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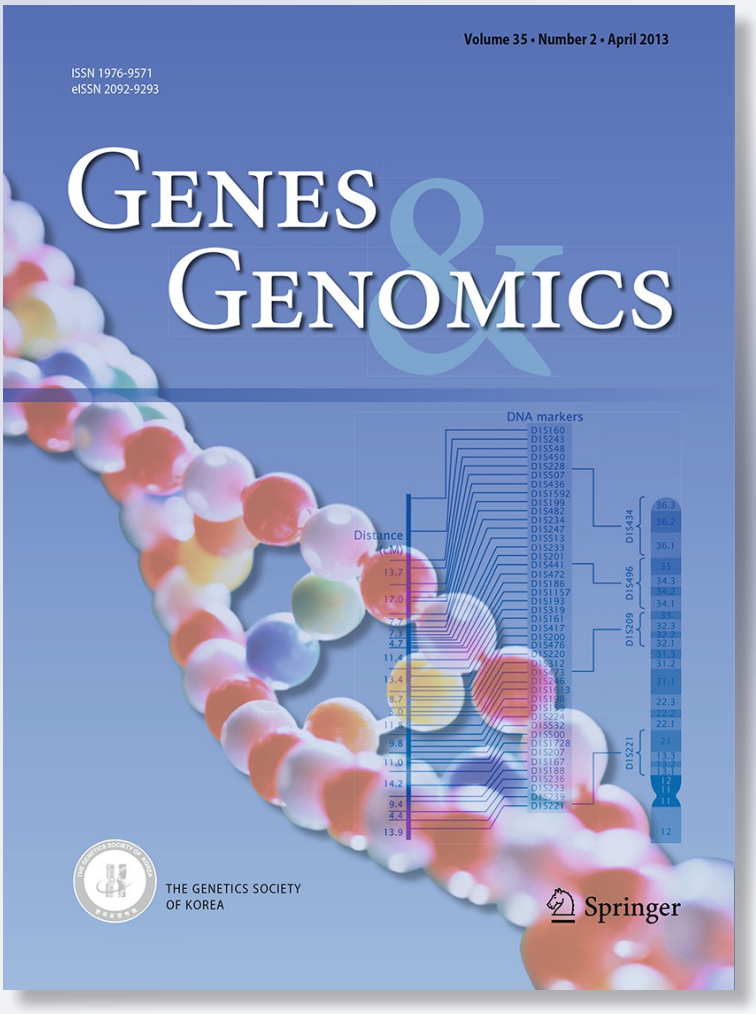

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RESEARCH ARTICLE

Nucleotide diversity of the upstream region of the putative MADS-box gene controlling soybean maturity

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Abstract MADS-box genes are involved in plant reproductive development. However, the role of gene nucleotide diversity in soybean flowering and maturity remains unknown. Therefore, in this study, the distribution of DNA polymorphisms in the putative MADS-box gene located near the quantitative trait loci (QTL) for flowering time and maturity was targeted for association analysis using *Glycine max* (cultivated soybean) and *Glycine soja* (wild soybean). Sixteen single nucleotide polymorphisms identified in the upstream region of the putative MADS-box gene around QTL *Pod mat* 13-7 and *Fflr* 4-2 on chromosome 7 were found to be highly associated with maturity in soybean. The genetic diversity between cultivated soybeans and the wild relative was comparable, although the early maturity group (EMG) was less diverse than the late maturity group (LMG) of the cultivated soybean. Population size changes of the MADS-box gene in this soybean germplasm appeared to result from non-random selection. A selective pressure seemed to act on this gene in the EMG, while the LMG and *G. soja* were in genetic equilibrium. Neutrality tests and the constructed neighbor-joining tree indicate that the EMG of *G. max* has experienced strong artificial selection for its domestication and genetic improvement.

Keywords *Glycine max* · *Glycine soja* · MADS-box gene · Nucleotide diversity · SNP (single nucleotide polymorphism)

Electronic supplementary material The online version of this article (doi:10.1007/s13258-013-0075-7) contains supplementary material, which is available to authorized users.

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Introduction

As a short-day crop, soybean depends on a variety-specific day length for the development of flowering initiation (Chen et al. 2004). The mechanisms that control the timing of flowering and maturity are crucial (Mansur et al. 1996) for the production of soybean seeds. The time of flowering and maturity in soybean varies among genotypes. Thus, understanding soybean maturity is essential for the selection of appropriate varieties with specific geographical adaptations.

For the last few decades, candidate genes responsible for traits of interest could not be identified due to the limitations of quantitative trait loci (QTL) mapping on broad genomic regions. However, a recent consensus soybean genetic map composed of various markers (Choi et al. 2007) has been constructed. More than 100 QTLs for traits related to soybean reproduction are now publicly accessible (http://soybeanbreederstoolbox.org/), including QTLs for flowering and maturity. Several QTL analyses for reproductive traits in soybean were progressively investigated to resolve questions of phylogeny (Lee et al. 1996; Orf et al. 1999; Tasma et al. 2001; Khan et al. 2008).

With the benefit of revolutionary sequencing technology, the genome sequencing projects of several plant species have published sequences and provided user-friendly web browsers. The genomes of cultivated soybean [*Glycine max* (L.) Merrill] and wild soybean (*G. soja* Sieb. and Zucc) have been sequenced (Kim et al. 2010; Lam et al. 2010; Schmutz et al. 2010). The soybean genome sequence is available on the Phytozome website (http://www.phytozome.net), where the sequence-based markers linked to specific QTLs can be located physically on the chromosomes (Schmutz et al. 2010). Therefore, a candidate gene with a potentially relevant role in a trait of interest, such as flowering and maturity, can be easily browsed on annotated genes of soybean and other sequenced genomes.

Several gene families influence the regulation of plant developmental processes. These include *GIGANTEA*, *CONSTANS*, and *FLOWERING LOCUS* (Li et al. 2008), specifically *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (Samach et al. 2000) and several Arabidopsis loss-of-function mutations of *FLOWERING LOCUS C* (Michaels and Amasino 1999). Other genes, designated MADS-box genes, have been well characterized. MADS-box genes encode a highly conserved N-terminal DNA-binding domain and include a family of transcription factors that are involved in various aspects of flowering plant development, such as the development of roots, flowers and fruits (Ng and Yanofsky 2001). Several MADS-box genes have been studied in plant species, including *MADS AFFECTING FLOWERING1* (Ratcliffe et al. 2001) and *AGAMOUS-LIKE24* (Michaels et al. 2003). The direct effect of *Le-MADS-RIN* on maturity in tomato was also investigated (Vrebalov et al. 2002). Sequence variability of the MADS-box genes in other plants, for instance *Zea mays*, *A. thaliana*, and *Brassica oleraceae* (Zhao et al. 2011), as well as the phylogeny of the MADS-box multigene family in eukaryotes and in lineages of angiosperms and gymnosperms (Becker et al. 2000), has been well investigated.

In soybean, numerous genes that control reproductive stages have been identified. The eight *E*-series genes from *E1* to *E8* show differential sensitivity to light quality and photoperiod, in which, dominant E alleles determine late flowering and maturity (Cober et al. 1996). Under natural day lengths, the dominant E alleles tend to delay flowering time and maturity, although the impact of each gene may vary (Tasma et al. 2001). Recently, MADS-box genes have been isolated from soybean (Han et al. 2010), and twelve MADS-box genes likely participate in soybean flowering, including *APETALA1* have been identified (Chi et al. 2011). However, the role of the MADS-box genes' nucleotide diversity on the reproductive stages of soybean remains to be elucidated.

Since MADS-box genes play critical roles in the gene networks that control plant development, a better understanding of the phylogeny of the soybean MADS-box genes may improve the understanding of soybean evolution and genetic relationships. Thus, based on the hypothesis that single nucleotide polymorphisms (SNPs) in the putative MADS-box genes surrounding QTLs for flowering and maturity are associated with the traits of interest, this study investigated the nucleotide variation of the gene in *G. max* and *G. soja*.

Materials and methods

Plant materials and DNA extraction

A total of 56 *G. max* and 20 *G. soja* genotypes originating from Asia, Europe, America and Africa were used in this study. The soybean genotypes were obtained from the U.S. Department of Agriculture (USDA) soybean germplasm collection. With respect to maturity, the *G. max* genotypes were classified into early maturity group (EMG) and late maturity group (LMG). EMG included 28 genotypes with MG-I (maturity group of I) and earlier, while LMG comprised 28 genotypes showing MG-V and later. The origin and maturity groups of the soybean genotypes used in this study are listed in Supplementary Table 1.

Young and healthy leaves of soybean were harvested for DNA extraction. Genomic DNA was extracted according to Shure et al. (1983).

Sequence identification of the target genomic region

Based on the linked position of the MADS-box gene and the QTLs for maturity and/or flowering, the targeted loci were identified by browsing the *G. max* genome sequence (Glyma1.01) at the Phytozome website (http://www.phytozome.net/soybean). The locations of the MADS-box genes were searched using a MADS-box specific domain coding sequence. QTLs for three traits of interest (first flower, flowering time and pod maturity) with high phenotypic variation ($R^2 > 10$) and logarithm of odds (LOD) scores higher than 5.0 were selected using Soybean Breeder's Toolbox (http://soybeanbreederstoolbox.org/). Principally, phenotypic variation underlines heritable genetic variation, while LOD is to estimate whether two gene loci to be inherited together as a package. These higher R^2 and LOD score give higher confidence of heritability and evidence for linkage being observed does not occur by chance (Collard et al. 2005).

Several simple sequence repeat (SSR) markers associated with the QTLs were found in the region of the chromosomes using the Basic Local Alignment Search Tool (*BLAST*) between the markers sequences searched against the *G. max* genome sequence. The putative MADS-box

genes located relatively close to the QTLs for first flower, flowering time and pod maturity were chosen for further PCR amplification and sequencing. To confirm the potential participation of these genes in the control of flowering and/or maturity, the flanking sequences (~ about 10 Kb) of the QTLs were aligned against all GenBank entries using the BLAST algorithm. Any putative genes were also investigated with the FGENESH software (http://linux1.softberry.com/berry.phtml) using the dicot plant option (Arabidopsis).

Primer design and PCR amplification

To amplify the putative MADS-box genes and their upstream sequences, 22 primer sets were designed based on William 82 sequence that was available from the Joint Genome Institute soybean database. The primers were generated using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), and primer quality was confirmed with the GeneRunner tool version 3.05 (Hastings Software, Hastings on Hudson, N.Y., USA). The primer sequences are listed in Supplementary Table 2.

The designed primers were initially tested for SNP discovery using four soybean genotypes in each maturity group, EMG (M1, M2, M3 and M4) and LMG (M37, M38, M39 and M40). Polymerase chain reaction (PCR) was performed with a Tetrad Thermal Cycler (MJ Research Inc., Watertown, MA, USA), and the reaction consisted of 50 ng of genomic DNA, 3.2 pmol of forward and reverse primers, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 1 × reaction buffer [10 mM Tris–HCl (pH 8.3), 50 mM KCl], and 1.6 U of *Taq* DNA polymerase (VIVAGEN, Sungnam, Korea) in a total reaction volume of 50 μl. The PCR reactions were performed with an initial denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 50–70 °C for 30 s and 72 °C for 1 min, and a final extension of 72 °C for 1 min. The primer sets possessing SNPs detected among the eight EMG and LMG genotypes were used for further amplification using the remaining 48 genotypes.

Sequencing analysis

The PCR products composed of a single band were purified with exonuclease I and shrimp alkaline phosphatase. The cleaned PCR product was directly sequenced using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). One initial amplification primer and an internal sequencing primer were used for sequencing. The sequence product was run on an ABI 3730xl DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Twenty wild soybeans (*G. soja*) were amplified and sequenced using only the primer sets that exhibited significant genetic diversity associated with maturity in the *G. max* genotypes. DNA amplification and sequencing analysis of the MADS-box genes in the *G. soja* accessions was conducted in the same manner as for *G. max*.

Sequence and data analysis

The resulting sequences in ABI trace files were aligned using SeqScape software v2.0 (Applied Biosystems, Foster City, CA, USA) to identify the SNPs. The association between SNP allele frequencies and the soybean maturity group (MG) was evaluated using standard contingency tests for 2 × 2 tables (two alleles, LMG vs. EMG). *P* values were determined via an approximate calculation on TASSEL version 2.1 (http://www.maizegenetics.net/tassel). Molecular population genetics statistics were estimated using DNASP 5.10.01 (Librado and Rozas 2009) and MEGA4 (Tamura et al. 2007). This included nucleotide diversity (π) (Tajima 1989), the expected heterozygosity per nucleotide site and nucleotide polymorphism (θ) (Watterson 1975), and the number of polymorphic sites in a genotypic sample corrected for sample size.

To test the neutrality of the polymorphisms, Tajima's D statistical test was used (Tajima 1989). This test considered the difference between theta Watterson (θ) and nucleotide diversity (π). The coalescence process defined by Fu and Li's D* and F* statistical tests (Fu and Li 1993) was used to evaluate evidence of the neutral theory of molecular evolution. A neighbor-joining tree was built using PHYLIP version 3.68 with slight modification (Felsenstein 1993). The sequence alignment for PHYLIP was performed with ClusterX version 1.81 (Thompson et al. 1997).

Results and discussion

Selection of the target genomic region

Approximately 100 QTLs associated with maturity and flowering in soybean were identified. The completion of the soybean genome sequences and advanced bioinformatics may facilitate the in silico cross-matching of candidate sequences with QTLs using approaches of positional cloning or association mapping (Salvi and Tuberosa 2005). However, the complicated genome structure of soybean (Shoemaker et al. 1996; Schmutz et al. 2010) presents difficulty in discriminating major genes from genes copied via duplication in the soybean genome. Considering the characteristics of the soybean genome, the present study applied the candidate gene approach in parallel with QTL mapping to identify specific genomic sequences associated with maturity in the cultivated and wild soybeans.

Forty-one of the 84 genetic loci that were searched from Soybean Breeder's Toolbox (http://soybeanbreederstoolbox.

org/) for maturity and flowering traits (first flower, *Fflr*; flowering time, *FT*; pod maturity, *Pod mat*) exhibited highly significant phenotypic variation and were selected for further analysis. These 41 QTLs were distributed over nine linkage groups/LGs (B1, C1, C2, G, I, K, L, M and O) of the soybean consensus genetic map (Choi et al. 2007). Based on the linkage position of MADS-box genes and the QTLs for maturity and/or flowering, several potential genes were chosen according to E-value and high similarity using BLAST algorithm. Further screening of the putative MADS-box genes was predicted using Arabidopsis genome (http://linux1.softberry.com/berry.phtml). In consideration of their potential relevance in the control of soybean flowering and maturity, finally 75 putative MADS-box genes were selected.

The sequence information of the SSR and SNP markers associated with the 41 QTLs was used to estimate the physical position of the targeted QTLs. The putative MADS-box genes were located around the markers associated with the QTLs by narrowing down the two genomic regions to chromosomes 7 and 11 with three QTLs on linkage group (LG) M and B1. The QTLs of *Pod mat 13-7* ($R^2 = 30$) and *Fflr 4-2* ($R^2 = 26$) were linked to the same SSR marker, satt150, and the QTL of *Pod mat 6-2* ($R^2 = 10.8$) was linked to the cr122_1 marker (Lee et al. 1996; Orf et al. 1999). Finally, two putative MADS-box genes located on chromosome 7 (Glyma07g03400) and chromosome 11 (Glyma11g07820) were identified as tightly associated with pod maturity and first flower, and the nucleotide polymorphisms of these genes were investigated. This approach demonstrated that a link between the MADS-box genes and the quantitative trait for maturity can be estimated based on the linkage information of the genes co-segregating with the QTL at positional genes. The genetic variation of these genes was identified among cultivated *G. max* based on the maturity and its wild relative, *G. soja*.

Nucleotide polymorphism

No nucleotide changes were found in the genic region of the putative MADS-box genes on chromosomes 7 (2390257–2394709 bp) and 11 (5489539–5493481 bp). Sequence polymorphisms were detected only in the upstream region of chromosome 7 (2383002–2384178 bp) with the predicted promoters on the cultivated and wild soybean genotypes as shown in Figs. 1 and 2, respectively. In the upstream region, a promoter initiates transcription of a particular gene. This study demonstrated that there were ten possible promoter regions predicted in the MADS-box gene on chromosome 7. Approximately 7 kb a part from the possible promoter region, the 16 SNPs with *P* value lower than 0.05 were identified using standard contingency test. The SNPs highly associated with maturity group (MG) were identified using the primer set indicated with an asterisk in Supplementary Table 2. The positions of polymorphic sites were counted from transcription starting point of putative MADS-box gene.

Although the SNPs existed within the upstream of the gene, the nucleotide variation proved the high association with maturity. These highly associated SNPs on the transcription factor binding sites on the putative promoter were expected to control the transcription of the putative MADS-box gene. This result signifies that MADS-box gene in chromosome 7 contributes more to control soybean maturity in comparison with that in chromosome 11. This result is in good agreement with previous QTL cloning studies which reported the major contribution of variants in the upstream region of the *Ap2*-like transcription factor involved in flowering (Salvi et al. 2007) and the *tb1* gene governing the increase in apical dominance in maize (Clark et al. 2004). Importantly, MADS-box gene highly associated with soybean maturity in this study was not included among 12 MADS-box genes for flowering/maturity in soybean identified in previous studies (Chi et al. 2011). Therefore, this MADS-box gene containing this variants associated with soybean maturity could be novel.

The SNP frequency in the upstream region of the MADS-box gene for all of the genotypes averaged one SNP per 73.5 bases, with nine identical allele pairs on 16 segregating sites. With alleles commonly found in cultivated soybean, the nucleotide polymorphism in LMG was higher than that of EMG. Interestingly, a small number of alternate alleles in EMG were found to be common alleles in *G. soja.* Diverse soybean accessions comprising modern soybean genotypes and the ancestor from different demographic regions may also contribute to detect more frequent SNPs in this gene. Based on the Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution (Tamura et al. 2007), the overall transition/tranversion ratio bias of the wild soybeans was 2.58 times higher than that of *G. max* (0.487). In this approach, the evolutionary distances are estimated independently of others, either by analytical formula or by likelihood methods with lower errors and expectedly is more accurate (Tamura et al. 2007). Clearly, although the selection was made for loci with a function, the nucleotide variation associated with the function may result from the non-coding region as shown in this study. The variation detected in upstream of the MADS-box gene may support the expectation based on previous QTL cloning studies.

Neutrality test

A neutrality test was conducted to assess the selection of mutations in this putative soybean MADS-box gene based on Tajima's D (Tajima 1989), and Fu and Li's D* and F*

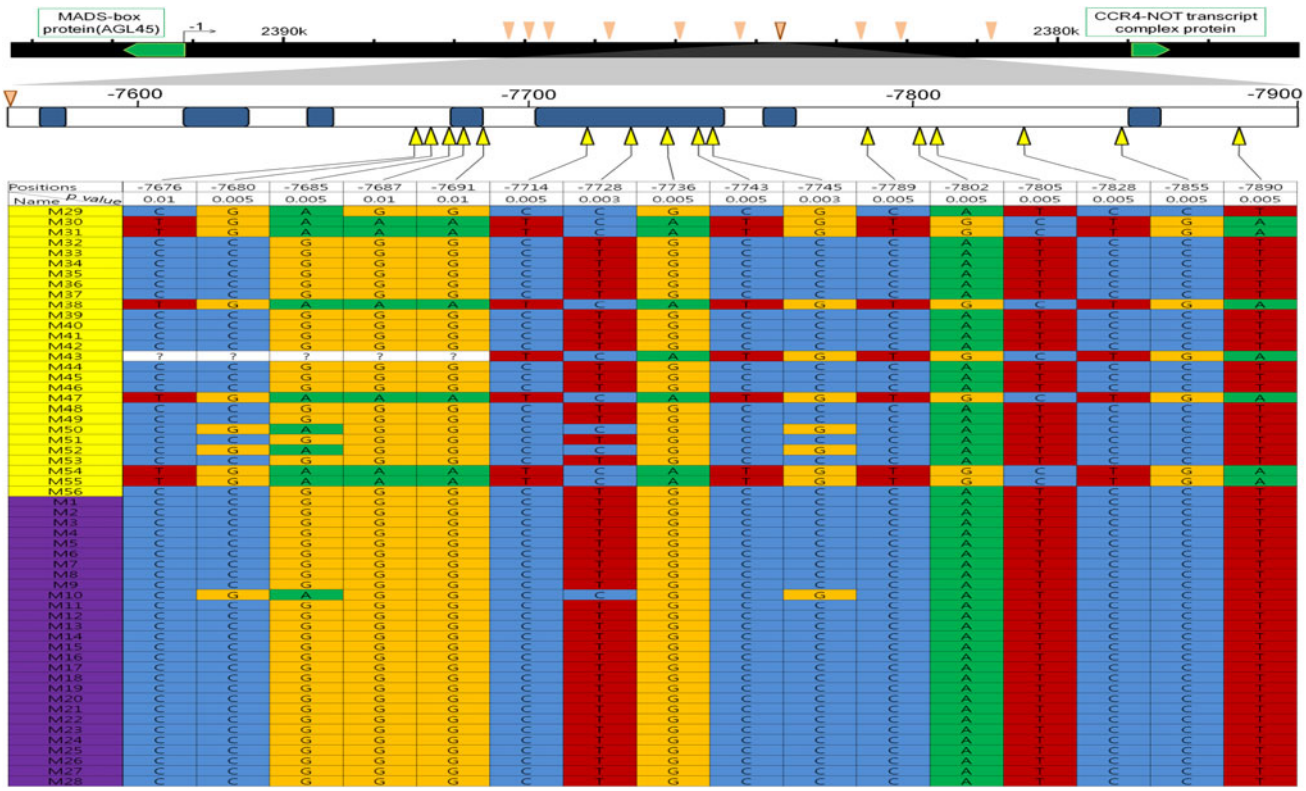

Fig. 1 Sequence polymorphisms and structure prediction in the genomic region associated with MG in 56 genotypes of *G. max*. Within the upstream region, ten possible promoter regions were predicted using TSSP (http://linux1.softberry.com/berry.phtml), which are indicated with *pink arrowheads*. At one of the possible promoter regions (highlighted arrowhead) as far as 7 Kb upstream of the putative MADS-box gene, sixteen polymorphisms with p values lower than 0.05 were identified. Codes of M1–M28 in the left column represent the EM group of *G. max*, and M29–M56 indicate the LM group of *G. max*. The positions of the polymorphic sites are indicated based on the transcription starting point of the putative MADS-box gene. *Green arrows* on the *black bold bar* represent the chromosome 7 predicted genes. *Yellow arrows* and *blue boxes* symbolize the exact location of polymorphisms and predicted transcription binding sites, respectively. *Question marks* represent the nucleotides that could not be sequenced. (Color figure online)

Position / Code name	-7074	-7078	-7083	-7085	-7089	-7112	-7126	-7134	-7141	-7143	-7187	-7200	-7203	-7226	-7253	-7288
S1	T	G	A	A	A	T	C	A	T	G	T	G	C	T	G	A
S2	T	G	A	A	A	T	C	A	T	G	T	G	C	T	G	A
S3	T	G	A	A	A	T	C	A	T	G	T	G	C	T	G	A
S4	T	G	A	A	A	T	C	A	T	G	T	G	C	T	G	A
S5	T	G	A	A	A	T	C	A	T	G	T	G	C	T	G	A
S6	T	G	A	A	A	T	C	A	T	G	T	G	C	T	G	A
S7	T	G	A	A	A	T	C	A	T	G	T	G	C	T	G	A
S8	C	C	G	G	G	C	C	G	T	G	C	A	T	C	C	T
S9	C	C	G	G	G	C	T	G	C	C	C	A	T	C	C	T
S10	T	G	A	A	A	C	C	G	T	G	T	G	C	T	G	A
S11	T	G	A	A	A	C	C	G	T	G	T	G	C	T	G	A
S12	T	G	A	A	A	T	C	A	T	G	T	G	C	T	G	A
S13	T	G	A	A	A	T	C	A	T	G	T	G	C	T	G	A
S14	T	G	A	A	A	T	C	A	T	G	T	G	C	T	G	A
S15	T	G	A	A	A	T	C	A	T	G	T	G	C	T	G	A
S16	C	C	G	G	G	C	T	G	C	C	C	A	T	C	C	T
S17	C	C	G	G	G	C	T	G	C	C	C	A	T	C	C	T
S18	T	G	A	A	A	T	C	A	T	G	T	G	C	T	G	A
S19	T	G	A	A	A	T	C	A	T	G	T	G	C	T	G	A
S20	C	C	G	G	G	C	T	G	C	C	C	A	T	C	C	T

Fig. 2 Sequence polymorphisms in *G. soja*. Code names of S1–S20 in the *left column* indicate *G. soja* genotypes. The *numbers* in the *first row* represent the distance from the transcription starting point of the putative MADS-box gene

tests (Fu and Li 1993). Tajima's D, and Fu and Li's D* and F* tests test are powerful statistical approaches for comparing the observed nucleotide diversity with the expected diversity in a neutral genetic model. Statistical analysis of the putative MADS-box gene sequence is presented in Table 1.

A positive Tajima's D (3.547, $P < 0.001$) for all of the observed genotypes suggests that this gene contains an excess of common variation. Significant positive Tajima's D for total soybeans signifies low levels of low and high frequency polymorphisms of the MADS-box gene. Based on this, it can be concluded that a decrease in the soybean population size may be influenced by balancing selection. A highly significant Tajima's D may also implies that the allele variation of the MADS-box genes in the total soybean accessions is not randomly selected (Tajima 1989), indicating that artificial selection may be acting upon this gene. In support of the conservative Tajima's D test results, positive significant Fu and Li's D* (1.615) and F* (1.957) values emphasize evidence of balancing selection or a population subdivision event. These results support the hypothesis that a demographic process may influence the diversity of *G. max* LMG and EMG, as well as the wild soybean during selection and domestication.

When each population of *G. soja* and *G. max* was tested individually for neutrality, the individual value of each conservative Tajima's D was non-significant positive, indicating a slight excess of intermediate frequency or long-lived polymorphism for this gene. This result was supported by similar positive values of Fu and Li's D* and F* tests for each population of *G. max* and *G. soja*. A non-significant negative value of Tajima's D (-1.889) in EMG of *G. max* proves that rare alleles that contribute to population growth, even under selective pressure, have occurred in these total soybean accessions (Tajima 1989). Accordingly, deleterious alleles that existed in the MADS-box gene on EMG may lead to population expansion. Demographic processes possibly affect the genome more evenly than selective pressures on the MADS-box gene itself on EMG (Stajich and Hahn 2005). This did not occur on LMG that showed a higher Tajima's D with a non-significant positive departure (D = 2.677). The frequency spectrum of polymorphic sites for this gene on LMG was skewed towards a deficit of low-frequency alleles relative to expectations. In the selection process, early maturity soybean genotypes are preferentially improved (Lee et al. 1996; Tasma et al. 2001). A non-significant Tajima's D of LMG specifies that both *G. max* and *G. soja* are in genetic equilibrium (Maia et al. 2009) but not for EMG as a target of artificial selection program. This clue provides evidence that *G. soja* and LMG have not been intentionally improved as strongly as EMG in the selection process. The MADS-box genes in the populations shaping these soybeans may be primarily contributed by the EMG.

Evolutionary divergence and phylogeny

The nucleotide diversity of 16 SNPs (Figs. 1, 2) was evaluated using two common measures, the expected heterozygosity per nucleotide site (π) and the difference between theta Watterson (θ). The theta estimates $4N_e\mu$, where N_e is the effective population size and μ is defined as the mutation rate per nucleotide (Watterson 1975). As expected, *G. max* exhibited lower nucleotide diversity ($\pi = 0.0179$) compared to the wild soybean ($\pi = 0.0253$). However, based on the θ value, *G. max* ($\theta = 24.1$) and *G. soja* ($\theta = 23.9$) were comparatively similar to one another. It is notable that LMG exhibited much higher nucleotide diversity estimators ($\pi = 0.0280$, $\theta = 0.0213$) than EMG ($\pi = 0.0030$,$\theta = 0.0109$), in spite of the same sample size.

Table 1 Sequence statistics of the putative MADS-box gene

Parameter	Total population	*G. max*	Early maturity group/ EMG (*G. max*)	Late maturity group/ LMG (*G. max*)	*G. soja*
N	76	56	28	28	20
S	16	16	4	16	16
$\pi \times 10^{-3}$	0.0470	0.0179	0.003	0.028	0.0253
$\theta \times 10^{-3}$	0.0204	0.0241	0.0109	0.0213	0.0239
Hd	0.716	0.592	0.071	0.767	0.568
k	5.175	2.958	0.026	5.842	2.231
D	3.547**	1.305^{ns}	-1.889^{ns}	2.677^{ns}	1.497^{ns}
D*	1.615*	1.438^{ns}	−2.934*	1.439*	1.211^{ns}
F*	1.957*	1.647^{ns}	−3.051*	2.125*	1.503^{ns}

N number of accession, *S* number of segregating sites, π nucleotide diversity, θ nucleotide diversity Watterson, *Hd* haplotype diversity, *k* average evolutionary divergence, *D* Tajima's D test, *D** Fu and Li's D*, *F** Fu and Li's F*

^{ns} non-significant at $P < 0.05$ level, * and ** significant at $P < 0.05$ and $P < 0.01$ levels, respectively

The mean diversities within the total population comprising *G. max* and *G. soja* were higher (2.587) than the mean inter population diversity (1.832). Measured parameters representing evolutionary divergence of this gene is shown in Table 1.

According to the base substitution for the overall sequence pairs, the mean pairwise distance used to estimate the average evolutionary divergence of *G. max* was higher than that within *G. soja.* Among soybean MGs, the EMG genotypes were less divergent (k = 0.016) than the LMG genotypes (k = 6.933). Moreover, the pairwise distance of LMG and *G. soja* (1.889) was less than that of EMG and *G. soja* (11.366). The putative MADS-box gene of EMG and LMG also exhibited higher divergence (3.049) compared to that of LMG and *G. soja*. The coalescent theory (Wall and Hudson 2001) was performed to predict an initial number of haplotypes for the MADS-box gene. Considering all of the accessions, the estimated number of haplotypes ranged from 5 to 11. Based on each group, *G. max* and *G. soja* had comparable haplotype diversity according to theta Watterson. However, the haplotype diversity of the total soybean accessions was still less than that in LMG. In contrast to LMG, EMG showed the lowest haplotype diversity, specifying lower genetic differentiation. Considering the genomic region of the SNPs, *G. max* retained only 71 % (π) and 66 % (Hd) of the nucleotide diversity of *G. soja*. Furthermore, the higher π value on LMG compared to EMG supports the Tajima's D value, indicating that strong selection for maturity affects the genetic diversity at the specific region. In addition, the lower θ value of EMG compared to *G. soja* suggests that the haplotypes of *G. soja* have been eliminated in *G. max* EMG by high pressure selection during the domestication of *G. soja* to *G. max*. The alternate alleles probably remained in the EMG that commonly existed in the wild progenitors due to purifying selection.

The neighbor-joining tree analysis with the genetic distance revealed two major clades. One of the clades encompassed all of the EMG and a number of the LMG with very minor *G. soja* genotypes. *G. soja* and the LMG of *G. max* were distributed evenly in both clades, regardless of their origins. In contrast, EMG was grouped dominantly in one branch of the second clade despite diverse origins (Fig. 3) that may be considered different haplotypes. In good agreement with neutrality tests and evolutionary divergence analysis, the neighbor-joining tree showed that the trace of strong selection identified on EMG pooled into one clade, signifying low genetic diversity. Consistent with the series of Tajima and Fu and Li tests results, the phylogenic analysis also demonstrates that selection on this genomic region has focused on early maturity with consideration of diverse origins such as Korea, China and Japan. The lower mean evolutionary distance of this gene found between LMG and *G. soja* compared to that between

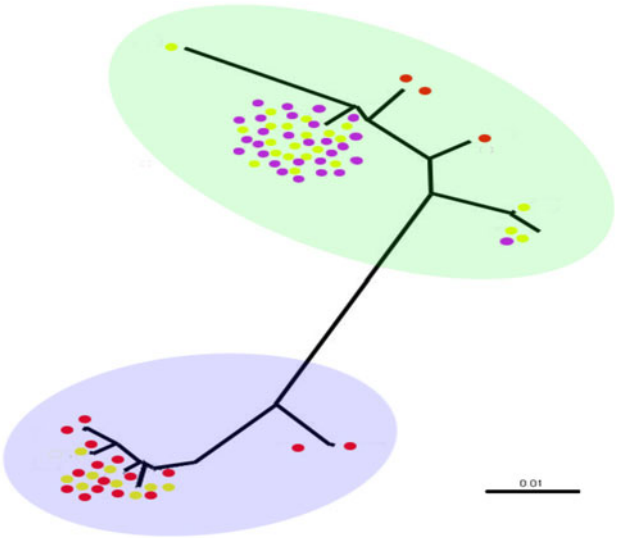

Fig. 3 Neighbor-joining tree representing the clade relationships of the MADS-box genes. *Yellow*, *purple* and *red dots* represent the LMG of *G. max*, the EMG of *G. max*, and *G. soja*, respectively. (Color figure online)

EMG and LMG also indicates that those genotypes may belong to the same molecular evolutionary lineage. Thus, the results of this study provide insight into the genetic relationship and evolutionary history of both cultivated and wild soybeans.

In this study, the candidate gene approach in parallel with QTL mapping proved effective at identifying genomic regions associated with QTLs. Considering the advantages of whole genome sequences (Varshney et al. 2009), a common domain of a gene family was detected around the QTL as a candidate gene for association analysis. A cladistic analysis for establishing future genetic association studies of the MADS-box gene may be validated. Moreover, further analysis on the putative MADS-box gene associated with soybean maturity using genetic engineering, mutagenesis or RNA interference will be valuable for gene regulation studies.

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