり，第 16AFLP マーカーからの距離は 4.0cM と推定された。

キーワード：AFLP，花芽分化，ソルガム，温度感応遺伝子