



Evolution and expression of tandem duplicated maize flavonol synthase genes

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Flavonoids are specialized compounds widely distributed and with diverse functions throughout the plant kingdom and with several benefits for human health. In particular, flavonols, synthesized by flavonol synthase (FLS), protect plants against UV-B radiation and are essential for male fertility in maize and other plants. We have recently characterized a UV-B inducible *ZmFLS1*, corresponding to the first to be described in monocot plants. Interestingly, the new assembly of the B73 maize genome revealed the presence of a second putative *FLS* gene (*ZmFLS2*), with very high identity with *ZmFLS1*. *ZmFLS*s expression was analyzed in different maize tissues, and by combining electrophoretic mobility shift assays and transient expression experiments, we show that both genes are direct targets of anthocyanin (C1/PL1 + R/B) and 3-deoxy flavonoid (P1) transcriptional regulators. *ZmFLS* expression analyses show higher levels of both transcripts in high altitude landraces than inbred lines, and both genes are regulated by UV-B radiation in all lines analyzed. Moreover, the high sequence conservation of the *ZmFLS* promoters between maize lines suggests that the differences observed in *ZmFLS* expression are due to allelic variations in the transcription factors that regulate their activities. Finally, we generated pFLS1::FLS1-RFP transgenic plants and analyzed *ZmFLS1* expression in different maize tissues; we found that this enzyme is localized in the ER and the perinuclear region.

Keywords: UV-B, duplication, grasses, natural variation, maize

INTRODUCTION

Flavonoids are widely distributed plant metabolites with diverse biological functions. There is considerable evidence showing a role for flavonoids in contributing to the human health associated to their antioxidant, anti-proliferative and anti-inflammatory properties, and consequently, their indication in prevention against cancer and cardiovascular disease (Knekt et al., 2000; Hirvonen et al., 2001; Mak et al., 2006; Vargo et al., 2006; Geleijnse and Hollman, 2008; Kaur et al., 2008; Kang et al., 2009). Flavonols, the most abundant and widespread subgroup of flavonoids, play important functions in plant physiology, growth, and development, including the modulation of basipetal auxin transport in *Arabidopsis* (Brown et al., 2001; Peer et al., 2004; Kuhn et al., 2011; Lewis et al., 2011), attraction of and defense against insects (Gronquist et al., 2001), pollen fertility (Mo et al., 1992; Taylor and Jorgensen, 1992; Ylstra et al., 1994; Taylor and Hepler, 1997), and UV-B protection (Solovchenko and Schmitz-Eiberger, 2003; Jaakola et al., 2004; Stracke et al., 2007, 2010a; Kusano et al., 2011). Moreover, flavonols have recently been shown to participate in the ethylene-signaling pathway (Lewis et al., 2011).

The biosynthesis of flavonols from dihydroflavonols is catalyzed by the enzyme flavonol synthase (FLS), a soluble 2-oxoglutarate-dependent dioxygenase (2-ODD). *FLS* cDNAs were cloned from a

large number of dicot plants, and they were functionally expressed in bacteria, yeast, and plants (Martens et al., 2010). However, the regulation of flavonol biosynthesis has only been well studied in *Arabidopsis thaliana*. In this species, both the regulators and the biosynthetic genes are mainly regulated at the level of transcription (Quattrocchio et al., 2006; Jenkins, 2008). One subgroup of R2R3-type MYB proteins, called PFG family for Production of Flavonol Glucosides, is constituted of PFG1/MYB12, PFG2/MYB11, and PFG3/MYB111, which exhibit differential spatial expression patterns and regulate flavonol accumulation in a tissue- and developmental-specific manner (Stracke et al., 2007). However, a PFG1-3-independent flavonol accumulation occurs in pollen and siliques/seeds (Stracke et al., 2010b). Moreover, it has been demonstrated that the bZIP transcription factor long HYpocotyl5 (HY5), an important participant in the UV-B-induced signal transduction cascade mediated by UVR8, regulates the expression of *PFG1/MYB12* under UV-B radiation (Stracke et al., 2010a). Thus, *AtFLS1* and other genes encoding enzymes involved in flavonol biosynthesis are targets of these regulators (Mehrtens et al., 2005; Stracke et al., 2007, 2010a).

We recently characterized an FLS enzyme from maize, *ZmFLS1*, which converts dihydroflavonols to the corresponding flavonols, partially complementing the flavonol deficiency of the *Arabidopsis*

fls1 mutant. In addition, we showed that the *ZmFLS1* transcript level is increased by UV-B radiation, induction that is at least in part mediated by the activation of the regulators P1 and C1/PL1 + R/B (Falcone Ferreyra et al., 2010). The most recent release (version 5b.60) of the maize genome (inbred B73) allowed us to identify a second *ZmFLS* gene with very high identity to *ZmFLS1*. Thus, the aim of this work is to investigate these two maize *FLS* genes, and comparatively evaluate this paralogous pair in evolutionarily closely related grasses. Here, we show the *ZmFLS*s expression pattern in different tissues of the B73 maize line, and demonstrate that both genes are regulated by the P1 and C1/PL1 + R/B regulators. We further analyze *ZmFLS* expression in different maize inbreds and landraces from high altitudes, and we find that *ZmFLS*s are induced by UV-B exposure in all lines analyzed. Higher transcript levels of both *ZmFLS* genes were found in high altitude lines compared to inbred lines in the absence of UV-B, indicating that higher flavonol levels may be a constitutive mechanism of protection against high irradiance in these landraces. Moreover, the high sequence conservation of the *ZmFLS* promoters between maize lines suggests that different expression levels are probably a result from allelic variations in the *trans*-acting regulatory machinery.

RESULTS

IDENTIFICATION AND ANALYSES OF FLAVONOL SYNTHASES IN MAIZE AND OTHER GRASSES

We recently described *ZmFLS1* (GRMZM2G152801), encoding the first monocot flavonol synthase enzyme (Falcone Ferreyra et al., 2010). Interestingly, the new assembly of the B73 genome (release 5b.60) revealed the presence of a second putative *FLS* gene (GRMZM2G069298, *ZmFLS2*) with very high identity to *ZmFLS1*. Both genes are located in chromosome 5 separated by ~50 kb (Figure 1A) and have identical structure, with two exons separated by an intron (Figure 1B). *ZmFLS1* and *ZmFLS2* share 96% identity, with higher identity in the coding regions (99%), and major differences between the respective 3'UTR regions. The predicted amino acid sequences exhibit 99% identity, with only two differences at positions 49 and 184, corresponding to isoleucine (I) and glycine (G) to methionine (M) and serine (S) for *ZmFLS1* and *ZmFLS2*, respectively. Their putative upstream regulatory regions (arbitrarily defined here as 1.5 kb upstream of the start codon) exhibit 61% identity (Figure A1 in Appendix) while 95% identity is observed for the region from -350 to the start codon. In comparison with *FLS* genes from other grasses with completed sequenced genomes, like sorghum (*Sorghum bicolor*), rice (*Oryza sativa*), and *Brachypodium distachyon*, a high percentage of identity is observed at the nucleotide level (70–89%) with the highest homology found with one of the *FLS* genes in sorghum (*SbFLS1*). Based on the available genome sequences, *Brachypodium* and rice contain only one *FLS* gene (Bradi3g57910 and Os02g52840, respectively), while two *FLS* genes are present in *S. bicolor* (BTx623 line; Figure 1B). But unlike what is found in maize, the two sorghum *FLS* genes are located in different chromosomes [4 and 3 for *SbFLS1* (Sb04g034240) and *SbFLS2* (Sb03g002040), respectively] and *SbFLS1* is more closely related to *ZmFLS*s (87–89% identity) than to *SbFLS2* (72% identity), which is consistent with synteny analyses (see below). The length and

the structure of the *FLS* genes are conserved between *ZmFLS*s, *SbFLS1*, and *BdFLS1*, each having two exons and one intron (Figure 1B), while *OsFLS1* has three exons and two introns, a structure similar to *AtFLS1* (Stracke et al., 2009). Although the annotation of *SbFLS2* suggests the presence of a second intron, this region includes a region that, in all *FLS*s from other grasses, encodes a part of the *FLS* protein. Thus, the deduced protein sequence of *SbFLS2* lacks 27 amino acids. In addition, the second intron of *OsFLS1*, *SbFLS2*, and the only one for *ZmFLS*s and *SbFLS1* are placed at the same position in all these genes (Figure 1B).

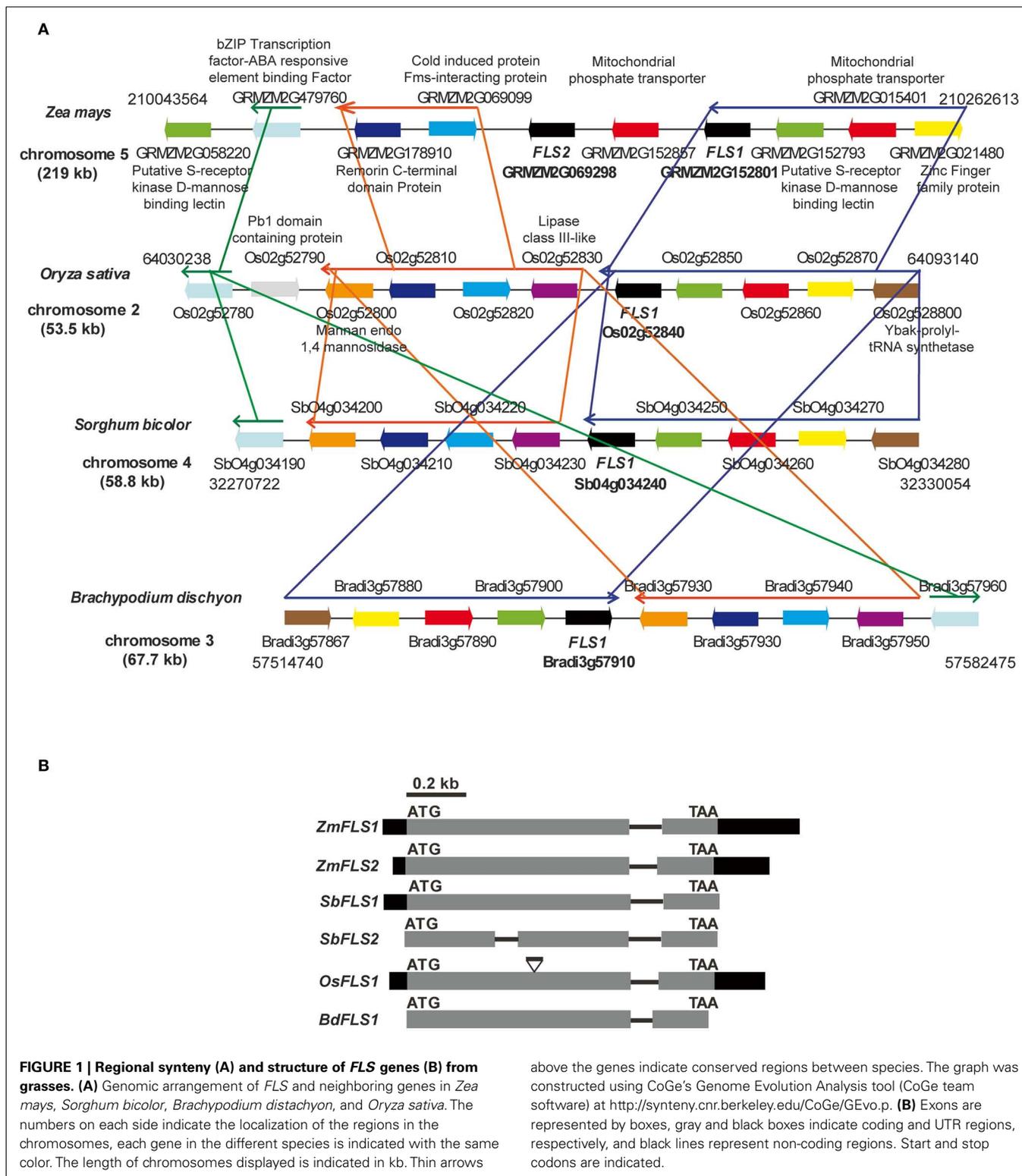
To verify the presence of the two tandemly arranged *ZmFLS* genes in the B73 genome, we designed primers to amplify fragments downstream of the 3'UTR of each gene, using the same forward primer that hybridizes in the 3'UTR of both genes, and specific reverse primers for each gene hybridizing elsewhere proximal in the chromosome. In addition, we amplified the intron of both *ZmFLS* genes using primers that hybridize in exons 1 and 2 of each gene (Figures A2B,C in Appendix). Moreover, to verify that both *ZmFLS* genes are localized in chromosome 5, we were able to amplify both genes using the same BAC clone (c0247D19) as a template (Figure A2D in Appendix). After sequencing the PCR products, we confirmed that the two different *ZmFLS* genes are present in chromosome 5 of the B73 maize inbred.

In order to analyze the orthologous *FLS* genes in detail, we explored the organization of the corresponding genes in the chromosomes. The analysis shows that *FLS* neighboring genes are conserved in maize (*ZmFLS1*, *ZmFLS2*), rice (*OsFLS1*), *Brachypodium* (*BdFLS1*), and sorghum (*SbFLS1*, with the exception of *SbFLS2* gene); however, some re-arrangements among blocks of linked genes are observed as well as inversions, duplication, and deletions of certain genes, particularly in maize (Figure 1A). Interestingly, synteny is spread over a region of 220 kb in maize while in other grasses this region is only 53–68 kb, as it was previously described for different regions of maize chromosomes (Li and Gill, 2002; Ilic et al., 2003; Bruggmann et al., 2006; Goette and Messing, 2009; Wei et al., 2009).

A phylogenetic tree generated with the amino acid sequences of plant *FLS* enzymes with demonstrated and predicted functionality showed a marked separation between enzymes from dicotyledonous and those from grasses monocotyledonous plants (Figure 2).

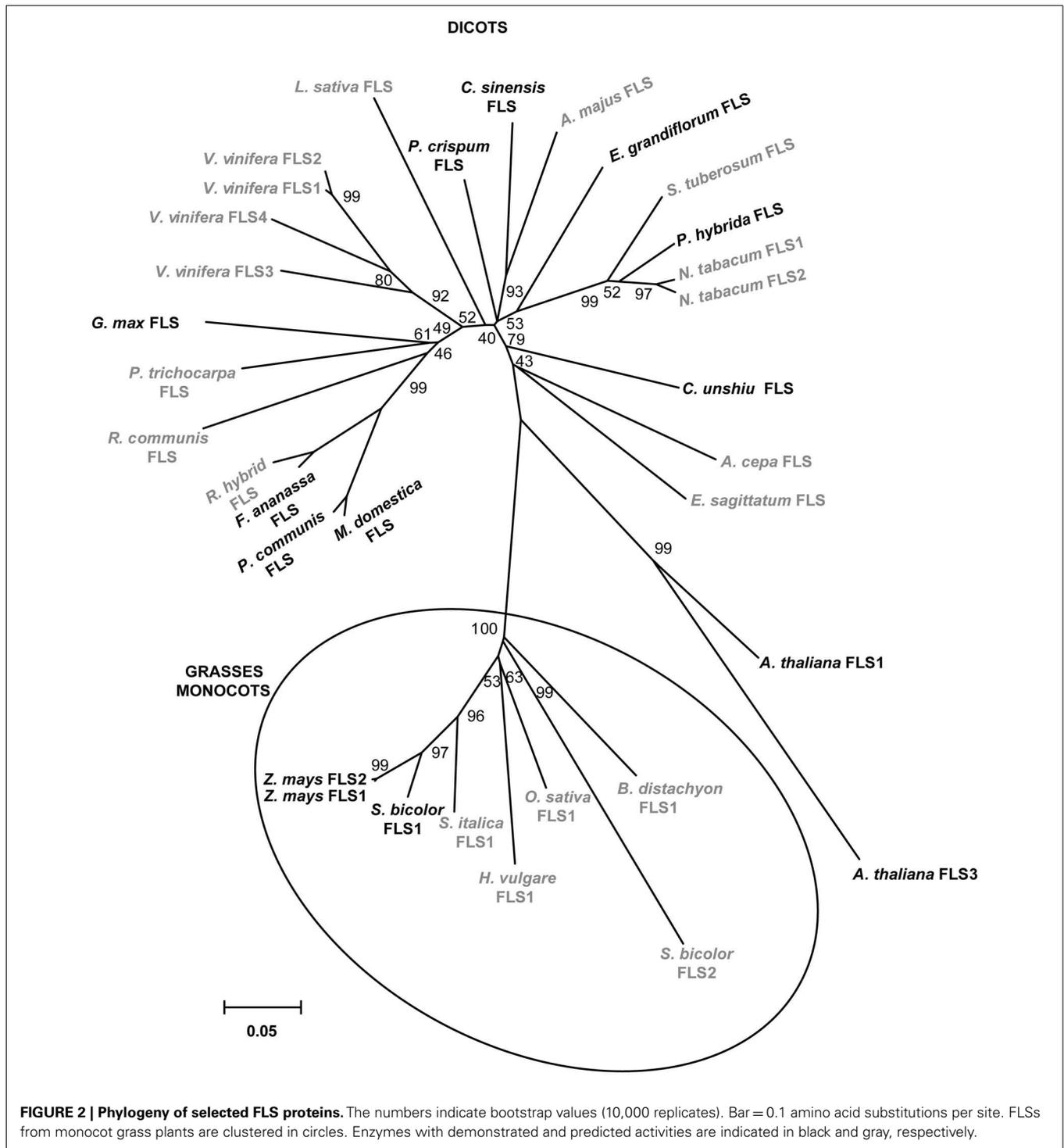
EXPRESSION ANALYSIS AND REGULATION OF *ZmFLS*s BY P1 AND C1 + R

To analyze the expression of *ZmFLS1* and *ZmFLS2*, we conducted quantitative RT-PCR (RT-quantitative PCR, qPCR) on RNA extracted from 7-day-old seedlings and juvenile leaves, roots, anthers (before anthesis), silks, 14 and 25 days after pollination (DAP) pericarps lacking (*PI-ww*) or accumulating (*PI-rr*) the phlobaphene pigments controlled by the maize *PI* gene (Grotewold et al., 1994), Black Mexican Sweet (BMS) maize cells, ectopically expressing the C1 + R anthocyanin regulators (BMS^{C1+R}, Grotewold et al., 1998), and untransformed control cells (BMS). Transcripts for *ZmFLS1* and *ZmFLS2* were detected in all the tissues analyzed, with the highest levels found in young seedlings



(Figure 3). It is worth mentioning that the expression patterns described are consistent with the microarray database from a genome-wide atlas of transcription in different tissues and developmental stage of maize (Sekhon et al., 2011).

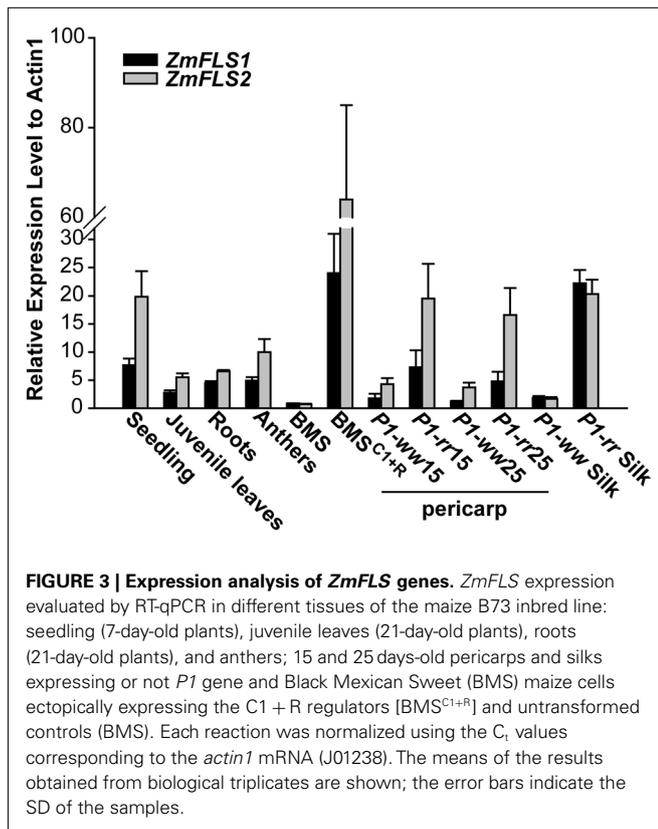
For both genes, transcripts were present at significantly higher levels in *PI-rr*, compared to *PI-ww* pericarps and silks, indicating that both genes are regulated by *PI*. Similarly, both *ZmFLS1* and *ZmFLS2* showed higher expression in BMS^{C1+R} than in BMS cells,



suggesting that both genes are also under the control of the C1 and R anthocyanin regulators (Figure 3).

Previously, we showed by transient expression experiments in BMS cells that *ZmFLS1* is positively regulated by P1 and C1 + R (Falcone Ferreyra et al., 2010). To investigate whether *ZmFLS2* is similarly regulated, we carried out transient co-transfection experiments of maize protoplast cells with the regulators driven

by the 35S promoter (p35S::P1 or p35S::C1 + p35S::R) and *ZmFLS2* promoter-luciferase reporter fusion (pZmFLS2::Luc; 1.5 kb upstream of the start codon). In addition, given that the proximal promoter regions (from -350 to the start codon) of *ZmFLS* genes are highly similar, we investigated to what extent the respective regions of *ZmFLS1* and *ZmFLS2* were sufficient for regulation by P1 and C1 + R.



Both *ZmFLS1* and *ZmFLS2* are robustly activated by C1 + R, with a significantly higher induction of *ZmFLS2*, compared to *ZmFLS1* ($P < 0.05$, **Figure 4A**). The analysis of candidate P1/C1 binding sites present in the *ZmFLS* promoters show additional candidate motifs in the distal region of the *ZmFLS2* promoter (**Figure 4A**). To investigate whether these binding sites could contribute to a major activation of *ZmFLS2* by C1 + R, we tested the proximal promoter regions up to -350 bp from the translation start codon; and, in this case, both genes were similarly activated, in accordance to the high identity of both *ZmFLS* proximal promoters (95%). For both genes, the 5' deletion of the promoter caused a significant reduction in their activation, indicating that the additional binding sites present in the distal promoter regions are important for C1 + R regulation. Additionally, when the most proximal binding site in both promoters was mutated (**Figure 4A**), regulation of both promoter::luciferase constructs by C1 + R remained the same, suggesting that this binding site is not the main contributor to C1 + R regulation. Thus, for *ZmFLS* genes, it is possible that the binding of C1 + R to DNA involves only one *cis*-regulatory element in the promoter (at -340 and -343 bp in *ZmFLS1* and *ZmFLS2*, respectively) or alternatively, another element could be involved in the binding and regulation, such as an E-box that is present in both promoters (at -296 and -293 bp, respectively), as it was suggested for the *Bz1* promoter (Roth et al., 1991).

Both *ZmFLS* genes were activated by P1, with higher levels of activation for *ZmFLS2* than for *ZmFLS1*. When the proximal

promoter regions were analyzed, *ZmFLS1* showed similar activation as when the full promoter was used, while a decreased activation was observed for *ZmFLS2*, suggesting that the additional P1-binding sites, absent in *ZmFLS1*, can contribute to *ZmFLS2* regulation by P1. Finally, when the most proximal binding site was mutated, the *ZmFLS* activation by P1 was almost completely lost, indicating an important and major contribution of this site in P1 regulation of *ZmFLS* genes (**Figure 4A**).

To verify which P1/C1 binding sites identified in the *ZmFLS* promoters are recognized by these transcription factors, we conducted electrophoretic mobility shift assays (EMSA). For these experiments, we expressed and affinity-purified from *E. coli* as N-terminal histidine-tagged fusions the P1 MYB domain (P1^{MYB}), as well as a version of the MYB domain of C1, C1^{SHMYB}, that binds DNA with higher affinity than C1^{MYB} and with comparable affinity to P1^{MYB} (Hernandez et al., 2004; N₆His-*ZmP1*^{MYB} and N₆His-*ZmC1*^{SHMYB}), obtaining 90–95% pure proteins (**Figure A3** in Appendix). C1^{SHMYB} and P1^{MYB} proteins bind to the APB1 probe, the positive control in our experiments that contains the ^{ha}PBS present in the *A1* promoter (**Figure 4B**), which was previously demonstrated to bind both proteins with high affinity (Hernandez et al., 2004). C1^{SHMYB} and P1^{MYB} proteins can also bind effectively FLSbind (**Figure 4C**), the labeled probe that contains the most proximal binding site present in both *ZmFLS* promoters at -248 and -250 bp for *ZmFLS1* and *ZmFLS2*, respectively (**Figure 4A**). Oligonucleotides containing other sites (comp 1–3) competed with the binding to FLSbind (**Figure 4C**), as FLSbind does itself. These results indicate that C1 and P1 can bind to all P1/C1 binding sites identified in the *ZmFLS1* and *ZmFLS2* promoters.

IN VIVO LOCALIZATION OF *ZmFLS1*

To confirm the *in vivo* expression pattern of *ZmFLS1*, and to investigate its subcellular localization, we generated transgenic maize plants expressing *ZmFLS1* fused to the red fluorescent protein (RFP) under its own promoter (p*ZmFLS1*::*ZmFLS1*-RFP). The 3'UTR region and introns can contain regulatory functions (Bailey-Serres and Dawe, 1996; Patel et al., 2004; Rose et al., 2008; Parra et al., 2011); thus, the construct included the endogenous 3'UTR. It is noteworthy that we only generated transgenic plants expressing *ZmFLS1* fused to RFP, since at the time these transgenic plants were constructed, the B73 maize genome was being sequenced and only the sequence of *ZmFLS1* was available in GenBank.

The FLS1-RFP fusion protein showed strong localization to the ER, as indicated by the “patchwork” pattern of fluorescence within leaf and root cells (**Figures 5A,C**), and was most easily detected in the perinuclear region in all cells in which fluorescence was detected (illustrated by **Figure 5B**). In developing inflorescence primordia, although expression was detected in all cells, higher levels of FLS1-RFP fluorescence could be observed in cells subtending spikelet pair (spm) and spikelet meristems (sp), as well as the L1 layer of suppressed bracts (sb; **Figures 5F–H**). In mature anthers, FLS1-RFP could be found in the degraded tapetum tissue surrounding pollen grains (**Figures 5D–E**), in accordance with the flavonol accumulation described in the later stages of maize anther development by Deboo et al. (1995). How the tissue and

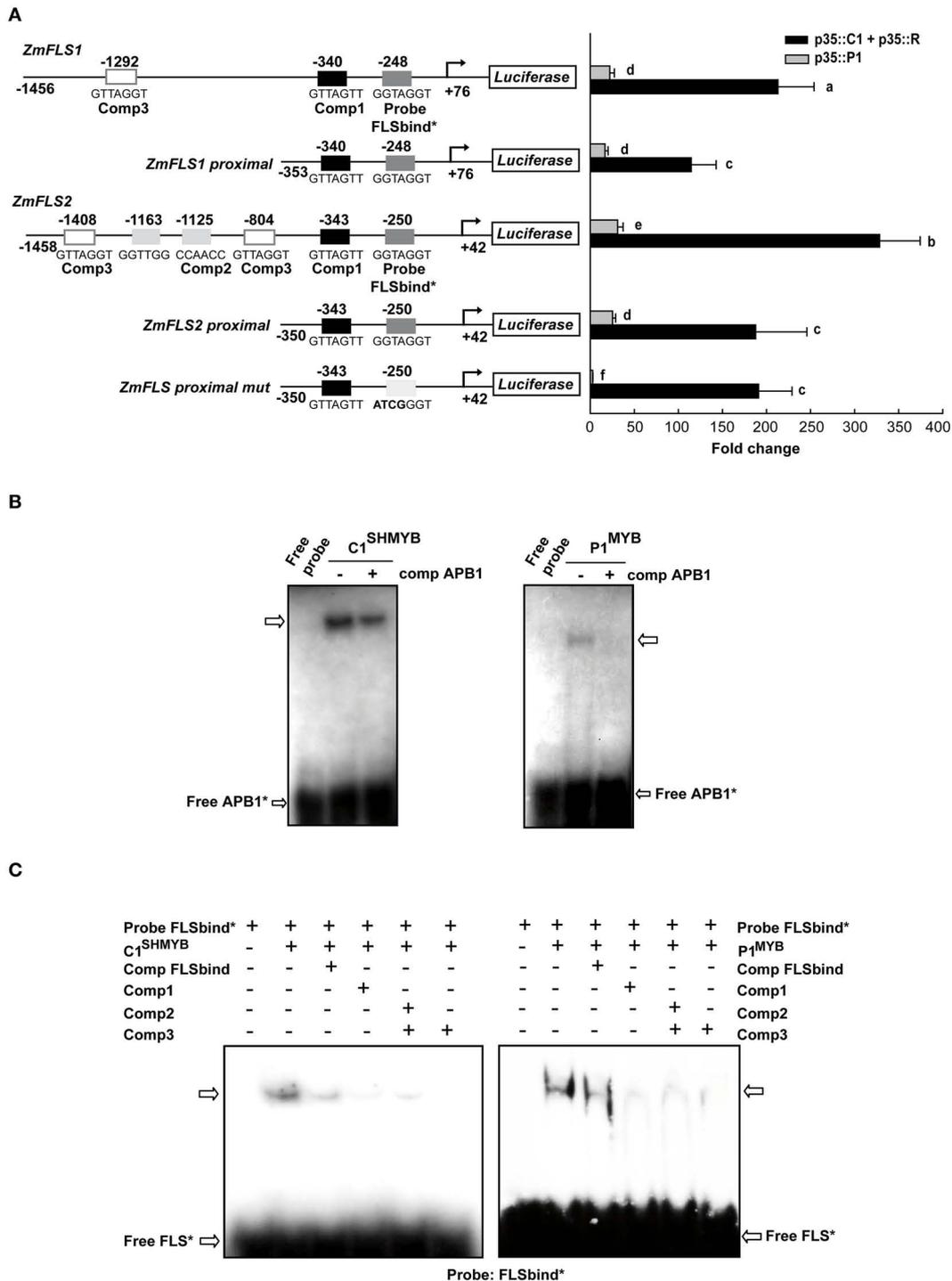
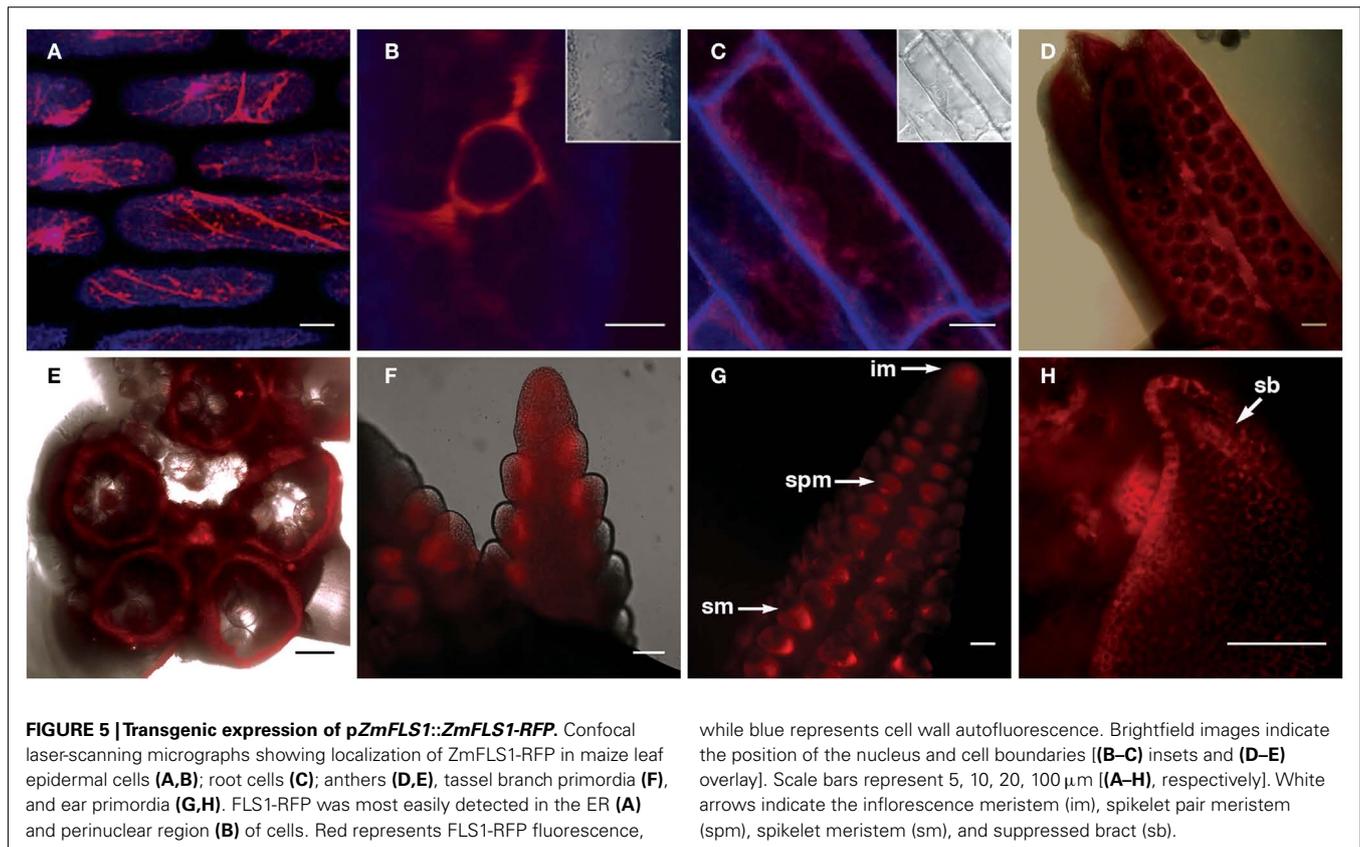


FIGURE 4 | Regulation of *ZmFLS* expression by P1 and C1 + R.

(A) Transient expression following the co-transfection of maize protoplasts with p35S::P1 or p35S::C1 + p35S::R along with the constructs shown on the left. Putative p1/C1 binding sites are indicated with boxes and the sequences of the oligonucleotide probes used for DNA-binding experiments are shown below the boxes. For each construct analyzed and for each regulator, different letters indicate a

significant difference at $P < 0.05$. (B) Binding of purified C1^{SHMYB} and P1^{MYB} proteins to ³²P-labeled APB1 probe containing the ^hAPBS of *A1* promoter analyzed by electrophoretic mobility shift assays (EMSA; Hernandez et al., 2004). (C) Gel mobility retardation analyses with purified C1^{SHMYB} and P1^{MYB} proteins and FLSbind* as probe. Free probe is indicated with an arrow. + and – indicate the presence or absence of different competitors, respectively.



subcellular localization of the ZmFLS1 fusion protein relates to the function of the enzyme, remains to be determined.

EXPRESSION OF ZmFLSs IN DIFFERENT MAIZE LINES

In order to investigate the evolution of *ZmFLS* genes, we amplified by PCR *ZmFLS1* and *ZmFLS2* genes in different maize lines, covering from position +739 (exon 1) to the 3'UTR using specific reverse primers for each gene. A high conservation at nucleotide level was observed between maize lines for both genes, showing minor differences mainly in the intron and in the 3'UTR (Figure A4 in Appendix). The phylogenetic tree generated from these sequences showed that *ZmFLS1* and *ZmFLS2* genes group in different clusters in all lines analyzed (Figure A5 in Appendix) indicating that paralogous genes arose by duplication before maize lines divergence.

Previously, we showed that the combined expression of *ZmFLS1* and *ZmFLS2* is induced by UV-B radiation in the B73 inbred, as well as in the W23 inbred line with (*W23^{B^{PL}}*) and without (*W23^{b^{pl}}*) the dominant alleles for the *B* and *PL1* anthocyanin regulators. We examined *ZmFLS1* and *ZmFLS2* expression levels in different maize inbred lines (A619, Mo17, W22, B73, and W23 with or without the dominant alleles for the *B* and *PL1* regulators) and in five maize landraces from high altitudes (Arrocillo Amarillo, Cacahuacintle, Conico, and Confite Puneño) by RT-qPCR using specific primers for each *FLS* gene covering the regions previously sequenced. Under control conditions without UV-B radiation, higher transcript levels of *ZmFLS1* and *ZmFLS2* were observed in 28-day-old seedlings from high altitude lines, compared to

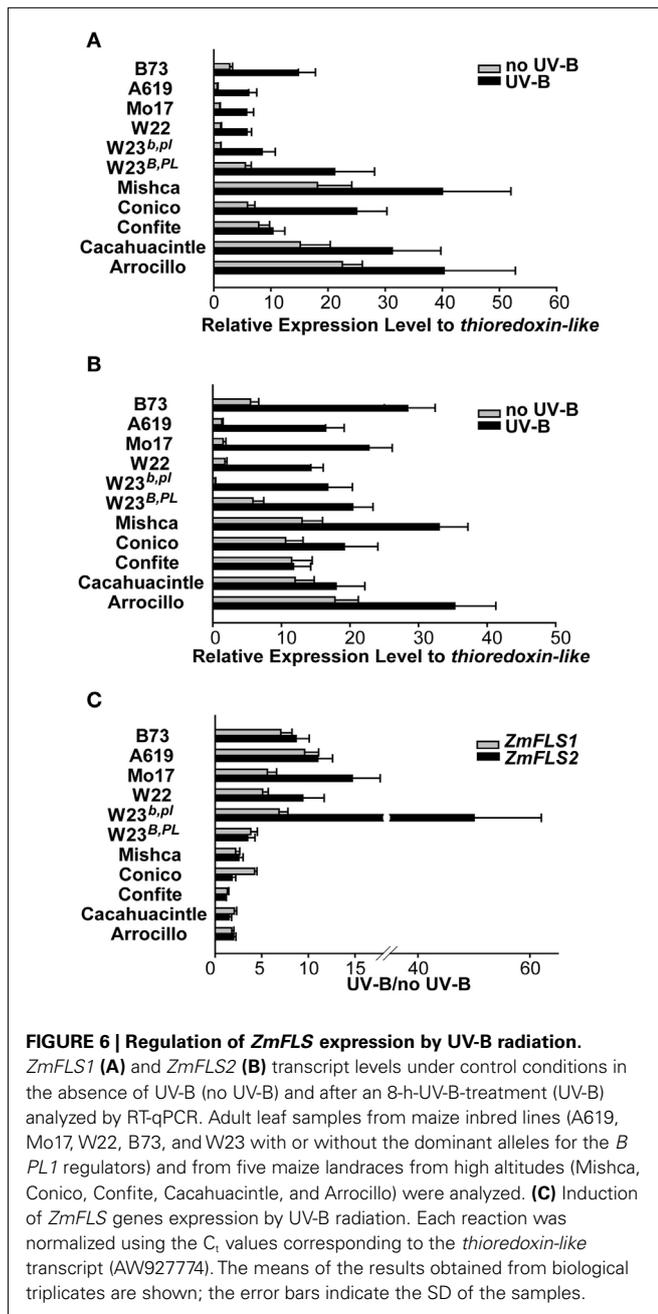
levels in the low-altitudes inbreds (Figures 6A,B). When the plants were irradiated with UV-B, *ZmFLS* transcripts increased in all lines, showing greater induction in the low-altitudes inbreds (Figure 6C). However, it is important to emphasize that despite the greater increase in *ZmFLS* expression by UV-B in low-altitudes inbreds, transcript levels, particularly for *ZmFLS1* after the UV-B treatment, are in general lower than those in high altitude plants.

CONSERVATION OF ZmFLS PROMOTERS BETWEEN MAIZE LINES

To study if the differences observed in *ZmFLS1* and *ZmFLS2* expression levels between the maize inbred lines and the landraces from high altitudes (Figure 6) are a consequence of allelic variations in their promoters, we amplified the proximal promoters of *ZmFLS* genes from these maize lines. A high similarity of sequence was found in both promoters in all lines, with the conservation of the C1/P1-binding sites present in the B73 line, showing only short insertions or deletions at the 3' promoter end, downstream of the putative TATA box (Figure A6 in Appendix). Consequently, the increased expression of *ZmFLS* genes in the maize landraces from high altitudes could be explained by differences in the activities and/or expression levels of C1 + R and P1 regulators in these landraces.

DISCUSSION

The recently completed maize genome sequence (release 5b.60) shows that two *FLS* genes are present in the B73 line, resulting from complete genome duplication during evolution (Schnable et al., 2009). Consequently, this study was aimed at characterizing



both *ZmFLS* genes and their patterns of expression and regulation, in order to analyze if duplication/divergence resulted in them being expressed under different conditions with functional specificity, or if there is functional redundancy between these genes. The analysis of both genes showed that there is an extremely high degree of identity between them, both at the nucleotide and amino acid levels, and also in their promoters. In addition, we found that there is a very high level of conservation in the sequences and genomic synteny with *FLS* genes from other grasses like rice, *Brachypodium*, and sorghum (*SbFLS1*, Figure 1). Although two *FLS* genes are also present in *S. bicolor* (*SbFLS1* and *SbFLS2*), unlike in *O. sativa* and *B. distachyon* which have only one *FLS* gene, *SbFLS1* is more closely

related to the maize counterparts than to *SbFLS2*, and the annotated amino acid sequence of *SbFLS2* lacks 27 amino acids. It is possible that *SbFLS2* is a pseudo-gene that has not yet been lost, perhaps a result of a whole-genome duplication of the common ancestor of the grasses (Salse et al., 2008; Paterson et al., 2009). This hypothesis is consistent with the fact that no matching transcript could be found in the sorghum EST collections, and no expression of *SbFLS2* could be observed under standard growth conditions, or upon exposure to salt stress or UV-B radiation in contrast to *SbFLS1* (unpublished data). Moreover, according to the evolution of grass genomes from a common ancestor (50–70 mya), maize, and sorghum diverged from each other ~12 mya, and maize underwent a complete genome duplication (~5 mya) followed by re-arrangements leading to the current chromosomes (Salse et al., 2008; Buell, 2009; Paterson et al., 2009). Based on this, we can hypothesize that *ZmFLS* genes resulted from gene duplication ~5 mya.

It is important to mention that, although we had previously reported an analysis of *ZmFLS1* expression by RT-qPCR (Falcone Ferreyra et al., 2010); because there was no evidence of a second closely linked gene with very high identity to *ZmFLS1*, the primers used recognized both transcripts. The two *ZmFLS* genes studied here are expressed in all maize tissues analyzed, showing higher levels of expression in tissues where the P1 and C1 + R regulators are present, indicating that both genes are regulated by these transcription factors (Figure 3). To validate this hypothesis, we combined *in vitro* DNA-binding experiments with *in vivo* transient co-expression studies in maize protoplasts, showing that *ZmFLS2* is more activated by the two regulators than *ZmFLS1*, probably due to the presence of additional P1/C1 binding sites in the distal region of its promoter (Figure 4).

Differences in regulatory elements present in *ZmFLS*-3'UTRs genes may contribute to their differential expression regulation. In humans, *C. elegans* and yeasts, regulatory elements present in the 5' and 3'UTRs affect mRNA stability and translation rate; however, information of this type of regulation in plants is scarce (Zubiaga et al., 1995; Yang et al., 2003; Wilusz and Wilusz, 2004; Shalgi et al., 2005; Merritt et al., 2008). The role of mRNA sequence determinants in posttranscriptional regulation of the *adh1* gene was investigated in maize protoplasts under hypoxia, demonstrating that the 5' and 3'UTRs are required for the regulation of the expression of this gene under this stress condition (Bailey-Serres and Dawe, 1996). Moreover, the 5' and 3'UTRs of the amaranth *RbcS* gene function as translational enhancers in different tissues (Patel et al., 2004). RNA binding proteins that complex with 5' or 3'UTR are involved in the stability and translation of chloroplast mRNA (Bruck and Mayfield, 1999). However, additional studies are needed to investigate the contribution of the *ZmFLS* UTRs to gene regulation, for example by transient experiments with constructs containing the 3'UTR of each *ZmFLS* gene. Furthermore, it is well-known that mRNA levels do not necessarily correlate with protein levels in plant cells (Kawaguchi et al., 2004; Branco-Price et al., 2008; Mustroph et al., 2009). It has been reported that mRNA translation state is perturbed by stressful conditions like hypoxia, dehydration, light availability, cadmium intoxication, elevated temperature and high salinity in *Arabidopsis* (Kawaguchi

et al., 2004; Muströph et al., 2009; Matsuura et al., 2010; Sormani et al., 2011; Juntawong and Bailey-Serres, 2012). Therefore, we cannot rule out that *ZmFLS* mRNAs could be differentially translated.

We also show that both *ZmFLS* genes are regulated by UV-B radiation in both high altitude landraces and low-altitudes inbreds. However, under control conditions in the absence of UV-B radiation, higher transcript levels for both genes are present in high altitude plants compared with levels in the low-altitudes inbreds (Figure 6).

Maize lines growing at high altitudes have developed mechanisms to prevent damage caused by high UV-B exposure, such as the accumulation of *C*-glycosyl flavones in leaves, maysin, and its biosynthetic precursor rhamnosylisoorientin, flavones commonly found in silks (Snook et al., 1993; Casati and Walbot, 2005). Previously, we also showed that *B*, *PL*, and *PI* are up-regulated by UV-B in W23 and B73 lines, while in high altitude landraces these regulators are also expressed in leaves at high levels (Casati and Walbot, 2005). Thus, given the protective role of flavonols to UV-B radiation (Stafford, 1990; Ryan et al., 2001, 2002; Solovchenko and Schmitz-Eiberger, 2003; Jaakola et al., 2004; Stracke et al., 2010a; Kusano et al., 2011; Pollastri and Tattini, 2011), it is possible that the high transcript levels of both *ZmFLS* genes may also contribute to the adaptation to this stress condition with higher UV-B fluxes.

The analysis of the *ZmFLS* proximal promoters in high altitude landraces and low-altitude inbreds showed a high degree of conservation in the distribution of *cis*-regulatory elements, perhaps suggesting allelic variations in the *trans*-regulating machinery, either provided by differences in the activities and/or in the expression levels of the transcription factors that regulate the expression of both *ZmFLS* genes. Previous reports have shown that flavonols are essential for pollen germination and conditional male fertility in maize and petunia (Mo et al., 1992; Taylor and Jorgensen, 1992; Ylstra et al., 1994; Taylor and Hepler, 1997), but not in *Arabidopsis* (Burbulis et al., 1996; Ylstra et al., 1996). However, maize plants lacking the *P1* and *R/B + C1/PL1* anthocyanin regulators are fertile (Coe and Neuffer, 1988; Dooner et al., 1991; Neuffer et al., 1997), suggesting that additional regulators, not yet identified, are involved in the regulation of *ZmFLS* genes in anthers.

The FLS1-RFP fusion protein was detected in all tissues analyzed in agreement with transcript pattern established by RT-qPCR (Figures 3 and 5). At the subcellular level, this enzyme was localized at the ER and the perinuclear region, but not in the nucleus, contrary to what was recently reported for FLS1 in *Arabidopsis* (Kuhn et al., 2011), and previously for other flavonoid enzymes (Saslowsky et al., 2005). Interestingly, FLS1-RFP was observed in tapetum tissue of mature anthers, suggesting a role in late pollen development. Future experiments of localization will be necessary to investigate the specific participation of FLS1 and FLS2 proteins in maize anther development and pollen viability by analyzing maize *fls* mutant plants and complementation. While we cannot guarantee the functionality of the fusion protein, additional experiments such as the complementation of *A. thaliana* mutant plants in the *fls1* gene with the construct pFLS1::FLS1-RFP would allow to demonstrate its functionality in planta. Initial

supporting evidence demonstrated that 35S:FLS1-GFP was able to complement *A. thaliana fls1* mutant plants, restoring the levels of anthocyanins and flavonols (Falcone Ferreyra et al., 2010). Finally, based on the high identity between *ZmFLS1* and *ZmFLS2*, we can speculate that *ZmFLS2* would be able to complement the *A. thaliana fls1* mutants similarly as *ZmFLS1*. These proteins show only two differences in their amino acid sequences at positions not participating in 2-oxoglutarate and substrate binding, or the coordination of the ferrous iron necessary for the enzyme activity.

In conclusion, although it is expected that paralogous genes may be expressed in different tissues, at different levels and also regulated by distinct factors, our results indicate that it is not the case for duplicated *ZmFLS* genes. Moreover, while pseudogenization process eliminates unnecessary genes, it is notably that none of the *ZmFLS* genes turned non-functional or were removed from the genome. It is possible that *ZmFLS* genes are still diverging to specific functions. However, we cannot rule out that unidentified regulators can be involved in the differential regulation of *ZmFLS* genes to meet the physiological needs of the plants under specific conditions not investigated in our study.

MATERIALS AND METHODS

PLANT MATERIAL, GROWTH CONDITIONS, AND UV TREATMENTS

The *Zea mays* highland lines Confite Puneño, Mischa, Conico, Arrocillo Amarillo, and Cacahuacintle were obtained from the Germplasm Resources Information Network (GRIN, <http://www.ars-grin.gov/cgi-bin/npgs/acc/>).

The two near isogenic maize (*Z. mays*) lines that differ in flavonoid content from the genotype W23 (W23^{B^{PL}} and W23^{b^{pl}}) correspond to those previously described (Casati and Walbot, 2003). B73 seeds were obtained from the Instituto Nacional de Tecnología Agropecuaria (INTA, Pergamino, Buenos Aires, Argentina) while A619, W22, Mo17 seeds were maintained as laboratory stocks by self-pollination. The generation and analysis of the BMS cells expressing p35S::C1 and p35S::R were previously described (Grotewold et al., 1998).

Maize plants were grown in greenhouse conditions with supplemental visible lighting to 1000 $\mu\text{E m}^{-2} \text{s}^{-1}$ with 15 h of light and 9 h of dark without UV-B for 28 days. UV-B treatments were performed by illuminating plants with UV-B lamps for 8 h using fixtures mounted 30 cm above the plants (TL 20 W/12; Phillips) at a UV-B intensity of 2 W m^{-2} and a UV-A intensity of 0.65 W m^{-2} . The bulbs were covered with cellulose acetate filters (100 mm extra clear cellulose acetate plastic, Tap Plastics, Mountain View, CA, USA); the cellulose acetate sheeting does not remove any UV-B radiation from the spectrum but excludes wavelengths lower than 280 nm. No UV-B-treated plants (control) were exposed for the same period of time under the same lamps covered with polyester filters (100 mm clear polyester plastic; Tap Plastics, 0.04 W m^{-2} , UV-A, 0.4 W m^{-2}), which absorbs both UV-B and wavelengths lower than 280 nm. Lamp outputs were recorded using a UV-B/UV-A radiometer (UV203 A β B radiometer; Macam Photometrics) to ensure that bulbs and filters provided the designated UV light dosage in all treatments. Adult leaf samples (leaf 9 or 10) were collected immediately after irradiation and stored at -80°C . The UV-B treatment experiments were repeated at least three times.

For gene expression analyses in tissues of the B73 maize line, plants were grown in greenhouse conditions as described above and samples were collected from anthers, roots (21-day-old plants), seedlings (7-day-old plants), and juvenile leaves (21-day-old plants). Pericarps and silks were obtained from maize A619^{P-tr} and A619^{P-wv} plants grown in the field under natural sunlight conditions.

GENE EXPRESSION ANALYZES BY RT-qPCR

Tissues from three independent biological replicates were frozen in liquid nitrogen and stored at -80°C . Total RNA was extracted using the RNeasy Plant Mini kit with optional DNase treatment (Qiagen) or alternatively with Trizol Protocol (Invitrogen) followed by DNase treatment (Promega). cDNAs were synthesized from 4 μg of total RNA using Superscript Reverse Transcription Enzyme II (Invitrogen) with oligo-dT as a primer. The resulting cDNAs were used as templates for qPCR amplification in a iCycler iQ detection system with the Optical System Software version 3.0a (Bio-Rad), using the intercalation dye SYBR Green I (Invitrogen) as a fluorescent reporter and Platinum Taq Polymerase (Invitrogen). Primers were designed to generate unique 150–250 bp-fragments using the PRIMER3 software (Rozen and Skaletsky, 2000). Three biological replicates were used for each sample plus negative control (reaction without reverse transcriptase). To normalize the UV treatment data, primers for *thioredoxin-like* transcript (AW927774) were used, while for tissue dependent expression studies, primers for *Actin1* (J01238) were used for maize species (Table A1 in Appendix). Amplification conditions were as follows: 2 min denaturation at 94°C ; 40–45 cycles at 94°C for 10 s, 57°C for 15 s, and 72°C for 20 s, followed by 5 min at 72°C . Melting curves for each PCR product were determined by measuring the decrease of fluorescence with increasing temperature (from 65 to 95°C). To confirm the size of the PCR products and to check that they corresponded to a unique and expected PCR product, the final PCR products were separated on a 2% (w/v) agarose gel and also sequenced. Primers used for *ZmFLS1* and *ZmFLS2* are listed in Table A1 in Appendix (*ZmFLS*-RT-forward, *ZmFLS1*-RT-reverse, and *ZmFLS2*-RT-reverse, respectively).

AMPLIFICATION OF ZmFLS GENES AND PROMOTERS, CLONING, AND MUTAGENESIS

To verify the presence of two *ZmFLS* genes in B73, primers were designed to amplify fragments downstream of the 3'UTR of each gene using the same forward primer (*ZmFLS*-3'UTR-forward, *ZmFLS1*-crom5-reverse, and *ZmFLS2*-crom5-reverse, Table A1 in Appendix). PCR reactions were made with *Platinum Pfx* Polymerase (Invitrogen) under the following conditions: 1 \times Pfx buffer, 1 \times enhancer, 2 mM MgSO_4 , 0.5 mM of each dNTP, 0.5 μM of each primer, 0.3 U *Platinum Pfx* Polymerase, and sterile water added to obtain a volume of 20 μl . Cycling conditions were as follows: 5 min denaturation at 95°C , 35 cycles at 20 s denaturation at 95°C , 30 s annealing at 50°C , 90 s amplification at 68°C .

The BAC clone (c0247D19) was obtained from Arizona Genomics Institute (AGI, Tucson, USA). *ZmFLS* genes were amplified from the BAC clone by PCR using the same forward primer (*ZmFLS*-cfs-forward) and specific primers for each

gene (*ZmFLS1*-RT-reverse and *ZmFLS2*-RT-reverse, Table A1 in Appendix) that hybridize in the 3'UTR. PCR reactions were performed with *Platinum Pfx* Polymerase (Invitrogen) as described above but with annealing at 60°C . The PCR products were purified from the gels and sequenced.

To amplify the introns of the *ZmFLS* genes from B73, primers were designed to hybridize in the exon 1 and 2 of genes (Table A1 in Appendix). PCR reaction were performed with GoTaq (Promega) and *Pfu* Polymerases (Invitrogen; 10:1) under the following conditions: 1 \times buffer, 2 mM MgCl_2 , 0.5 μM of each primer, 0.5 mM of each dNTP and 0.5 U of enzyme, in 25 μl of final volume under the following cycling condition: 5 min denaturation at 94°C ; 35 cycles at 94°C for 20 s, 57°C for 25 s, and 72°C for 40 s, followed by 7 min at 72°C .

To amplify *ZmFLS1* and *ZmFLS2* genes in different maize lines, PCR reaction were performed as described above for B73 using primers that hybridize in the exon 1 and 3'UTR of each gene (*ZmFLS*-intron2-forward, *ZmFLS1*-RT-reverse, and *ZmFLS2*-RT-reverse, respectively, Table A1 in Appendix).

To clone the promoter of *ZmFLS2* from the B73 genotype, primers were designed to amplify a 1.5-kb fragment upstream of the start codon, as predicted from www.maizesequence.com release 5b.60. Restriction sites *NotI* and *KpnI* were included in the forward and reverse primers, respectively (*NotI*-*ZmFLS2*-prom-forward and *KpnI*-*ZmFLS2*-prom-reverse, Table A1 in Appendix). Genomic DNA was isolated from leaf tissue using a DNA isolation kit (Qiagen). PCR reactions were performed with *Platinum Taq* Polymerase (Invitrogen) under the following condition: 1 \times buffer, 0.3% DMSO, 2 mM MgCl_2 , 0.5 μM of each primer, 0.5 mM of each dNTP, 100 ng genomic DNA, and 0.3 U *Platinum Taq* Polymerase in a volume of 25 μl . Cycling conditions were as follows: 30 s denaturation at 95°C , 30 s annealing at 68°C , 2 min amplification at 72°C , with a 1°C decrement of annealing temperature in each cycle until it reached 58°C , followed by 25 cycles of 30 s denaturation at 95°C , 30 s annealing at 58°C , 2 min amplification at 72°C . The PCR products were purified from the gels, digested with the corresponding restriction enzymes and purified. The *pZmFLS1::Luc* construct (pMSZ011; Falcone Ferreyra et al., 2010) was restricted with *NotI* and *KpnI* and the *ZmFLS1* promoter was replaced by the *ZmFLS2* promoter, resulting in the *pZmFLS2::Luc* construct. Proximal promoters of *ZmFLS1* and *ZmFLS2* from B73 were amplified by PCR using the *pZmFLS1::Luc* and *pZmFLS2::Luc* constructs as templates with *Platinum Taq* Polymerase and *Pfu* Polymerases (Invitrogen; 10:1) under the following conditions: 1 \times buffer, 2 mM MgCl_2 , 0.5 μM of each primer, 0.5 mM of dNTPs and 0.5 U of enzyme, in 25 μl of final volume under the following cycling condition: 5 min denaturation at 94°C ; 35 cycles at 94°C for 20 s, 59°C for 30 s, and 72°C for 50 s, followed by 7 min at 72°C . The PCR products were purified from the gels, digested with the corresponding restriction enzymes and purified, and the *ZmFLS* promoters (1.5 kb) in pMSZ011 were replaced by the *ZmFLS* proximal promoters. For *ZmFLS* proximal promoter analysis from different maize lines, DNA was extracted from leaves using the CTAB Method, and PCR reactions were performed with *Platinum Taq* Polymerase under the following conditions: 1 \times buffer, 2 mM MgCl_2 , 0.4% DMSO, 0.5 μM of each primer, 0.5 mM of each dNTP and 0.5 U of enzyme,

in 25 μ l final volume. Amplification conditions were as follows: 5 min denaturation at 94°C; 35 cycles at 94°C for 25 s, 59°C for 35 s, and 72°C for 50 s, followed by 5 min at 72°C. PCR products were purified, cloned into pGEM®-T-Easy vector (Promega) and sequenced.

Mutagenesis of the P1/C1 binding site at –250 bp in *ZmFLS* promoters was made by PCR using the same forward primer described above for amplification of proximal promoters but using a reverse oligonucleotide with the mutated binding site (*ZmFLS*prom-mut-reverse; **Table A1** in Appendix). PCR reactions were performed with GoTaq (Promega) and *Pfu* Polymerases (Invitrogen; 10:1) under the following conditions: 1 \times buffer, 2 mM MgCl₂, 0.5 μ M of each primer, 0.5 mM of each dNTP, and 0.5 U of enzyme, in 25 μ l final volume under the following cycling condition: 5 min denaturation at 94°C; 35 cycles at 94°C for 20 s, 50°C for 35 s, and 72°C for 50 s, followed by 7 min at 72°C. The PCR product (mega primer) was purified from the gel and then used as a forward primer (0.16 μ M) in a new PCR with the reverse primer (1 μ M) described above for proximal promoters (**Table A1** in Appendix). The PCR products were purified from the gels, digested with the corresponding restriction enzymes and purified, and the *ZmFLS1* proximal promoter in pMSZ011 was replaced by the *ZmFLS* mutated proximal promoter. Finally, mutations in the P1/C1 binding site were confirmed by sequencing.

CLONING, EXPRESSION AND PURIFICATION OF RECOMBINANT PROTEINS

The MYB domain of P1 (P1^{MYB}) cloned in pET19b (Invitrogen) was used for its expression in *E. coli* (Williams and Grotewold, 1997). The plasmid for the expression of the MYB domain of C1^{SH} was obtained by subcloning from pTYB2 vector (Hernandez et al., 2004) to pET19b. Thus, the C1^{SH} MYB domain was cut with *Nde*I and *Xho*I restriction enzymes and inserted in pET19b previously digested with the same enzymes. BL21(DE3)pLys cells were transformed with the clones described; P1^{MYB} expression was achieved by induction of the cell culture (250 ml) with 1 mM IPTG at 37°C for 3 h, while C1^{SHMYB} expression was done at 30°C with 0.5 mM IPTG for 6 h. Protein purification was essentially done as described by Williams and Grotewold (1997) with the following modifications. After binding the proteins to a Ni-NTA resin (Invitrogen) by rocking at 4°C for 1 h, the resin was loaded onto a column, washed three times with 15 volumes of binding buffer (50 mM sodium phosphate, pH 8.0, 500 mM NaCl, 20 mM imidazole, 5% glycerol and 1 mM phenylmethylsulfonyl fluoride), followed by three washes with seven volumes of washing buffer (50 mM sodium phosphate, pH 8.0, 500 mM NaCl, 5% glycerol, 50 mM imidazole). Elution was carried out by seven sequential additions of 1.5 ml of elution buffer (50 mM sodium phosphate, pH 8.0, 500 mM NaCl, 5% glycerol, and 200 mM imidazole). Finally, recombinant proteins were desalted in desalting buffer (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 5% glycerol, and 1 mM DTT) by four cycles of concentration and dilution using Amicon Ultra-15 3K (Millipore) and stored at –80°C. Protein levels were estimated both by comparison with dilution series of bovine serum albumin on a Coomassie Blue-stained SDS-PAGE and also using the Bradford reagent (Bio-Rad, Bradford, 1976). The yield of 90–95%

pure recombinant proteins obtained in these conditions was 4 and 6 mg/L of culture for P1^{MYB} and C1^{SHMYB}, respectively.

ELECTROPHORETIC MOBILITY SHIFT ASSAYS

End labeling of synthetic oligonucleotide probes (APB10, FLSbind-forward, comp1-forward, comp2-forward, and comp3-forward, **Table A1** in Appendix) was carried out using T4 polynucleotide kinase (Invitrogen) in the presence of a 2-M excess of [γ -³²P]ATP (>8,000 Ci/mmol). The labeled oligonucleotides were then annealed to equal amounts of complementary oligonucleotides (APB01, FLSbind-reverse, comp1-reverse, comp2-reverse, and comp3-reverse) by heating to 95°C and slowly cooling down to room temperature to generate APB1, FLSbind, comp1, comp2, and comp3 probes, respectively. A fraction of the double-stranded labeled oligonucleotides was precipitated on glass filters for quantification by scintillation of the radiation incorporated. The probe used as positive control (APB1) contains the high affinity P1-binding sites from the *a1* gene promoter, Protein-DNA incubations were performed essentially as described previously (Heine et al., 2004) with the following modifications. Approximately 2 μ g of purified proteins were incubated in a buffer containing 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 5% glycerol, 2 μ g of salmon sperm DNA, 1 mM DTT with 1 ng of end-labeled, double-stranded DNA probes, for 1 h at 4°C in the presence or absence of competitors as indicated in the Figures. Protein-DNA complexes were resolved on 8% polyacrylamide gels (30:0.8 acrylamide:bis-acrylamide) in 0.25 \times Tris-borate/EDTA (22.5 mM Tris-Borate and 0.5 mM EDTA) at 100 V for 90 min at 4°C. The gels were then dried onto Whatman paper and subjected to autoradiography at –80°C overnight.

TRANSIENT EXPRESSION EXPERIMENTS IN MAIZE PROTOPLASTS

The p35S::C1 + p35S::R, p35S::P1, p35S::Renilla, and p35S::BAR and p35S::GFP plasmids have all been previously described (Grotewold et al., 1994; Sainz et al., 1997; Hernandez et al., 2004, 2007).

Protoplasts from 11- to 13-day-old etiolated maize seedlings were obtained from kernels of B73xMo17 plant. After chopping second or third leaves into small pieces, leaf stripes were digested in 3% cellulase RS, 0.6% macerozyme R10 (both from Yakult Honsha Co., Japan), 0.6 M mannitol, 10 mM MES (pH 5.7), 5 mM CaCl₂, and 0.1% (w/v) BSA for 15 min under vacuum followed by 2:30 h gentle shaking (40 rpm) at 25°C in the dark. After releasing the protoplasts at 80g, the protoplasts were filtered through a 35- μ m nylon mesh and collected by centrifugation at 150g for 1 min. The protoplasts were washed in ES buffer (0.6 M mannitol, 5 mM MES, pH 5.7, 10 mM KCl) and counted with a hemocytometer. Electroporation was carried out on ~105 protoplasts with 40 μ g of total DNA per transformation, using 100 V/cm, 10 ms, and one pulse with a BTX Electro-Square-Porator T820. After electroporation, protoplasts were incubated for 18–22 h in the dark at RT before performing the luciferase reaction. Transformation efficiency was estimated following GFP expression by fluorescence microscopy. Transient expression assays for Luciferase and Renilla were performed essentially as previously described (Sheen, 1991; Feller et al., 2006; Hernandez et al., 2007).

GENERATION OF MAIZE TRANSGENIC PLANTS EXPRESSING pZmFLS1::ZmFLS1-mRFP

Construct was generated using the MultiSite Gateway® Pro 3.0 system (Invitrogen).

Two sets of primers (P1/P2, P3/P4; **Table A1** in Appendix) were designed for the amplification of two genomic fragments. The first set of primers amplified a fragment (P1–P2) that extends 2.483 kb upstream of the start codon to the tag insertion site within the coding sequence of *ZmFLS1* (just before the stop codon). The second set of primers amplified a fragment (P3–P4) from the tag insertion site to 2.1 kb downstream of the gene to include 3' UTR and regulatory sequences. P1 and P4 contained, in addition to gene-specific sequences, sequences partially overlapping the attB1 and attB2 Gateway forward and reverse primers, respectively (used for a second PCR). P2 and P3 contained sequences partially overlapping the RFP primers. A second PCR reaction, designated triple-template PCR (TT-PCR), utilized two primers containing the complete attB1 and attB2 Gateway sequences and partially overlapping the P1 and P4 primers. Thus, the RFP fluorescent tag was introduced into the *ZmFLS1* gene (C-terminal) and resulted in an internally tagged full-length gene sequence flanked by attB1 and attB2 sites ready for Gateway recombination cloning. The construct was recombined into the maize pTF101.1 binary vector, tagging *ZmFLS1* with *mRFP1* at the C-terminus. *Agrobacterium*-mediated transformation of maize Hill was performed by the Iowa State University Plant Transformation Facility. T0s were crossed to the inbred line B73 and T1s imaged live using a Zeiss 510 or 710 confocal laser-scanning microscope. Construct sequences and image metadata are available at <http://maize.jcvi.org/cellgenomics/index.shtml>.

PHYLOGENETIC ANALYSIS

The trees were constructed using MEGA 4.0 Software with the Neighbor-Joining method based on ClustalW multiple alignments (Tamura et al., 2007). For analysis of FLS proteins from monocot and dicot plants the following sequences were analyzed: *Pyrus communis* FLS (ABB70118), *Fragaria x ananassa* FLS (ABH07784.1), *Rosa hybrid* FLS (BAC66468), *Ricinus communis* FLS (XP_002513774), *Populus*

trichocarpa (XP_002301003.1), *Glycine max* FLS (AB246668.1), *Vitis vinifera* FLS1 (XP_002285838.1), *V. vinifera* FLS2 (BAE75809.1), *V. vinifera* FLS3 (XP_002284410.1), *V. vinifera* FLS4 (XP_002285839.1), *Lactuca sativa* FLS (BAG12186.1), *Petroselinum crispum* FLS (AAP57395.1), *Camellia sinensis* FLS (ACL98052), *Antirrhinum majus* FLS (ABB53382.1), *Eustoma grandiflorum* FLS (BAD34463), *Solanum tuberosum* FLS (ACN81826.1), *Petunia x hybrida* FLS (Q07512.1), *Nicotiana tabacum* FLS1 (ABE28017.1), *N. tabacum* FLS2 (BAF96939.1), *Citrus unshiu* FLS (BAA36554.1), *Allium cepa* FLS (AAO63023.1), *Epimedium sagittatum* FLS (ABY63659.1), *Hordeum vulgare* FLS (BAJ98444.1), *Setaria italica* FLS (Si017742m), *S. bicolor* FLS1 (Sb04g034240), *S. bicolor* FLS2 (Sb03g002040), *B. distachyon* FLS1 (Bd5g57910), *O. sativa* FLS1 (Os02g52840).

STATISTICAL ANALYSIS

Data presented were analyzed using one-way analysis of variance (ANOVA). Minimum significant differences were calculated by the Bonferroni, Holm–Sidak, Dunnett, and Duncan tests ($P < 0.05$) using the Statgraphics Plus 5.0 Software.

ACCESSION NUMBERS

Sequence data from this article can be found in the maize genome sequence (version 3b.60 at maizesequence.org), sorghum genome sequence (release Sbi1.4 at gramene.org), TIGR Rice Genome Annotation Project (release 7 at rice.plantbiology.msu.edu), *Brachypodium* genome sequence (GBrowse v1.0 at brachypodium.org), and GenBank databases under the following accession numbers: *ZmFLS1* (GRMZM2G152801); *ZmFLS2* (GRMZM2G069298); *SbFLS1* (Sb04g034240); *SbFLS2* (Sb03g002040); *OsFLS1* (Os02g52840); *BdFLS1* (Bradi3g57910); *Z. mays thio redoxin-like*, AW927774; *Z. mays actin1*, J01238.

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APPENDIX

Table A1 | Primers used for cloning, sequencing, RT-qPCR generation of maize transgenic plants, and EMSA.

Name	Sequence
<i>ZmFLS</i> -3'UTR-forward	5'TGTAAGGGCACTAATACATG3'
<i>ZmFLS</i> -cfs-forward	5'ATGGGGGGCGAGACGCACCTGAG3'
<i>ZmFLS1</i> -crom5-reverse	5'GAAAAGGCTCACTCGGTCTA3'
<i>ZmFLS2</i> -crom5-reverse	5'GCCCTGCAGTTCAGTAACTT3'
<i>ZmFLS</i> -intron2-forward	5'GTCAATGGTACGAGGCCAAG3'
<i>ZmFLS</i> -intron2-reverse	5'CATGGGGAGCTTGTGATCT3'
<i>NotI</i> - <i>ZmFLS2</i> prom-forward	5'CACCGCGCCGCGAGCCATGAAAAATGACAGT3'
<i>KpnI</i> - <i>ZmFLS2</i> prom-reverse	5'CTATGGTACCCGCGAGCAGGAAGCAGGACC'3
<i>NotI</i> - <i>ZmFLS</i> prom-int-forward	5'CACCGCGCCGCGCTATTACTCGTTAGTTGG'3
<i>ZmFLS</i> prom-mut-reverse	5'GGCCGTACGACGCACCCGATAGAAACTCGTTTA'3
<i>KpnI</i> - <i>ZmFLS1</i> prom-reverse	5'CTATGGTACCCGCGAGCAGGAAGCAGGGCC'3
<i>ZmFLS</i> -RT-forward	5'GACGGTGAACAAGGAGAAGAC'3
<i>ZmFLS1</i> -RT-reverse	5'GGCCATGCATGCGACTGGAAT'3
<i>ZmFLS2</i> -RT-reverse	5'ACAGAAGCGGGTGCACACTGC'3
<i>ZmActine1</i> -forward	5'CTTCGAATGCCAGCAAT3'
<i>ZmActine1</i> -reverse	5'CGGAGAATAGCATGAGGAAG3'
<i>ZmThioredoxine</i> -like-forward	5'GGACCAGAAGATTGCAGAAG3'
<i>ZmThioredoxine</i> -like-reverse	5'ACGGATGTCCCATGAAGA3'
APB10	5'GATCCGGGTCAGTGACTACCAACCTTAAACAC3'
APB01	5'GATCGTGTTTAAGTTGGTAGTACACTGACCCG3'
FLSbind-forward	5'GAGTTTCTGGTAGGTGCGTACGTCAGCCAG3'
FLSbind-reverse	5'CTGGCCGTACGACGCACCTACCAGAAACTC3'
Comp1-forward	5'CTATTACTCGTTAGTTGGATTTTAGTTT3'
Comp1-reverse	5'AAACTAAAATCCAACCTAACGAGTAATAG3'
Comp2-forward	5'CCAAGTCAAAATCCAACCATTATCTCTTGA3'
Comp2-reverse	5'TCAAGAGATAATGGTTGGATTTTGACTTGG3'
Comp3-forward	5'TAGATGTGATTCGTTAGGTGTGTTTTATAA3'
Comp3-reverse	5'TTATAAAAACACACCTAACGAATCACATCTA3'
P1	5'ggggacaagttgtacaaaaagcaggcttaGCGACGAATCCATTCTTGA3'
P2	5'ggggacaactttgatagaaaagttgggtgCATGGGAGCTTGTGATCTTGC3'
P3	5' ggggacaactttgtataataaagttgcaTAATTATGTAGCTCGGGTTC3'
P4	5'ggggaccactttgtacaagaagctgggtaTATTGCTGGCGTGAATAACC3'

A

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GRMZM2G152801 -----AGATAGGCCACTAGACACGTTTGGTGCATATTAGACCAA -1417
GRMZM2G069298 AGCCATGAAAAATGACAGTAAAATTAGATCCCTTCACCAACACCTGAGGGGTTAGGTTAA -1398
                        *   * * *   * * *   * *
                        * * * * * * * * * * * * * * * * * * * * * *

GRMZM2G152801 AGAGTTCAATTTACATGCTCTCTCTAAGAGAAAACAATATTATATAATTAGACCTTCGGT -1357
GRMZM2G069298 AAACATCATTTGAACA-----ATTAATAAAGGAAAAATCTCAACTTACTCAAATGCTTTAT -1343
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

GRMZM2G152801 TCA---AGCATGCAAGCGAGCCTCCTATATGTTAGATCATGATCGAACCTAGATATAGA -1300
GRMZM2G069298 TAATTGGACAAAAAGATGAGCTGCTCAACTAGTGTCTACAAGATCCAAC-----AACTATA -1288
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

GRMZM2G152801 TGTAGTTCGTTAGGTGTGTTTTTTTATAAGTCTACATAAGTTATGGCAGAAGTGTTTAAG -1240
GRMZM2G069298 TGTGATGCGCTAGAAGCTCTCTAATCGCACCCAAATGATGAACTGCTAGATGAAATGAG -1228
*** * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

GRMZM2G152801 TGTAAATGTGATATATGATTGAATCAGTAT-CTAACGGACCAAGAGCCTCACTTATAGG -1181
GRMZM2G069298 TGAAATGTGTTTCTCTAGTCTCTAATAGGTGTTCTCAAGTGTTAGGAG--TTAAGGAAAC -1170
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

GRMZM2G152801 GTGTTTGGTTTGAGGAATAAGCTAGTCCACCATCT--TCTCACTTCTCACTTTTTTTTGT -1124
GRMZM2G069298 TAGCCAAGGTTGGACAAAGGGTCTATTTATAGCCAAGTCAAATCCAACCATTTCTCT -1110
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

GRMZM2G152801 TTGATTTGTGGAATGGAATGGGTTGATCCATCACACCTTATTTCTTATAGTTAATAATA -1064
GRMZM2G069298 TGAAGGGATAAAAATGGAGGCATCAAAC-ATGTCTATGCACTATCAGTTTTTGCACCAG -1051
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

GRMZM2G152801 ACATGAGGAATGAGGTCATCCC-ACCAAATTTGA---GGAATAAATCATGATGAACCA -1008
GRMZM2G069298 ACACATCCAGTGTCTTACAATGACTATATTTCAACTGGTATAAATAGTTGTTAATTG -991
*** * * * * * * * * * * * * * * * * * * * * * * * * * * * *

GRMZM2G152801 CATCATATTAGATGGAGTGATTACACAACCAAAACACCCC-TTAGGCAATCTCTAGCAGG -949
GRMZM2G069298 TGCCATAT---ATCTGGTG---CATCCACAGATATGCTCACTAGACTGTTTGGTGCATA -938
***** * * * * * * * * * * * * * * * * * * * * * * * * * *

GRMZM2G152801 CCGTGTAAAAGATCGTGCAAAGTAC-TGTTTTGTAACGTAGATTACACTATTTCTAGAGT -890
GRMZM2G069298 TTAGACCAAAGA--GTTCAATTTTACATGCTCTCTAAGAGAAAACAATATT-----AT -886
***** * * * * * * * * * * * * * * * * * * * * * * * * * *

GRMZM2G152801 GATGTTTGAATAATGAGCGAGATTGCATTTAAAGCCACATTGCTGTTAGAGATGGCTAA -830
GRMZM2G069298 GTAATTAGGCATTAGGTTTAAGCGTCAAGTGA-GCCTCCTATCTGTTAGATCA---TGA -830
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

GRMZM2G152801 ACGGGCCGCCCGGCCCGGCCCGGCCGTTTGGAGTCTGGCCCGCAAGCACGGTTAGAAA -770
GRMZM2G069298 TCGTACCCTAGATATAGATGTGATTCGT--TAGGTGTGTTTTTATAAATCTGCATAAGAT -772
** * * * * * * * * * * * * * * * * * * * * * * * * * * * *

GRMZM2G152801 ATCGGGTCGGACCGTCTAAGCACGCGAGCTCAATTTTCATGCTCTGAGTTCGGCTCGCAGG -710
GRMZM2G069298 GGTGG-TGGAAGGGTTAAGTGT-TAAATGTAACAT-ATGATTGAAT-CAATATCTAACG -716
* * * * * * * * * * * * * * * * * * * * * * * * * * * * *

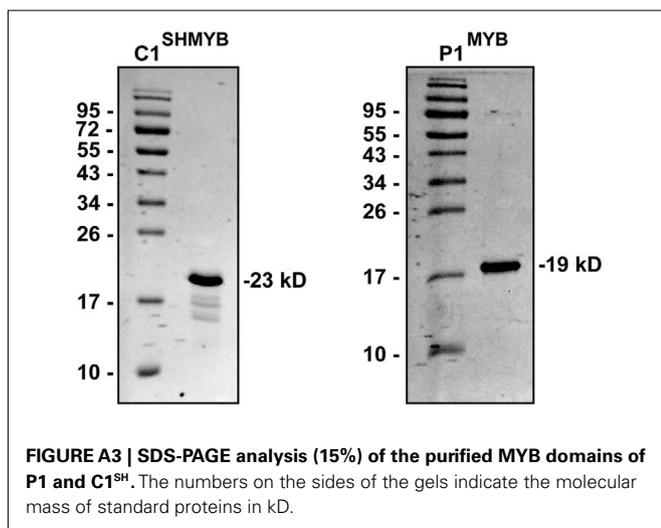
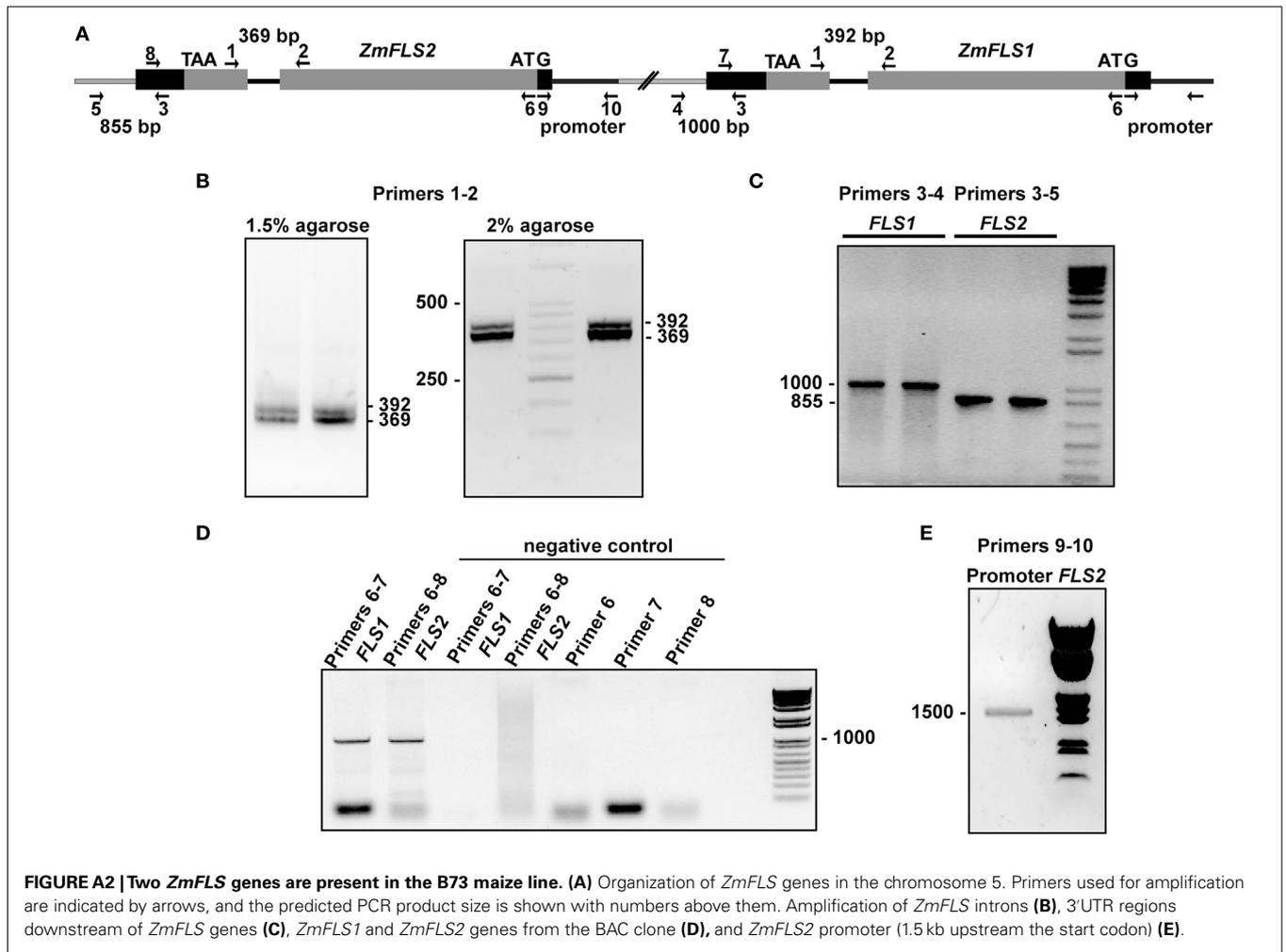
GRMZM2G152801 TGCCGAAAAGCGGGCTATACGGGCTGTAACCACGTTTTAGTGTAAAAGCAGGCTTAAC -650
GRMZM2G069298 GAACAAGAGCCTCACTTAAAGGGTGTGG-----TTTGAGGAATAAGCTAGCTCATT -663
* * * * * * * * * * * * * * * * * * * * * * * * * * * * *

GRMZM2G152801 GGTCTTAGAGATAAACGGTCTGTCGGGGCTAGCGCTA---GCCACCAT-GCCTAGTT -595
GRMZM2G069298 ATCTTCTCACTTCTCAGTTTTTTGTTGATTTGTGGAATGGAATCCATCACACCTTATT -603
* * * * * * * * * * * * * * * * * * * * * * * * * * * * *

GRMZM2G152801 TCCTGTCTGAGCCCGACCCGTATAGAACCTGGTCTGCGGACTCGGGCCGATCCAAC -535
GRMZM2G069298 CCCTATAGTAATT-AGTTAGTATTAACATGAGAAATGAGGTCATCTACCAAATTTGAAG -544
*** * * * * * * * * * * * * * * * * * * * * * * * * * * * *

GRMZM2G152801 AACGGGCT--TCGTAC-CGGCCTCGCGGACTCGTGCTTATT--GGCCATCTATAATTGC -480
GRMZM2G069298 AATAAATCATGATACACCACATCATATTAGATGGAGTGATCCACACACCAACACCCAT -484
** * * * * * * * * * * * * * * * * * * * * * * * * * * * *
    
```

FIGURE A1 | (Continued).



A

Mishca CCAAGTACGTGCCCGACGCACTCATCGTCCATATCGGGGATCAGATGAGGCAAGCGACC 60
W22-1 CCAAGTACGTGCCCGACGCACTCATCGTCCATATCGGGGATCAGATCGAGGCAAGCGACC 60
Cachuacintle CCAAGTACGTGCCCGACGCACTCATCGTCCATATCGGGGATCAGATCGAGGCAAGCGACC 60
W23b,pl CCAAGTACGTGCCCGACGCACTCATCGTCCATATCGGGGATCAGATCGAGGCAAGCGACC 60
Mo17 CCAAGTACGTGCCCGACGCACTCATCGTCCATATCGGGGATCAGATCGAGGCAAGCGACC 60
Arrocillo CCAAGTACGTGCCCGACGCACTCATCGTCCATATCGGGGATCAGATCGAGGCAAGCGACC 60
A619 CCAAGTACGTGCCCGACGCACTCATCGTCCATATCGGGGATCAGATCGAGGCAAGCGACC 60
B73 CCAAGTACGTGCCCGACGCACTCATCGTCCATATCGGGGATCAGATCGAGGCAAGCGACC 60
Confite CCAAGTACGTGCCCGACGCACTCATCGTCCATATCGGGGATCAGATCGAGGCAAGCGACC 60
Conico CCAAGTACGTGCCCGACGCACTCATCGTCCATATCGGGGATCAGATCGAGGCAAGCGACC 60
***** * *****

Mishca TACGTAGCATCTTTTTTTTTTTCATTCTATTGTGTTGTGATGATCTTGCTCCC-TCAAGTA 119
W22 TACGTAGCATCTTTTTTTTTT-CATTCTATTGTGTTGTGATGATCTTGCTCCC-TCAAGTA 118
Cachuacintle TACGTAGCATCTTTTTTTTTTTCATTCTATTGTGTTGTGATGATCTTGCTCCC-TCAAGTA 118
W23b,pl TACGTAGCATCTTTTTTTTTT-CATTCTATTGTGTTGTGATGATCTTGCTCCC-TCAAGTA 118
Mo17 TACGTAGCATCTTTTTTTTTTTCATTCTATTGTGTTGTGATGATCTTGCTCCC-TCAAGTA 119
Arrocillo TACGTAGCATCTTTTTTTTTTTCATTCTATTGTGTTGTGATGATCTTGCTCCC-TCAAGTA 115
A619 TACGTAGCATCTTTTTTTTTTTCATTCTATTGTGTTGTGATGATCTTGCTCCC-TCAAGTA 119
B73 TACGTAGCATCTTTTTTTTTTTCATTCTATTGTGTTGTGATGATCTTGCTCCC-TCAAGTA 119
Confite TACGTAGCATCTTTTTTTTTTTCATTCTATTGTGTTGTGATGATCTTGCTCCC-TCAAGTA 118
Conico TACGTAGCTTCTTTTTTTTTT-CATTCTATTGTGTTGTGATGATCTTGCTCCC-TCAAGTA 118
***** * *****

Mishca TTTC TTGATGTCGGACAAC TACTA ACTGGTACGAACCGATCGAGCAGATTTTCAGCAAC 179
W22 TTTC TTGATGTCGGACAAC TACTA ACTGGTACGAACCGATCGAGCAGATTTTCAGCAAC 178
Cachuacintle TTTC TTGATGTCGGACAAC TACTA ACTGGTACGAACCGATCGAGCAGATTTTCAGCAAC 178
W23b_pl TTTC TTGATGTCGGACAAC TACTA ACTGGTACGAACCGATCGAGCAGATTTTCAGCAAC 178
Mo17 TTTC TTGATGTCGGACAAC TACTA ACTGGTACGAACCGATCGAGCAGATTTTCAGCAAC 179
Arrocillo TTTC TTGATGTCGGACAAC TACTA ACTGGTACGAACCGATCGAGCAGATTTTCAGCAAC 152
A619 TTTC TTGATGTCGGACAAC TACTA ACTGGTACGAACCGATCGAGCAGATTTTCAGCAAC 179
B73 TTTC TTGATGTCGGACAAC TACTA ACTGGTACGAACCGATCGAGCAGATTTTCAGCAAC 179
Confite TTTC TTGATGTCGGACAAC TACTA ACTGGTACGAACCGATCGAGCAGATTTTCAGCAAC 178
Conico TTTC TTGATGTCGGACAAC TACTA ACTGGTACGAACCGATCGAGCAGATTTTCAGCAAC 178
***** * *****

Mishca GGGGCATACAAGGCGGTGCTGCACCGTACGACGGTGAACAAGGAGAAGACGCGGATGTCA 239
W22 GGGGCATACAAGGCGGTGCTGCACCGTACGACGGTGAACAAGGAGAAGACGCGGATGTCA 238
Cachuacintle GGGGCATACAAGGCGGTGCTGCACCGTACGACGGTGAACAAGGAGAAGACGCGGATGTCA 238
W23b,pl GGGGCATACAAGGCGGTGCTGCACCGTACGACGGTGAACAAGGAGAAGACGCGGATGTCA 238
Mo17 GGGGCATACAAGGCGGTGCTGCACCGTACGACGGTGAACAAGGAGAAGACGCGGATGTCA 239
Arrocillo GGGGCATACAAGGCGGTGCTGCACCGTACGACGGTGAACAAGGAGAAGACGCGGATGTCA 212
A619 GGGGCATACAAGGCGGTGCTGCACCGTACGACGGTGAACAAGGAGAAGACGCGGATGTCA 239
B73 GGGGCATACAAGGCGGTGCTGCACCGTACGACGGTGAACAAGGAGAAGACGCGGATGTCA 239
Confite GGGGCATACAAGGCGGTGCTGCACCGTACGACGGTGAACAAGGAGAAGACGCGGATGTCA 238
Conico GGGGCATACAAGGCGGTGCTGCACCGTACGACGGTGAACAAGGAGAAGACGCGGATGTCA 238
***** * *****

Mishca TGGCCGATGTTCTGGAGCCGCCGGGGAGCTCGTCTCGGGCCGCACCCCAAGCTGGTC 299
W22 TGGCCGATGTTCTGGAGCCGCCGGGGAGCTCGTCTCGGGCCGCACCCCAAGCTGGTC 297
Cachuacintle TGGCCGATGTTCTGGAGCCGCCGGGGAGCTCGTCTCGGGCCGCACCCCAAGCTGGTC 298
W23b,pl TGGCCGATGTTCTGGAGCCGCCGGGGAGCTCGTCTCGGGCCGCACCCCAAGCTGGTC 298
Mo17 TGGCCGATGTTCTGGAGCCGCCGGGGAGCTCGTCTCGGGCCGCACCCCAAGCTGGTC 299
Arrocillo TGGCCGATGTTCTGGAGCCGCCGGGGAGCTCGTCTCGGGCCGCACCCCAAGCTGGTC 272
A619 TGGCCGATGTTCTGGAGCCGCCGGGGAGCTCGTCTCGGGCCGCACCCCAAGCTGGTC 299
B73 TGGCCGATGTTCTGGAGCCGCCGGGGAGCTCGTCTCGGGCCGCACCCCAAGCTGGTC 299
Confite TGGCCGATGTTCTGGAGCCGCCGGGGAGCTCGTCTCGGGCCGCACCCCAAGCTGGTC 298
Conico TGGCCGATGTTCTGGAGCCGCCGGGGAGCTCGTCTCGGGCCGCACCCCAAGCTGGTC 298
***** * *****

FIGURE A4 | (Continued).

Mishca	ACGGAGGAGAGCCCGCCAAGTACAAGGCCAAGAAGTACAAGGACTACCAGCACTGCAAG	359
W22	ACGGAGGAGAGCCCGCCAAGTACAAGGCCAAGAAGTACAAGGACTACCAGCACTGCAAG	357
Cachuacintle	ACGG---AGAGCCCGCCAAGTACAAGGCCAAGAAGTACAAGGACTACCAGCACTGCAAG	355
W23b,pl	ACGGAGGAGAGCCCGCCAAGTACAAGGCCAAGAAGTACAAGGACTACCAGCACTGCAAG	358
Mo17	ACGGAGGAGAGCCCGCCAAGTACAAGGCCAAGAAGTACAAGGACTACCAGCACTGCAAG	359
Arrocillo	ACGGAGGAGAGCCCGCCAAGTACAAGGCCAAGAAGTACAAGGACTACCAGCACTGCAAG	332
A619	ACGGAGGAGAGCCCGCCAAGTACAAGGCCAAGAAGTACAAGGACTACCAGCACTGCAAG	359
B73	ACGGAGGAGAGCCCGCCAAGTACAAGGCCAAGAAGTACAAGGACTACCAGCACTGCAAG	359
Confite	ACGGAGGAGAGCCCGCCAAGTACAAGGCCAAGAAGTACAAGGACTACCAGCACTGCAAG	358
Conico	ACGGAGGAGAGCCCGCCAAGTACAAGGCCAAGAAGTACAAGGACTACCAGCACTGCAAG	358
	**** *	
Mishca	ATCAACAAGCTCCCCATG TAA TTATGTAGCTCGGGTCTACTGTCGTGTGCACCCGCTTC	419
W22	ATCAACAAGCTCCCCATG TAA TTATGTAGCTCGGGTCTACTGTCGTGTGCACCCGCTTC	417
Cachuacintle	ATCAACAAGCTCCCCATG TAA TTATGTAGCTCGGGTCTACTGTCGTGTGCACCCGCTTC	415
W23b,pl	ATCAACAAGCTCCCCATG TAA TTATGTAGCTCGGGTCTACTGTCGTGTGCACCCGCTTC	418
Mo17	ATCAACAAGCTCCCCATG TAA TTATGTAGCTCGGGTCTACTGTCGTGTGCACCCGCTTC	419
Arrocillo	ATCAACAAGCTCCCCATG TAA TTATGTAGCTCGGGTCTACTGTCGTGTGCACCCGCTTC	392
A619	ATCAACAAGCTCCCCATG TAA TTATGTAGCTCGGGTCTACTGTCGTGTGCACCCGCTTC	419
B73	ATCAACAAGCTCCCCATG TAA TTATGTAGCTCGGGTCTACTGTCGTGTGCACCCGCTTC	419
Confite	ATCAACAAGCTCCCCATG TAA TTATGTAGCTCGGGTCTACTGTCGTGTGCACCCGCTTC	418
Conico	ATCAACAAGCTCCCCATG TAA TTATGTAGCTCGGGTCTACTGTCGTGTGCACCCGCTTC	418
	***** *	
Mishca	TGTAATTTTCAGTCTACCATATTTAAGTCACATAACCGTTATTCCAGTCGCATGCATGGCC	479
W22	TGTAATTTTCAGTCTACCATATTTAAGTCACATAACCGTTATTCCAGTCG GGGG GATGG GG	477
Cachuacintle	TGTAATTTTCAGTCTACCATATTTAAGTCACATAACCGTTATTCCAGTCG GGGG GATGGCC	475
W23b,pl	TGTAATTTTCAGTCTACCATATTTAAGTCACATAACCGTTATTCCAGTCG GGGG GATGGCC	478
Mo17	TGTAATTTTCAGTCTACCATATTTAAGTCACATAACCGTTATTCCAGTCG AGGG GATGGCC	479
Arrocillo	TGTAATTTCA T TCTACCATATTTAA T TCACATAACCGTTATTCCAG A CGCATGGATGGCC	452
A619	TGTAATTTTCAGTCTACCATATTTAAGTCACATAACCGTTATTCCAGTCGCATGGATGGCC	479
B73	TGTAATTTTCAGTCTACCATATTTAAGTCACATAACCGTT ATTCCAGTCGCATGCATGGCC	479
Confite	TGTAATT - CAGTCTACCATATTTAA AA CATATGACCA A ATTCCAGTCGCATGCATGGCC	477
Conico	TGTAATT - CAGTCTACCATATTT CT GTCT CT TCACCGTAATTCCAG A CGCATGCT T GGCC	477
	***** ** *	

FIGURE A4 | (Continued).

B

B73 CCAAGTACGTGCCGACGCACTCATCGTCCATATCGGCGATCAGATCGAGG-CAAGCGAC 59
 Arrocillo CCAAGTACGTGCCGACGCACTCATCGTCCATATCGGCGATCAGATCGAGG-CAAGCGAC 59
 A619 CCAAGTACGTGCCGACGCACTCATCGTCCATATCGGCGATCAGATCGAGG-CAAGCGAC 59
 Cacahuacintle CCAAGTACGTGCCGACGCACTCATCGTCCATATCGGCGATCAGATCGAGG-CAAGCGAC 59
 Conico CCAAGTACGTGCCGACGCACTCATCGTCCATATCGGCGATCAGATCGAGG-CAAGCGAC 59
 Confite CCAAGTACGTGCCGACGCACTCATCGTCCATATCGGCGATCAGATCGAGG-CAAGCGAC 59
 W23b,pl CCAAGTACGTGCCGACGCACTCATCGTCCATATCGGCGATCAGATCGAGG-CAAGCGAC 59
 Mishca CCAAGTACGTGCCGACGCACTCATCGTCCATATCGGCGATCAGATCGAGG-CAAGCGAC 59
 Mo17 CCAAGTACGTGCCGACGCACTCATCGTCCATATCGGCGATCAGATCGAGG-CAAGCGAC 60
 W22 CCAAGTACGTGCCGACGCACTCATCGTCCATATCGGCGATCAGATCGAGG-CAAGCGAC 59

B73 CTACG----TAGCATCTTTTTTTTT-CTTCTATTGTGTTGTGATGATCTTGCTCCCT 113
 Arrocillo CTACG----TAGCATCTTTTTTTTT-CTTCTATTGTGTTGGATGATCTTCTCCCT 113
 A619 CTACG----TAGCATCTTTTTTTTT-CTTCTATTGTGTTGTGATGATCTTGCTCCCT 113
 Cacahuacintle CTACGCTACGTAGCATCTTTTTTTTT-CTTTTATTGTGTTGTGATGATCTTGCTCCCT 118
 Conico CTACG----TAGCATCTTTTTTTTT-CTTCTATTGTGTTGTGATGATCTTGCTCCCT 113
 Confite CTACG----TAGCATCTTTTTTTTT-CTTCTATTGTGTTGTGATGATCTTGCTCCCT 113
 W23b,pl CTACG----TAGCATCTTTTTTTTT-CAATTCATTGTGTTGTGATGATCTTGCTCCCT 112
 Mishca CTACG----TAGCATCTTTTTTTTT-CAATTCATTGTGTTGTGATGATCTTGCTCCCT 112
 Mo17 CTACG----TAGCATCTTTTTTTTTTCAATTCATTGTGTTGTGATGATCTTGCTCCCT 114
 W22 CTACG----TAGCATCTTTTTTTTT-CTTCTATTGTGTTGTGATGATCTTGCTCCCT 113

B73 CAAGTATTTCTTTGATGTC-----CCGATCGAGTAGATTTTC 150
 Arrocillo CAAGTATTTATGTGATGTC-----CCGATCAAGTAGATTTTC 150
 A619 CAAGTATTTCTTTGATGTC-----CCGATCGAGTAGATTTTC 150
 Cacahuacintle CAAGTATTTCTTTGATGTC-----CCGATCGAGTAGATTTTC 155
 Conico CAAGTATTTCTTTGATGTC-----CCGATCGAGTAGATTTTC 150
 Confite CAAGTATTTCTTTGATGTC-----CCGATCGAGTAGATTTTC 150
 W23b,pl CAAGTATTTCTTTGATGTCGGACAACACTACTAAGTGGTACGAA CCGATCGAGCAGATTTTC 172
 Mishca CAAGTATTTCTTTGATGTCGGACAACACTACTAAGTGGTACGAA CCGATCGAGCAGATTTTC 172
 Mo17 CAAGTATTTCTTTGATGTCGGACAACACTACTAAGTGGTACGAA CCGATCGAGCAGATTTTC 174
 W22 CAAGTAAATTTGATGTTA-----CGATCGAGTAGATTTTC 150

B73 AGCAACGGGGCATAACAAGGCGGTGCTGCACCGTACGACGGTGAACAAGGAGAAGACGCGG 210
 Arrocillo AGCAACGGGGCATAACAAGGCGGTGCTGCACCGTACGACGGTGAACAAGGAGAAGACGCGG 210
 A619 AGCAACGGGGCATAACAAGGCGGTGCTGCACCGTACGACGGTGAACAAGGAGAAGACGCGG 210
 Cacahuacintle AGCAACGGGGCATAACAAGGCGGTGCTGCACCGTACGACGGTGAACAAGGAGAAGACGCGG 215
 Conico AGCAACGGGGCATAACAAGGCGGTGCTGCACCGTACGACGGTGAACAAGGAGAAGACGCGG 210
 Confite AGCAACGGGGCATAACAAGGCGGTGCTGCACCGTACGACGGTGAACAAGGAGAAGACGCGG 210
 W23b,pl AGCAACGGGGCATAACAAGGCGGTGCTGCACCGTACGACGGTGAACAAGGAGAAGACGCGG 232
 Mishca AGCAACGGGGCATAACAAGGCGGTGCTGCACCGTACGACGGTGAACAAGGAGAAGACGCGG 232
 Mo17 AGCAACGGGGCATAACAAGGCGGTGCTGCACCGTACGACGGTGAACAAGGAGAAGACGCGG 234
 W22 AGCAACGGGGCATAACAAGGCGGTGCTGCACCGTACGACGGTGAACAAGGAGAAGACGCGG 210

B73 ATGTCATGGCCGATGTTCTGTTGGAGCCGCGGGGGAGCTCGTCGTCGGGCCGACCCCAAG 270
 Arrocillo ATGTCATGGCCGATGTTCTGTTGGAGCCGCGGGGGAGCTCGTCGTCGGGCCGACCCCAAG 270
 A619 ATGTCATGGCCGATGTTCTGTTGGAGCCGCGGGGGAGCTCGTCGTCGGGCCGACCCCAAG 270
 Cacahuacintle ATGTCATGGCCGATGTTCTGTTGGAGCCGCGGGGGAGCTCGTCGTCGGGCCGACCCCAAG 275
 Conico ATGTCATGGCCGATGTTCTGTTGGAGCCGCGGGGGAGCTCGTCGTCGGGCCGACCCCAAG 270
 Confite ATGTCATGGCCGATGTTCTGTTGGAGCCGCGGGGGAGCTCGTCGTCGGGCCGACCCCAAG 270
 W23b,pl ATGTCATGGCCGATGTTCTGTTGGAGCCGCGGGGGAGCTCGTCGTCGGGCCGACCCCAAG 292
 mishca ATGTCATGGCCGATGTTCTGTTGGAGCCGCGGGGGAGCTCGTCGTCGGGCCGACCCCAAG 292
 Mo17 ATGTCATGGCCGATGTTCTGTTGGAGCCGCGGGGGAGCTCGTCGTCGGGCCGACCCCAAG 294
 W22 ATGTCATGGCCGATGTTCTGTTGGAGCCGCGGGGGAGCTCGTCGTCGGGCCGACCCCAAG 270

FIGURE A4 | (Continued).

```

B73          CTGGTCACGGAGGAGAGCCCGGCCAAGTACAAGGCCAAGAAGTACAAGGACTACCAGCAC 330
Arrocillo    CTGGTCACGGAGGAGAGCCCGGCCAAGTACAAGGCCAAGAAGTACAAGGACTACCAGCAC 330
A619         CTGGTCACGGAGGAGAGCCCGGCCAAGTACAAGGCCAAGAAGTACAAGGACTACCAGCAC 330
Cacahuacintle CTGGTCACGGAGGAGAGCCCGGCCAAGTACAAGGCCAAGAAGTACAAGGACTACCAGCAC 335
Conico       CTGGTCACGGAGGAGAGCCCGGCCAAGTACAAGGCCAAGAAGTACAAGGACTACCAGCAC 330
Confite      CTGGTCACGGAGGAGAGCCCGGCCAAGTACAAGGCCAAGAAGTACAAGGACTACCAGCAC 330
W23b,pl     CTGGTCACGGAGGAGAGCCCGGCCAAGTACAAGGCCAAGAAGTACAAGGACTACCAGCAC 352
Mishca      CTGGTCACGGAGGAGAGCCCGGCCAAGTACAAGGCCAAGAAGTACAAGGACTACCAGCAC 352
Mo17        CTGGTCACGGAGGAGAGCCCGGCCAAGTACAAGGCCAAGAAGTACAAGGACTACCAGCAC 354
W22         CTGGTCACGGAGGAGAGCCCGGCCAAGTACAAGGCCAAGAAGTACAAGGACTACCAGCAC 330
*****

B73          TGCAAGATCAACAAGCTCCCCATGTAATTATGTAGCTCGGGTTCTACTGTCGTG----- 384
Arrocillo    TGCAAGATCAACAAGCTCCCCATGTAATTATGTATTGTCGGTTTCTACTGCGGTGTGCAGG 390
A619         TGCAAGATCAACAAGCTCCCCATGTAATTATGTAGCTCGGGTTCTACTGTCGTG----- 384
Cacahuacintle TGCAAGATCAACAAGCTCCCCATGTAATTATGTAGCTCGGGTTCTACTGTCGTG----- 389
Conico       TGCAAGATCAACAAGCTCCCCATGTAATTATGTAGCTCGGGTTCTACTGTCGTG----- 384
Confite      TGCAAGATCAACAAGCTCCCCATGTAATTATGTAGCTCGGGTTCTGCTGCCATG----- 384
W23b,pl     TGCAAGATCAACAAGCTCCCCATGTAATTATGTACCTCGGGTTCTACTG----- 401
Mishca      TGCAAGATCAACAAGCTCCCCATGTAATTATGTAGCTCGGGTTCTACTG----- 401
Mo17        TGCAAGATCAACAAGCTCCCCATGTAATTATGTAGCTCGGGTTCTACTGTCGTG----- 408
W22         TGCAAGATCAACAAGCTCCCCATGTAATTATGTAGCTCGGGTTCTATTGT----- 380
*****

B73          ---CAGTGTGCACCCGCTTCTGT 404
Arrocillo    CTTCAGTGTGCACCCGCTTCTGT 414
A619         ---CAGTGTGCCCCGGCTTCTGT 404
Cacahuacintle ---CAGTGTGCACCCCCTTCTGT 409
Conico       ---CAGTGTGCCCCCCTTCTGT 404
Confite      ---CAATGTGCACCCGCTTCTGT 404
W23b,pl     ---CAGTGTGCACCCCCTTCTGC 421
Mishca      ---CAGTGTGCACCCCCTTTTTT 421
Mo17        ---CAGTGTGCACCCGCTTCTGT 431
W22         ---AGTGTGCACCCGCTTCTGT 399
* * * * *
    
```

FIGURE A4 | Sequence comparison of partial *ZmFLS1* (A) and *ZmFLS2* (B) genes in different maize lines. The regions of *ZmFLS1* and *ZmFLS2* genes range from +740 bp (exon 1) to +1218 and +1143 bp, respectively (3'UTR). The introns in *ZmFLS* genes are

highlighted in gray, different nucleotides and insertions or deletions are highlighted in yellow. Stop codons are indicated in bold-underlined letters. Primers used for RT-qPCR are in bold-underlined letters on B73 sequences.

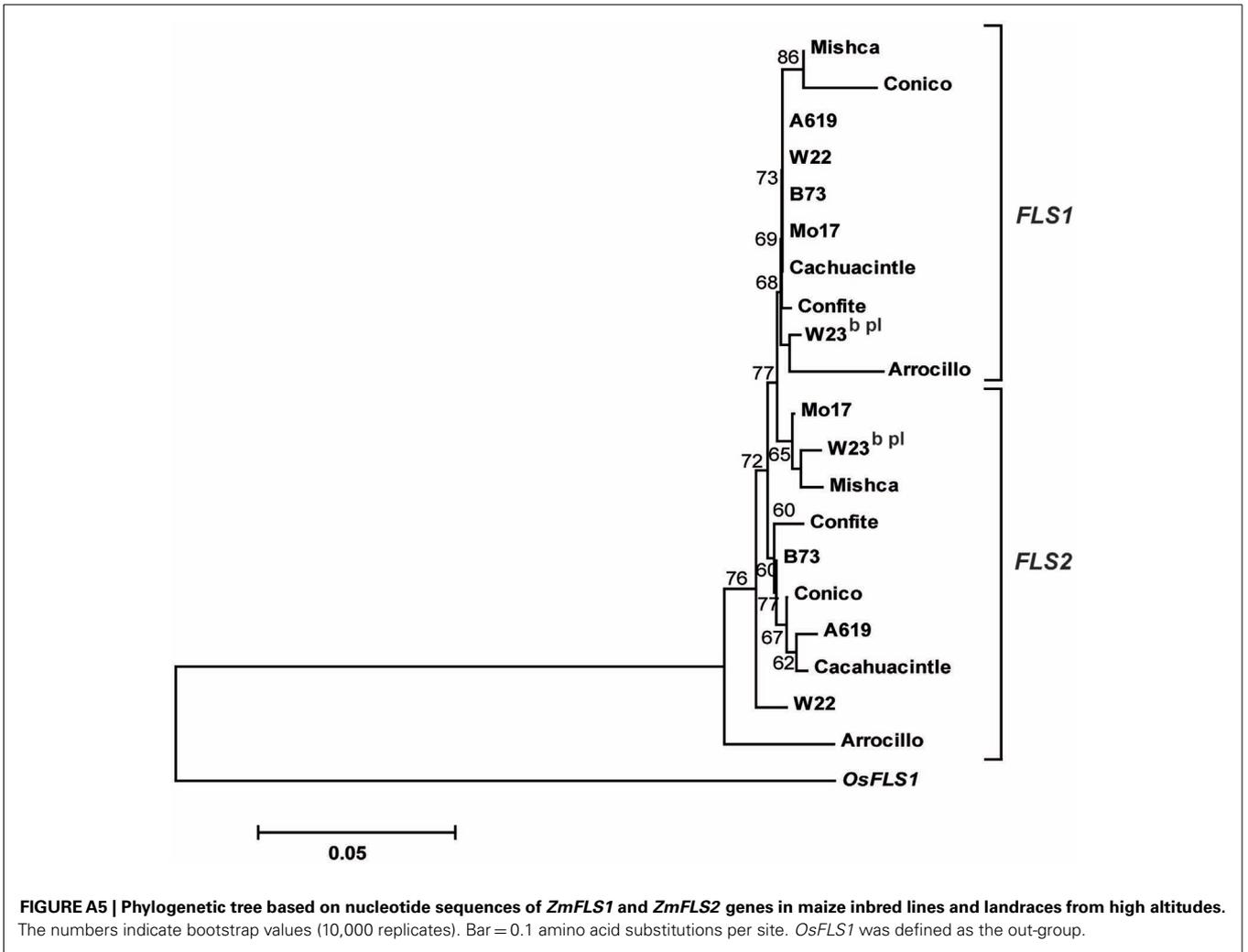


FIGURE A5 | Phylogenetic tree based on nucleotide sequences of *ZmFLS1* and *ZmFLS2* genes in maize inbred lines and landraces from high altitudes. The numbers indicate bootstrap values (10,000 replicates). Bar = 0.1 amino acid substitutions per site. *OsFLS1* was defined as the out-group.

A

```

B73          GCTATTACTCCTTAGTGGATTTTAGTTTTCTAAGAAGTTGAAGTAGGGTCAGGGCACAG 60
W23b,pl     GCTATTACTCCTTAGTGGATTTTAGTTTTCTAAGAAGTTGAAGTAGGGTCAGGGCACAG 60
Mo17        GCTATTACTCCTTAGTGGATTTTAGTTTTCTAAGAAGTTGAAGTAGGGTCAGGGCACAG 60
Confite     CGTTATTACTCCTTAGTGGATTTTAGTTTTCTAAGAAGTTGAAGTAGGGTCAGGGCACAG 60
Conico      GCTATTACTCCTTAGTGGGTTTTAGTTTTCTAAGAAGTTGAAGTAGGGTCAGGGCACAG 60
Mishca      GCTATTACTCCTTAGTGGGTTTTAGTTTTCTAAGAAGTTGAAGTAGGGTCAGGGCACAG 60
            *****

B73          TTGAAGCTGGTGACATGTCGCAGCGAACATAAAAGAGTTTCTCTTAGTGCCTCGTACGG 120
W23b,pl     TTGAAGCTGGTGACATGTCGCAGCGAACATAAAAGAGTTTCTCTTAGTGCCTCGTACGG 120
Mo17        TTGAAGCTGGTGACATGTCGCAGCGAACATAAAAGAGTTTCTCTTAGTGCCTCGTACGG 120
Confite     TTGAAGCTGGTGACATGTCGCGCGAACATAAACAGAGTTTCTCTTAGTGCCTCGTACGG 120
Conico      TTGAAGCTGGTGACATGTCGCAGCGAACATAAAAGAGTTTCTCTTAGTGCCTCGTACGG 120
Mishca      TTGAAGCTGGTGACATGTCGCAGCGAACAAAAAGAGTTTCTTAGTGCCTCGTACGG 120
            *****

B73          CCAGAAACACACAACGGCGGCTATCTTCGCCTCGCTCTCGGGTCTGTTCCGTGACCAG 180
W23b,pl     CCAGAAACACACAACGGCGGCTATCTTCGCCTCGCTCTCGGGTCTGTTCCGTGACCAG 180
Mo17        CCAGAAACACACAACGGCGGCTATCTTCGCCTCGCTCTCGGGTCTGTTCCGTGACCAG 180
Confite     CCAGAAACACACAACGGCGGCTATCTTCGCCTCGCTCTCGGGTCTGTTCCGTGACCAG 180
Conico      CCAGAAACACACAACGGCGGCTATCTTCGCCTCGCTCTCGGGTCTGTTCCGTGACCAG 180
Mishca      CCAGAAACACACAACGGCGGCTTTTTTCGCCTCGCTCTCGGGTCTGTTCCGTGACCAG 180
            *****

B73          CATGGCAAGGCTGCCACGTTCCAGCCACCCCTGGGC-----ACCAAGTAACCGGCCA 234
W23b,pl     CATGGCAAGGCTGCCACGTTCCAGCCACCCCTGGGC-----ACCAAGTAACCGGCCA 234
Mo17        CATGGCAAGGCTGCCACGTTCCAGCCACCCCTGGGC-----ACCAAGTAACCGGCCA 234
Confite     CATGGCAAGGCTGCCACGTTCCAGCCACCCCTGGGCCTGGGCACCAAGCAACCGGCCA 240
Conico      CATGGCAAGGCTGCCACGTTCCAGCCACCCCTGGGC-----ACCAAGCAACCGGCCA 234
Mishca      CATGGCAAGGCTGCCACGTTCCAGCCACCCCTGGGC-----ACCAAGCAACCGGCCA 234
            *****

B73          CACAATCCGCACCGCGTCTGTGCCCTACGCCATACCCAAACGCAGTTTCCAGCGCAGC 294
W23b,pl     CACAATCCGCACCGCGTCTGTGCCCTACGCCATACCCAAACGCAGTTTCCAGCGCAGC 294
Mo17        CACAATCCGCACCGCGTCTGTGCCCTACGCCTATACCCAAACGCAGTTTCCAGCGCAGC 294
Confite     CACAATCCGCACCGCGTCTGTGCCCTACGCCATACCCAAACGCAGTTTCCAGCGCAGC 300
Conico      CACAATTCGCACCGCGTCTGTGCCCTACGCCATACCCAAACGCAGTTTCCAGCGCAGC 294
Mishca      CACAATTCGCACCGCGTCTGTGCCCTACGCCATACCCAAACGAACGTTTCCAGGCGCAGC 294
            *****

B73          GGCCGGTCTACCACACCACAGCCACTCACTCCTGTCCCGTGCCCCACAA-----CA 346
W23b,pl     GGCCGGTCTACCACACCACAGCCACTCACTCCTGTCCCGTGCCCCACAA-----CA 346
Mo17        GGCCGGTCTACCACACCACAGCCACTCACTCCTGTCCCGTGCCCCACAA-----CA 346
Confite     GGCCGGTCTACCACACCACAGCCACTCACTCCTGTCCCGTGCCCCACAA-----CA 352
Conico      GGCCGGTCTACCACACCACAGCCACTCGCTTTGTCCCGTGCCCCACAAGGCCACAACA 354
Mishca      GGCCGGTCTACCACACCACCCCACTCCATTCTGTCCCGTGCCCCGCCTGGCCACAACA 354
            *****

B73          CACAACACTATTAATTCAGTGCTCTTCCCTTCGTCTGCGTCTGCGT-----GCAGCGTG 400
W23b,pl     CACAACACTATTAATTCAGTGCTCTTCCCTTCGTCTGCGTCTGCGT-----GCAGCGTG 400
Mo17        CACAACACTATTAATTCAGTGCTCTTCCCTTCGTCTGCGTCTGCGT-----GCAGCGTG 400
Confite     CA--ACAATATTAATTCAGTGCTCTTCCCTGCGTCTGCGTCTGCGTTGCCTGCGGTG 410
Conico      CACAACACTATTAATTCAGTGCTCTTCCCTGCGTCTGCGT-----TCGTG 399
Mishca      CACAACACTATTAATTCAGTGCTCTTCCCTGCGTCTGCGT-----GCATCCGTG 402
            **  *

B73          CTCGCGAGGCCCTGCTTCTGCTGCG 426
W23b,pl     CTCGCGAGGCCCTGCTTCTGCTGCG 426
Mo17        CTCGCGAGGCCCTGCTTCTGCTGCG 426
Confite     CTCGCGAGGTCCTGCTTCTGCTTGGG 436
Conico      CTCGCGAGGTCCTGCTTCTGCTTGGG 425
Mishca      CTCGCGAGGCCCTGCTTCTGCTGCG 428
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FIGURE A6 | (Continued).

B

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Arrocillo      GCTATTACTCCTTAGTTGGATTTTAGTTTCTAAGAAGTTGAAGTAGGGTCAGGGCA 60
Cacahuacintle GCTATTACTCCTTAGTTGGATTTTAGTTTCTAAGAAGTTGAAGTAGGGTCAGGGCA 60
Mo17           GCTATTACTCCTTAGTTGGATTTTAGTTTCTAAGAAGTTGAAGTAGGGTCAGGGCA 60
W23b,pl       GCTATTACTCCTTAGTTGGATTTTAGTTTCTAAGAAGTTGAAGTAGGGTCAGGGCA 60
Mishca        GCTATTACTCCTTAGTTGGATTTTAGTTTCTAAGAAGTTGAAGTAGGGTCAGGGCA 60
B73           GCTATTACTCCTTAGTTGGATTTTAGTTTCTAAGAAGTTGAAGTAGGGTCAGGGCA 60
*****

Arrocillo      TTCAAAGCTGGTGACACGTACAGCGAACATAAATGAGTTTCTCTAGGTCACGTCGTACG 120
Cacahuacintle TTCAAAGCTGGTGACACGTACAGCGAACATAAATGAGTTTCTCTAGGTCACGTCGTACG 120
Mo17           TTCAAAGCTGGTGACACGTACAGCGAACATAAATGAGTTTCTCTAGGTCACGTCGTACG 120
W23b,pl       TTCAAAGCTGGTGACACGTACAGCGAACATAAATGAGTTTCTCTAGGTCACGTCGTACG 120
Mishca        TTCAAAGCTGGTGACACGTACAGCGAACATAAATGAGTTTCTCTAGGTCACGTCGTACG 120
B73           TTCAAAGCTGGTGACACGTACAGCGAACATAAATGAGTTTCTCTAGGTCACGTCGTACG 120
*****

Arrocillo      GCCAGAAACACAACAACGGCCTATCTTCGCGCTCGCATAGCAAGGCTACCACGTTCCGAG 180
Cacahuacintle GCCAGAAACACAACAACGGCCTATCTTCGCGCTCGCATAGCAAGGCTACCACGTTCCGAG 180
Mo17           GCCAGAAACACAACAACGGCCTATCTTCGCGCTCGCATAGCAAGGCTACCACGTTCCGAG 180
W23b,pl       GCCAGAAACACAACAACGGCCTATCTTCGCGCTCGCATAGCAAGGCTACCACGTTCCGAG 180
Mishca        GCCAGAAACACAACAACGGCCTATCTTCGCGCTCGCATAGCAAGGCTACCACGTTCCGAG 180
B73           GCCAGAAACACAACAACGGCCTATCTTCGCGCTCGCATAGCAAGGCTACCACGTTCCGAG 180
*****

Arrocillo      CCACCCCGG-----GCACCAAGCAACCGGCCACACAATTTCGACCCGGGTCTGTGCCCA 234
Cacahuacintle CCACCCCGG-----GCACCAAGCAACCGGCCACACAATTTCGACCCGGGTCTGTGCCCA 234
Mo17           CCACCCCGG-----GCACCAAGCAACCGGCCACACAATTTCGACCCGGGTCTGTGCCCA 234
W23b,pl       CCACCCCGG-----GCACCAAGCAACCGGCCACACAATTTCGACCCGGGTCTGTGCCCA 234
Mishca        CCACCCCGG-----GCACCAAGCAACCGGCCACACAATTTCGACCCGGGTCTGTGCCCA 234
B73           CCACCCCGGCCCCGGGCACCAAGCAACCGGCCACACAATTTCGACCCGGGTCTGTGCCCA 240
*****

Arrocillo      TACCCAAACGCACGTTTCCAGCACAGCGGCCGGTCTACCACACCACAGCCACTCACTCC 294
Cacahuacintle TACCCAAACGCACGTTTCCAGCACAGCGGCCGGTCTACCACACCACAGCCACTCACTCC 294
Mo17           TACCCAAACGCACGTTTCCAGCACAGCGGCCGGTCTACCACACCACAGCCACTCACTCC 294
W23b,pl       TACCCAAACGCACGTTTCCAGCACAGCGGCCGGTCTACCACACCACAGCCACTCACTCC 294
Mishca        TACCCAAACGCACGTTTCCAGCACAGCGGCCGGTCTACCACACCACAGCCACTCACTCC 294
B73           TACCCAAACGCACGTTTCCAGCACAGCGGCCGGTCTACCACACCACAGCCACTCACTCC 300
*****

Arrocillo      TGTCCCGTGCCCCACAACACAACACTATTAATTCAGTGTCTCTCC-TGCCTGCGTCTGCG 353
Cacahuacintle TGTCCCGTGCCCCACAACACAACACTATTAATTCAGTGTCTCTCCCTGCCTGCGTCTGCG 354
Mo17           TGTCCCGTGCCCCACAACACAACACTATTAATTCAGTGTCTCTCCCTGCCTGCGTCTGCG 354
W23b,pl       TGTCCCGTGCCCCACAACACAACACTATTAATTCAGTGTCTCTCCCTGCCTGCGTCTGCG 354
Mishca        TGTCCCGTGCCCCACAACACAACACTATTAATTCAGTGTCTCTCCCTGCCTGCGTCTGCG 354
B73           TGTCCCGTGCCCCACAACACAACACTATTAATTCAGTGTCTCTCCCTGCCTGCGTCTGCG 360
*****

Arrocillo      TGCAGCGTGCTCGCGAGGTCCTGCTTCCTGCTGCG 389
Cacahuacintle TGCAGCGTGCTCGCG-AGGTCCTGCTTCCTGCTGCG 389
Mo17           TGCAGCGTGCTCGCG-AGGTCCTGCTTCCTGCTGCG 389
W23b,pl       TGCAGCGTGCTCGCG-AGGTCCTGCTTCCTGCTGCG 389
Mishca        TGCAGCGTGCTCGCG-AGGTCCTGCTTCCTGCTGCG 389
B73           TGCAGCGTGCTCGCG-AGGTCCTGCTTCCTGCTGCG 395
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FIGURE A6 | Alignment of *ZmFLS1* (A) and *ZmFLS2* (B) proximal promoters of maize inbred lines and landraces from high altitudes.

C1/P1-binding sites are highlighted in different colors. Insertions or deletions

in promoters are highlighted in gray, while differences in nucleotides from B73 are highlighted in yellow. The putative TATA boxes are indicated in bold-underlined letters.