Transcriptional, Posttranscriptional, and Posttranslational Regulation of SHOOT MERISTEMLESS Gene Expression in Arabidopsis Determines Gene Function in the Shoot Apex

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The activity of SHOOT MERISTEMLESS (STM) is required for the functioning of the shoot apical meristem (SAM). STM is expressed in the SAM but is down-regulated at the site of leaf initiation. STM is also required for the formation of compound leaves. However, how the activity of STM is regulated at the transcriptional, posttranscriptional, and posttranslational levels is poorly understood. We previously found two conserved noncoding sequences in the promoters of STM-like genes across angiosperms, the K-box and the RB-box. Here, we characterize the function of the RB-box in Arabidopsis (Arabidopsis thaliana). The RB-box, along with the K-box, regulates the expression of STM in leaf sinuses, which are areas on the leaf blade with meristematic potential. The RB-box also contributes to restrict STM expression to the SAM. We identified FAR1-RELATED SEQUENCES-RELATED FACTOR1 (FRF1) as a binding factor to the RB-box region. FRF1 is an uncharacterized member of a subfamily of four truncated proteins related to the FAR1-RELATED SEQUENCES factors. Internal deletion analysis of the STM promoter identified a region required to repress the expression of STM in hypocotyls. Expression of STM in leaf primordia under the control of the JAGGED promoter produced plants with partially undifferentiated leaves. We further found that the ELK domain has a role in the posttranslational regulation of STM by affecting the nuclear localization of STM.

The shoot apical meristem (SAM) contains a group of undifferentiated cells from which the aerial plant lateral organs derive. Several genes important for the functioning of the SAM have been characterized (for review, see Barton, 2010; Aichinger et al., 2012). In Arabidopsis (Arabidopsis thaliana), WUSCHEL (WUS) regulates the formation and maintenance of the SAM (Laux et al., 1996; Mayer et al., 1998) and acts in a negative feedback loop with CLAVATA (CLV) genes. WUS promotes stem cell activity, while CLV genes suppress this activity (Fletcher et al., 1999; Brand et al., 2000; Schoof et al., 2000). SHOOT MERISTEMLESS (STM), a class I KNOTTED-LIKE HOMEobox (KNOX1) gene, is required for the maintenance of the SAM to prevent cell differentiation (Endrizzi et al., 1996; Long et al., 1996).

STM and WUS act in different pathways (Lenhard et al., 2002). The KNOXI genes KNOTTED1-LIKE IN ARABIDOPSIS THALIANA/BREVIPEDICELLUS (KNAT1/BP), KNAT2, and KNAT6 show expression in the SAM and are partially redundant with STM (Byrne et al., 2002; Scofield and Murray, 2006). CLIP-SHAPED COTYLEDON (CUC) genes demarcate the boundaries between emerging leaf primordia and the SAM and are required for the initial expression of STM during embryogenesis (Aida et al., 1997, 1999; Takada et al., 2001).

In addition to these regulatory genes, plant hormones such as cytokinins (CKs) and GAs play an important role in the activity of the SAM. CKs are required for the maintenance of the meristem cells in an undifferentiated state, whereas GAs promote differentiation. STM promotes CK expression by activating the CK biosynthesis gene ISOPENTENYL TRANSFERASE7 (Jasinski et al., 2005; Yanai et al., 2005). Simultaneously, STM activates a GA-deactivating gene, AtGA20ox2 (Jasinski et al., 2005), and represses the expression of the GA biosynthesis gene AtGA20ox1 (Hay et al., 2002). In turn, increased CK levels enhance the expression of STM (Rupp et al., 1999; Kurakawa et al., 2007).

Leaves are formed on the flanks of the SAM. This process is characterized by the accumulation of auxins...
at the site of leaf primordia initiation, the plastochron 0 (P₀) site (Reinhardt et al., 2000; Benková et al., 2003), and by a down-regulation of STM (Long and Barton, 2000). ASYMMETRIC LEAVES1 (AS1) and AS2 are expressed in leaf primordia, but the activity of STM represses their expression in the SAM (Byrne et al., 2000).

STM also plays a role in the control of leaf morphology. Leaves of plants can be classified as simple or compound, with simple leaves having a single lamina and compound leaves having a lamina divided into leaflets. In simple-leaved species such as Arabidopsis, STM is not expressed in leaf primordia; however, STM expression is found in leaflet primordia of compound-leaved species (Bharathan et al., 2002), with the exception of species within a derived clade of legumes, where the function of STM-like genes in generating leaflet primordia is taken over by LEAFY-like genes (Hofer et al., 1997, 2001; Bharathan et al., 2002; Champagne et al., 2007). Furthermore, ectopic expression of STM-like genes in compound-leaved species produces leaves with an increased degree of leaf complexity (Hareven et al., 1996; Janssen et al., 1998). Conversely, a reduction in the expression of STM-like genes in compound-leaved species reduces leaf complexity (Hay and Tsiantis, 2006). The function of STM in leaf morphology is in part controlled by its interaction with the truncated form of the KNOX1 factor PETROSELINUM/KNOX ARABIDOPSIS THALIANA MEINOX (PTS/KNATM) through competition for the formation of dimers with BEL-LIKE (BELL) homeodomain (HD) factors (Kimura et al., 2008; Magnani and Hake, 2008). KNOX1 levels also can be controlled at the network level by upstream factors in the PTS network module (Ichihashi et al., 2014).

Despite the importance of STM function in plant development, the regulation of STM expression and the subsequent transcriptional, posttranscriptional, and posttranslational controls that determine effective STM levels are poorly understood. Gene regulation at the transcriptional level is mediated through the binding of transcription factors to cis-regulatory sequences. These regulatory sequences often are embedded in conserved noncoding sequences (CNSs), stretches of noncoding DNA that are evolutionarily preserved. Cis-regulatory sequences also can be located in introns. Importantly, intronic sequences show direct regulation of KNOX1 genes; several dominant mutants in maize (Zea mays) knotted1 (kat1) that confer ectopic expression of the gene are caused by events of transposon insertion in intron 3 in a region particularly rich in CNSs (Greene et al., 1994; Inada et al., 2003). In grasses, a CNS has been characterized in intron 2 of the class I KNOX genes liguleless3 (lg3), lg4a, and lg4b (Bauer et al., 2004). In rice (Oryza sativa), ORYZA SATIVA HOMEBOX1 (OSH1) positively regulates its own expression through binding to CNSs in intron 3 (Tsuda et al., 2011). In barley (Hordeum vulgare), the hooded mutation increases the expression of BARLEY KNOX3 due to a duplication of 305 bp in intron 4 (Müller et al., 1995). Yeast (Saccharomyces cerevisiae) one-hybrid experiments showed a number of factors that bind this intronic region (Santi et al., 2003; Osnato et al., 2010). In rice, exonic sequences of class I KNOX genes also have regulatory functions (Ito and Kurata, 2008). In general, however, most of the cis-regulatory sequences are located in the 5’ upstream region of the genes. We have previously described two CNSs in the STM promoter, the K-box and the RB-box (Uchida et al., 2007). Transgenic plants harboring a deletion in the K-box did not affect the expression of STM in the SAM or its down-regulation in P₀, but they showed an expansion of the STM expression pattern outside of these domains with consequences on leaf shape (Uchida et al., 2007). We determined that class I TEOSINTE BRANCHED1-CYCOIDEA-PROLIFERATING CELL FACTOR1 (TCP) transcription factors can bind to the K-box region (Aguilar-Martínez and Sinha, 2013). However, a role for the RB-box in Arabidopsis has not been defined, and other promoter regions important for STM regulation have yet to be characterized.

The regulation of STM at the protein level has been investigated more extensively. STM interacts with BELL factors through the MEINOX domain to access the nucleus and regulate the transcription of target genes (Bellouai et al., 2001; Smith and Hake, 2003; Bhatt et al., 2004; Cole et al., 2006). The HD is required for DNA binding (Smith et al., 2002; Viola and Gonzalez, 2009) and intercellular trafficking (Lucas et al., 1995; Kim et al., 2005; Winter et al., 2007b; Bolduc and Hake, 2009). However, the role of the ELK domain is less understood, although it is considered to be required for protein-protein interaction (Vollbrecht et al., 1991; Kerstetter et al., 1994) and for nuclear localization signal (NLS) sequences (Meisel and Lam, 1996). Cole et al. (2006) indicated that STM does not contain an NLS, and in the rice KNOX1 gene OSH15, the ELK domain is not required for nuclear localization, DNA binding, or homodimer formation, although it was shown to have a role in suppressing transactivation activity (Nagasaki et al., 2001).

Here, we analyze the regulation of STM through the study of promoter-reporter constructs and gene-specific misexpression: we (1) investigate the role of the RB-box in Arabidopsis; (2) identify a binding factor to the RB-box region that belongs to a group of uncharacterized proteins; (3) delineate other important regions in the STM promoter for the proper expression of the gene; (4) study the down-regulation of STM in P₀, using a gene-specific misexpression form of STM, and (5) also show that the regulation of the activity of STM in the cell nucleus is mediated by the ELK domain.

RESULTS

The RB-Box Has a Role in the Control of STM Regulation in Arabidopsis

We have previously shown that the STM gene promoter contains two CNSs, the K-box and the RB-box (Uchida et al., 2007). The K-box is present in all species analyzed and has a role in STM regulation (Uchida et al., 2007). A sequence analysis determined that the RB-box is present in promoter regions of STM genes in...
all of the species we analyzed in this study (Fig. 1A; Uchida et al., 2007). In most of the species analyzed, the RB-box is located in a region around 500 bp upstream from the translation initiation codon. However, in the Brassicaceae species analyzed, the RB-box is located relatively far from the translation initiation codon, ranging from $-1,412$ bp in *Cardamine hirsuta* to $-3,484$ bp in *Thellungiella halophila* (Fig. 1B).

In Arabidopsis, STM expression is seen in the whole SAM except in the new leaf primordium initiation site (Long et al., 1996; Long and Barton, 2000). The deletion of the core K-box expands the normal expression domain of STM to the base of leaf primordia and the midvein of leaf petioles (Uchida et al., 2007). Through reporter fusion experiments using the $\beta$-Glucuronidase (*uidA*) gene, we wanted to determine the expression patterns produced by deletions of RB-box elements alone and in combination with deletions of elements of the K-box (Fig. 2A; Table I). We analyzed 8-d-old seedlings of representative T3 lines. A promoter fragment of 3,379 bp (*ProSTM:GUS*) recapitulates the endogenous STM expression (Uchida et al., 2007; Fig. 2, B and C). Plants transformed with the construct *ProSTM-ΔK:GUS* lack the large K-box and show a GUS expression pattern similar to that of plants with just the construct *ProSTM:GUS* (Fig. 2, D and E). In older plants, GUS expression is found on the abaxial side of leaves in *ProSTM-ΔK:GUS* just as in the construct *ProSTM-ΔK:GUS* that lack the core K-box (Supplemental Fig. S1A; Uchida et al., 2007). In *ProSTM-ΔRB:GUS* plants, there is a deletion in the core RB-box, the GUS expression pattern is expanded to the abaxial side of the growing leaves, and some expression is also seen in the hypocotyl (Fig. 2, F and G). A similar pattern, except for the expression in the hypocotyl, is found in plants with deletions in both the core RB-box and the core K-box (*ProSTM-ΔRB-ΔK:GUS*; Fig. 2, H and I). When the large RB-box is deleted (*ProSTM-ΔLRB:GUS*), the expression of GUS is detected in patches in the hypocotyl and the root (Fig. 2J). Analysis of transverse sections indicated that GUS expression in the pericycle corresponded with the two xylem poles (Fig. 2K). No GUS expression is detected in the shoot apex. A combined deletion of the large RB-box and the core K-box (*ProSTM-ΔLRB-ΔK:GUS*) produced an expression similar to the pattern of GUS in hypocotyls seen in *ProSTM-ΔLRB:GUS* (Fig. 2, L and N). Furthermore, GUS is located in the sinuses, or spaces between lobes or teeth, of the growing leaves (Fig. 2, L, M, and O). Deleting both the large RB-box and the large K-box (*ProSTM-ΔLRB-ΔLK:GUS*; Fig. 2, P and Q) showed a pattern of GUS expression similar to the construct with both core RB-box and core K-box deleted (Fig. 2, H and I). GUS expression was not evident in plants harboring constructs with deletions in the 5' region of the STM promoter up to the RB-box using a native promoter (*ProSTM-ΔRB:GUS*) or with the additional deletion of the core K-box (*ProSTM-ΔRB-ΔK:GUS*; Table I; Supplemental Fig. S1, B and C), indicating that regions farther upstream from the RB-box are needed for the wild-type expression of STM.

In summary, this promoter analysis shows that the CNS in the RB-box is required for proper STM expression and that there is interaction between RB-box and K-box regulatory functions.
Figure 2. Expression of GUS in Arabidopsis STM promoter constructs harboring combinations of RB-box and K-box deletions. A, Schemes of the STM promoter fragments used. Numbers indicate positions relative to the translation initiation codon ATG. Solid lines indicate fragments of the promoters used, dashed boxes indicate the promoter regions deleted for every construct, and vertical dashed lines connect the boxes between constructs for clarification. B to Q, Whole-mount and transverse sections of 8-d-old T3 seedlings harboring the constructs depicted in A. B and C, Staining of plants with the construct ProSTM::GUS with the native fragment of 3,379 bp. Seedling (B) and transverse section (C) through the shoot apex are shown. D and E, Staining of
Aguilar-Martínez et al.

| Table 1. Names of the constructs of the STM promoter and fragments deleted |
|--------------------------|------------------|
| Construct Name           | Fragment Deleted |
| ProSTM-ΔL:GUS            | −355 to −220     |
| ProSTM-ΔCRB:GUS          | −2,004 to −1,987 |
| ProSTM-ΔCRB-CK:GUS       | −2,004 to −1987  |
| ProSTM-ΔLRB:GUS          | −2,114 to −1,907 |
| ProSTM-ΔLRB-CK:GUS       | −2,114 to −1,907 |
| ProSTM-ΔLRB-LK:GUS       | −2,114 to −1,907 |
| ProSTM-ΔRB:GUS           | −3,379 to −1,907 |
| ProSTM-ΔRB-CK:GUS        | −3,379 to −1,907 |
| ProSTM-ΔF1:GUS           | −3,119 to −2,784 |
| ProSTM-ΔF2:GUS           | −2,784 to −2,449 |
| ProSTM-ΔF3:GUS           | −2,449 to −2,114 |
| ProSTM-ΔF4:GUS           | −1,907 to −1,519 |
| ProSTM-ΔF5:GUS           | −1,519 to −1,131 |
| ProSTM-ΔF6:GUS           | −1,131 to −743   |
| ProSTM-ΔF7:GUS           | −743 to −335     |
| ProSTM-ΔF8:GUS           | −220 to 0        |

*Relative position, in bp, with respect to the start codon.

FARI-RELATED SEQUENCES-RELATED FACTOR1

Binds the RB-Box

In order to gain insight into STM gene regulation, we used yeast one-hybrid screening to identify factors that interact with the STM promoter region. We previously identified class I TCP gene products binding to the K-box region (Aguilar-Martínez and Sinha, 2013). Using a similar approach, we wanted to study factors that bind the RB-box. For the yeast one-hybrid analysis, we used a fragment of the STM promoter containing the large RB-box (−2,133 to −1,907 bp from the start codon). From the putative binding factors identified (Supplemental Table S1), we focused on At3g59470, which encodes for a putative DNA-binding factor (see below). We used an electrophoretic mobility shift assay (EMSA) and determined that this factor interacts with the RB-box (Fig. 3A) and with the ΔCRB fragment (representing a 17-bp deletion of the core; Supplemental Fig. S2). At3g59470 is related to the FARI-RELATED SÉQUENCES (FRS) genes, a family of transposase-derived transcription factors (Hudson et al., 1999; Wang and Deng, 2002; Lin and Wang, 2004). Well-known members of this family are FAR-RED ELONGATED HYPOCHOTYL3 (FYH3; Whitelam et al., 1993) and FAR-RED IMPAIRED RESPONSE1 (FAR1; Hudson et al., 1999). We named At3g59470 FARI-RELATED SEQUENCES-RELATED FACTOR1 (FAR1). FRS factors are characterized by having an N-terminal C2H2-type chelating motif of the WRKY-Glial Cell Missing1 family, a central core transposase domain of Mutator-like element transposases, and a C-terminal SWIM (named after SWI2/SNF2 and MuDR transposases) zinc-finger domain. The N-terminal domain acts as a DNA-binding domain, while the C-terminal domain is required for transcriptional factor activity (Lin et al., 2007). There are four FRS-like genes in the Arabidopsis genome. Interestingly, FRF1, FRF2, FRF3, and FRF4 share only the N-terminal motif with FRS proteins (Fig. 3B; Supplemental Data Set S1) and form a well-supported, though not well-resolved, clade. We looked for FRS-like genes in species other than Arabidopsis (Fig. 3C; Supplemental Fig. S3) and found that these genes are represented across the dicots (tomato [Solanum lycopersicum], Medicago truncatula, and grape [Vitis vinifera]) and monocots such as rice.

We wanted to study the function of FAR1 by analyzing insertion alleles for this gene. We characterized one allele, frf1-1, that had a tandem insertion located at −700 bp from the putative start codon. frf1-1 plants had the same phenotype as wild-type plants. We then made RNA interference (RNAi) lines for FAR1, and these transgenic plants showed a normal phenotype. Additionally, we studied an allele of FRF2, frf2-1, that had a tandem insertion at −140 bp from the putative start codon. The frf2-1 allele also showed no phenotypic defects when compared with the wild type. To the best of our knowledge, no insertion alleles are available in the coding region for FAR1 or FRF2. Insertion alleles for FRF3 were not analyzed in this study. For FRF4, we analyzed an allele named frf4-1 with an insertion at 338 bp from the putative start codon. The phenotype of frf4-1 plants was similar to that of the wild type. The small size and low conservation between these gene sequences precludes simultaneous knockdown by RNAi or artificial microRNA strategies.

We looked at the expression pattern of FRF genes in the Arabidopsis eFP Browser at Bio-Array Resource (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Winter et al., 2007a), AtGenExpress (http://jsp.weigelworld.org/expviz/expviz.jsp; Schmid et al., 2005), and GeneCAT (http://genecat.mpg.de/899/genecat.html;
Figure 3. Characterization of FRF1 and related factors. A, FRF1 is capable of binding the RB-box through EMSA. C+ indicates the positive control from the manufacturer’s kit. Arrows mark the shifted bands, and arrowheads mark the free probe of 246 bp for the RB-box and 60 bp for C+. B, FRF factors are related to FRS factors. The protein domain structures of FRF and FRS factors are shown. The FAR1 DNA-binding domain is indicated by pale gray boxes, the Mutator-like element transposase domain by dark gray boxes, and the zinc finger of the SWIM type by black boxes. Numbers indicate the size of each factor. C, Phylogenetic analysis of FRF factors in Arabidopsis, tomato, M. truncatula, grape, and rice. A neighbor-joining tree with 1,000 bootstrap replications using the conserved FAR1 DNA-binding domain of the FRF factors is shown. Branch lengths are indicated. The tree was generated using PAUP* 4.0 (Swofford, 2003; paup.csit.fsu.edu).
Mutwil et al., 2008). FRF1 and FRF4 are broadly expressed in Arabidopsis tissues, particularly in shoot apices (Supplemental Fig. S4). For FRF2 and FRF3, there are no data available in these databases. For these two genes, EST and complementary DNA (cDNA) matches are described in The Arabidopsis Information Resource (www.arabidopsis.org). According to the Cell eFP Browser (http://bar.utoronto.ca/cell_efp/cgi-bin/cell_efp.cgi), FRF1 and FRF2 are located in the nucleus, while FRF3 and FRF4 are present in the nucleus and cytoplasm.

In summary, we determined that FRF1 binds the RB-box and belongs to a small family of phylogenetically conserved and FRS-related transcription factors. Limited analysis of mutant alleles and RNAi lines suggests that these genes may function redundantly.

**Deletion Analysis of the Promoter of Arabidopsis STM Reveals Important Regions for Its Proper Regulation**

We further analyzed the role of other fragments of the gene promoter in regulating STM expression. We divided the STM promoter into fragments of about the same size without including the RB- and K-boxes, generated internal deletions, fused them to the uidA gene, and analyzed the GUS expression pattern in the transgenic plants from T3 lines (Fig. 4; Table I). We divided the fragment upstream of the RB-box into three parts, F1, F2, and F3, of 335 bp each, and the fragment between the RB- and K-boxes into four parts, F4 to F7, of 388 bp each, and used the fragment F8 between the K-box and the starting codon of 220 bp (Fig. 4A). Compared with the normal expression of STM in the shoot apex (Long et al., 1996; Long and Barton, 2000; Uchida et al., 2007; Fig. 2, B and C), the deletion of fragment F1 (ProSTM-ΔF1:GUS) showed no changes in the shoot apex localization of GUS (Fig. 4, B and C). Deleting fragment F2 (ProSTM-ΔF2:GUS) lightly expanded the GUS expression pattern through the hypocotyls (Fig. 4, D and E). When the F3 fragment was removed (ProSTM-ΔF3:GUS), a strong GUS signal was observed throughout the hypocotyl, while no expression was found in the shoot apex (Fig. 4F). Analysis of cross sections of the hypocotyls showed staining markedly confined to the stele (Fig. 4G). Light expression in the shoot apex and the hypocotyls was found in constructs that lack the F4 fragment (ProSTM-ΔF4:GUS; Fig. 4, H and I) or the F5 fragment (ProSTM-ΔF5:GUS; Fig. 4, J and K). An expression similar to that of the native promoter was found in plants with the F6 fragment deleted (ProSTM-ΔF6:GUS; Fig. 4, L and M). GUS expression in plants with the construct ProSTM-ΔF7:GUS (Fig. 4, N and O) was similar to that in plants with the constructs ProSTM-ΔF4:GUS and ProSTM-ΔF5:GUS. The deletion of the F8 fragment (ProSTM-ΔF8:GUS) produced an expansion of the GUS expression pattern mainly to the abaxial side of growing leaves and petioles (Fig. 4, P and Q).

We also looked for conserved regions other than the K-box and RB-box in the promoters of STM-like genes. We focused on the Brassicaceae species with sequenced genomes (Brassica rapa, T. halophila, Capsella rubella, Arabidopsis, Arabidopsis lyrata, and C. hirsuta) and in regions from the RB-box up to -6,000 bp from the translation initiation codon (Fig. 5A). Three regions, Fa, Fb, and Fc, appear greatly conserved. Fa is the most distant (Fig. 5, A and B), Fb is located between fragments F1 and F2 (Fig. 5, A and C), and Fc is located in fragment F3 close to the RB-box (Fig. 5, A and D). The functional significance of these conserved sequences remains to be elucidated.

We also studied the sequence conservation in intronic regions of STM-like genes in the Brassicaceae species analyzed. Interestingly, sequences of introns I and II are greatly conserved between species, while sequences of intron III are poorly preserved (Supplemental Fig. S5A). In comparison, sequences of introns I, II, and IV of BP-like genes are not conserved, and only one fragment of intron III is well conserved between species (Supplemental Fig. 4B).

From this analysis, we determined that the sequences in the F3 fragment greatly contribute to the correct expression of STM, while the role of the other fragments analyzed appears to be less prominent. Sequence conservation analysis suggests that farther upstream sequences as well as introns I and II also may harbor STM regulatory sequences.

**STM Expression in Initiating Leaf Primordia**

One of the most striking features of STM expression is its down-regulation in the incipient leaf primordium, P0. P0 is characterized as the region containing a group of leaf founder cells located at the flanks of the SAM. The down-regulation of STM at P0 is required for the formation of new leaf primordia in a process that appears to be regulated at the transcriptional level (Long et al., 1996; Long and Barton, 2000). We wanted to determine the developmental consequences of the expression of STM in P0, a site where the gene is normally down-regulated. Based on mRNA in situ hybridization, the JAGGED (JAG) gene is expressed in P0 (Dinneny et al., 2004; Ohno et al., 2004). JAG is also expressed in young leaf primordia. In reproductive development, JAG is expressed in all flower organ primordia (Dinneny et al., 2004; Ohno et al., 2004). We found that 2.2 kb of the 5' region of JAG recapitulates the endogenous expression of the gene (Fig. 6, A and B; Supplemental Fig. S6). We generated transgenic plants harboring 2.2 kb of the JAG promoter fused to the coding sequences of GFP and STM (Pro[AG]:GFP-STM) and analyzed the phenotype (Fig. 6, C–H). We expected that the expression of STM in P0 would lead to defects in leaf primordia initiation, as STM promotes the maintenance of the cells in an undifferentiated stage. Compared with the wild type (Fig. 6C), the transgenic plants Pro[AG]:GFP-STM recovered with a strong phenotype were of reduced size and with epinastic cotyledons (Fig. 6D). Although leaf primordia emerged and showed characteristics of differentiation such as the presence of
leaf trichomes, leaf growth and expansion were arrested (Fig. 6D). These transgenic plants did not produce seeds. Transgenic plants with a milder phenotype had bigger rosettes and small highly lobed leaves with broad petioles (Fig. 6E; Supplemental Fig. S7A). Ectopic meristems were not observed on these leaves. We used confocal microscopy to characterize GFP distribution in the transgenic plants generated. In transgenic plants carrying ProSTM:GFP-STM, GFP is located in the SAM (Fig. 6F; Supplemental Fig. S7, B and C), similar to ProSTM:STM-VENUS plants that use VENUS instead of GFP (Heisler et al., 2005). However, in plants with STM under the control of the JAG promoter, we observed a proliferation of stipule-like structures that highly express GFP (Fig. 6G; Supplemental Fig. S7, D–F). In ProfJAG:GFP-STM plants with a less severe phenotype, GFP signal

Figure 4. GUS expression pattern in constructs with internal deletions in the Arabidopsis STM promoter. A, Schemes of the STM promoter with the internal deletion fragments used. Numbers indicate positions relative to the translation initiation codon ATG. The positions of the RB-box and the K-box are also indicated. Solid lines indicate the fragment of the promoter used, dashed boxes indicate the internal deletion on each construct, and vertical dashed lines connect the boxes between constructs for clarification. B to R, Whole-mount and transverse sections of 8-d-old T3 seedlings harboring the constructs indicated in A. B and C, Staining of plants with the construct ProSTM-D1:F1::GUS. Seedling (B) and transverse section through the shoot apex (C) are shown. D and E, Staining of plants with the construct ProSTM-D2::GUS. Seedling (D) and cross section through the shoot apex (E) are shown. F and G, Seedling (F) and cross section through the hypocotyl (G) of stained plants with the construct ProSTM-D3::GUS. H and I, GUS expression pattern in ProSTM-D4::GUS plants. Whole-mount stained seedling (H) and cross section through the shoot apical region (I) are shown. J and K, Staining of plants with the construct ProSTM-D5::GUS. Seedling (J) and cross section through the shoot apex (K) are shown. L and M, GUS expression pattern in ProSTM-D6::GUS plants. Seedling (L) and cross section through the shoot apex (M) are shown. N and O, Staining of plants with the construct ProSTM-D7::GUS. Seedling (N) and cross section through the shoot apical region (O) are shown. P to R, Staining of plants with the construct ProSTM-D8::GUS. Seedling (P) and cross sections through the shoot apex (Q) and through the hypocotyl (R) are shown. Leaf primordia are outlined with dashed lines. Bars = 0.5 mm in B, D, F, H, J, L, N, and P, 25 μm in C, E, and O, 50 μm in G, K, M, and R, and 100 μm in I and Q.
was more distributed throughout the base of developing leaf primordia (Fig. 6H), and later in development stipule-like structures also were observed (Supplemental Fig. S7, G–I). These results indicate that ectopic expression of STM in early leaf primordia disrupts the normal development of these primordia.

The ELK Domain Is Required for the Correct Nuclear Localization and Activity of STM

The STM protein is characterized by having four well-conserved domains, MEINOX, subdivided into the KNOX1 and KNOX2 subdomains, GSE, ELK, and HD (Fig. 7A; Vollbrecht et al., 1991; Kerstetter et al., 1994; Nagasaki et al., 2001). Despite the conservation of the ELK domain, its role is poorly understood. To study the function of this domain, we focused on the Ser residue at position 272 (Supplemental Fig. S8).

Ser residues are a common target of protein post-translational modification through phosphorylation, and additionally, the Ser residue at position 272 lies in a putative 14-3-3 protein-binding site (http://scansite.mit.edu). We performed a site-directed substitution of Ser-272 to an Ala in ProSTM:GFP-STM (Uchida et al., 2007) to generate transgenic ProSTM:GFP-STM-S272A

Figure 5. CNSs in STM-like gene promoters of Brassicaceae species upstream of the RB-box. A, mVISTA alignment of the B. rapa STM promoter compared with the corresponding genes of T. halophila, C. rubella, Arabidopsis, A. lyrata, and C. hirsuta. Three regions, Fa, Fb, and Fc, are identified. B, Sequence alignment in the Fa region. C, Alignment of the sequences for the Fb region. D, Sequence alignment in the Fc region. Sequence alignments were made with MUSCLE and displayed with Boxshade.
plants and analyzed the phenotype. Compared with control ProSTM::GFP-STM plants that behave as the wild type (Uchida et al., 2007), plants carrying ProSTM::GFP-STM-S272A were characterized by having lobed rosette leaves (Fig. 7B) and highly lobed cauline leaves (Fig. 7C), while other organs were not affected (Fig. 7D). This phenotype was different from that of Pro-35S::STM-GR plants that ectopically express STM in an inducible manner using the glucocorticoid receptor (GR) and are characterized by producing multiple and very small leaves and ectopic stipules (Gallois et al., 2002; Cole et al., 2006). Interestingly, plants that express both the BELL factor BLH9 (Pro-35S::BLH9) that interacts with STM to direct the complex to the cell nucleus and the STM factor fused to the GR (Pro-35S::STM-GR; Cole et al., 2006) have deeply lobed leaves, resembling ProSTM::GFP-STM-S272A plants. Furthermore, transgenic plants that overexpress STM fused to an NLS (Pro-35S::NLS-GFP-STM; Cole et al., 2006) also have a similar phenotype to ProSTM::GFP-STM-S272A plants. We then investigated if the target mutated STM-S272A had an altered nuclear localization of STM that could correlate with the observed phenotype. We transiently expressed the constructs GFP-STM and GFP-STM-S272A in Nicotiana benthamiana leaves and observed the localization of STM using confocal laser scanning microscopy. For the control construct GFP-STM, the STM protein was found to be located in the cytoplasm as described previously (Fig. 7E; Cole et al., 2006). However, the location of STM in STM-S272A was mainly nuclear (Fig. 7F). Taken together, these results suggest that the Ser-272 residue in the ELK domain has a role in the control of the nuclear localization of STM.

Nuclear localization of BELL factors depends on interaction with the nuclear export receptor CHROMOSOME REGION MAINTENANCE1 (AtCRM1)/EXPORTIN1 through the BELL domain, which harbors both a nuclear export signal and the STM-binding site (Rutjens et al., 2009). Treatment with leptomycin B (LMB) specifically inhibits CRM1 activity, and BELL factors accumulate in the nucleus (Rutjens et al., 2009). We found that in N. benthamiana leaves, treatment with LMB also determined the nuclear accumulation of STM (Supplemental Fig. S9). However, in a yeast two-hybrid assay, AtCRM1 does not interact with STM (Rutjens et al., 2009), suggesting that yeast endogenous factors might interfere with this interaction or that other plant factors might be required for the interaction.

In conclusion, our results highlight the importance of the promoter region RB-box in STM regulation. The RB-box is required for the expression of STM in the SAM and works with the K-box in controlling STM expression in leaves. A putative transcription factor, FRF1, from a previously uncharacterized subfamily of
FRS proteins shows binding to the RB-box. An initial scanning for important regulatory regions in STM other than the K-box and the RB-box showed that region F3 is required to repress the expression of STM in hypocotyls. The expression of STM in leaf primordia using the JAG promoter does not completely arrest leaf initiation, suggesting either that expression of STM in the P₀ domain does not have drastic consequences on leaf initiation or that STM is down-regulated posttranscriptionally in this domain. We also show that the ELK domain is required for the correct posttranslational regulation of STM. Combined, these results show that the regulation of STM expression is complex, with interplay between several promoter-binding transcription factors like the TCPs, FRFs, and other as yet unknown factors in the regions of the promoter upstream from the RB-box and between the RB- and K-boxes. Further analysis of subcellular STM protein localization and protein levels in the P₀ region of ProJAG:STM plants is needed to determine the role that posttranscriptional regulation plays in determining STM expression in this domain.

**DISCUSSION**

Role of the RB-Box in STM Transcriptional Regulation

In Arabidopsis, STM expression is normally restricted to the SAM. Our promoter-deletion analyses have shown that, in certain constructs lacking the RB-box or both the RB-box and the K-box, the expression of STM is expanded to the abaxial side of leaves. Interestingly, the endogenous STM promoter of a species with dissected leaves, such as C. hirsuta, drives a similar expression pattern in either C. hirsuta or Arabidopsis, a simple-leafed species (Hay and Tsiantis, 2006). Those authors proposed that the differences in STM expression between species, and therefore in leaf morphology, can be due to differences in regulatory regions of the STM promoters, reinforcing a role for the RB-box and the K-box in this process. Although both species possess
RB-box and K-box, subtle sequence differences, including different spacing between regulatory sequences or different responses of trans-acting factors, can account for different promoter activities.

We found that transgenic plants harboring a construct with a deletion of both the large RB-box and the core K-box showed strong GUS expression in leaf sinuses. KNOX1 genes can be expressed in leaf sinuses. For instance, in as1-1 and as2-2 mutants, the genes KNAT1/BP and KNAT2 are expressed in the sinuses (Ori et al., 2000). This expression is enhanced in the double mutants as1-1 serrate (se), as2-2 se, and as1-1 auxin resistant1-3, where deeper sinuses are formed (Ori et al., 2000; Hay et al., 2006). In these double mutants, ectopic structures resembling stipules are formed (Ori et al., 2000). STM has been found to be expressed at low rates and intensities in leaf sinuses, as indicated by the analysis of a construct with the uidA gene fused to the native STM promoter (ProSTM:GUS; Kawamura et al., 2010). STM is required for leaf serration (Depuydt et al., 2008; Kawamura et al., 2010). CUC2 is also expressed in leaf sinuses (Nikovics et al., 2006), and mutants with increased expression of CUC2 show the expression of STM as small spots in some sinuses where stipule-like organs are often observed (Kawamura et al., 2010). Leaf sinuses can be considered as quiescent domains with the potential for meristem formation (Hu et al., 2011). In fact, certain Kalanchoë spp. form plantlets on leaf sinuses (Garcés et al., 2007). Our results stress the importance of both the RB-box and the K-box in determining leaf shape through the regulation of leaf lobes and sinuses.

We have determined that different combinations of deletions of the RB-box and K-box render different GUS expression patterns. For instance, the expression pattern found when we combined deletions in the large RB-box and the core K-box is abolished when we used the large RB-box and the large K-box. This indicates that other sequences close to but different from the core sequences are also important in STM regulation.

Transcription regulation operates in part through DNA bending, which closely places regulatory factors located relatively distant to the core promoter with the RNA polymerase. It is possible that factors that bind the RB-box and the K-box participate in this bending mechanism and that specific factors can cause interactions between these sites.

FRF Proteins as Putative Binding Factors at the RB-Box

Through yeast one-hybrid analysis and EMSA, we determined that FRF1 is a putative binding factor at the RB-box. This factor defines a group of uncharacterized proteins related to the FRS transposase-derived transcription factors. Interestingly, FRF factors are conserved across dicots, suggesting a conserved role for them in plant development. Furthermore, FRF factors are short or truncated versions of FRS factors, with only the N-terminal C2H2 zinc-finger domain sequence conserved. This domain is essential for direct DNA binding (Lin et al., 2008). One possibility is that FRF factors could regulate the action of FRS factors through competitive binding for the same DNA-binding motifs. LONG HYPOCOTYL5 interacts with FHY3 and FAR1, interfering with their DNA-binding capabilities (Li et al., 2010). Interestingly, these interactions are mediated by the N-terminal C2H2 of FHY3 and FAR1. Also, FHY3 interacts with CIRCADIAN CLOCK-ASSOCIATED1, LATE ELONGATED HYPOCOTYL, and PHYTOCHROME-INTERACTING FACTOR1 through the N-terminal C2H2 domain (Li et al., 2011b; Tang et al., 2012). Thus, another possibility is that FRF factors could interact with factors other than FRS factors. Further functional studies on FRF factors would help to analyze these possibilities.

The F3 Region as Controller of the Stem and Root Expression of STM

Our promoter-deletion analysis showed that deletion of certain promoter fragments leads to STM expression located at the pericycle. This is particularly noticeable for fragment F3 and also is found in part in constructs without the large RB-box. The RB-box and fragment F3 are adjacent, indicating that sequences from this region, in particular from F3, are important to repress the expression of STM in the pericycle. The pericycle is a primary meristem tissue and a place for shoot regeneration (Atta et al., 2009). In plants that have secondary growth, tissue of the pericycle contributes to the development of the vascular cambium. Furthermore, the pericycle has the capacity to produce lateral roots. Interestingly, the pattern of lateral root distribution is altered in the mutant stem-1 (Casimiro et al., 2001). Indeed, the STM orthologs in tomato LYCopersicum ESCULENTUM T6/TOMATO KNOTTED2 (LeT6/TKN2) and in M. truncatula MtKNOXI are found to be expressed in lateral roots (Koltai and Bird, 2000; Koltai et al., 2001). The class I KNOX gene KNAT6 acts redundantly with STM in SAM maintenance and is expressed in roots (Dean et al., 2004; Belles-Boix et al., 2006). Similarly, KNAT1 and the class II KNOX genes KNAT3, KNAT4, and KNAT5 show root-specific tissue expression (Truernit et al., 2006). Furthermore, KNAT1 has a specific role in root skewing (Qi and Zheng, 2013).

Arabidopsis STM expression is found not only in the SAM but in stem tissue, although not uniformly (Long et al., 1996). In tomato, LeT6 is expressed in the SAM and in vascular tissue, and in poplar (Populus spp.), the KNOX gene ARBOROXX1 (ARK1) is expressed in the SAM and in the vascular cambium (Groover et al., 2006). Transcripts from the STM-like gene of the parasitic plant Cuscuta pentagona are found in the haustoria, specialized organs derived from stems (Alakonya et al., 2012). However, stem tissue expression is more characteristic of KNAT1-like genes, as in Arabidopsis KNAT1 (Lincoln et al., 1994), tomato TKNI (Hareven et al., 1996), poplar ARK2 (Du et al., 2009), maize kn1 (Smith et al., 1992; Jackson et al., 1994), and peach (Prunus persica) KNOPE1 (Testone et al., 2012), with

New Insights on the Regulation of STM Gene Function

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these genes having a more prominent role in the regulation of stem tissue differentiation (Townsley et al., 2013).

We also located three CNSs in the promoter of STM-like genes upstream of the RB-box. It will be interesting to analyze if Fa has some function in the regulation of STM expression. Fb could be the place for enhancer sequences, as constructs with deletions in this region, ProSTM-4F1:GUS and ProSTM-4F2:GUS, have decreased GUS signal in shoot apices. Fc, included in the F3 fragment, could contain sequences for the repression of STM expression in hypocotyls. Functional analyses will determine these possibilities. Likewise, functional analyses of the ProSTM-ACRB: STM and ProSTM-4F3:STM transgenic lines will help us to understand the roles of RB- and F3-boxes in SAM and leaf development.

Expression of STM in Leaf Primordia and Mechanisms for the Regulation of STM Expression

Leaves of plants expressing ProJAG:STM with a weak phenotype resemble the leaves of plants expressing Pro-35S:KNAT1 or Pro-35S:kn1 (Lincoln et al., 1994; Chuck et al., 1996), while plants expressing ProAG:STM with a strong phenotype resemble plants expressing Pro-35S: STM (Williams, 1998; Gallois et al., 2002) or plants that express STM or kn1 in a GR-inducible system (Pro-35S: STM-GR and Pro-35S:kn1-GR) grown from germination on dexamethasone-containing medium (Gallois et al., 2002; Hay et al., 2003). AINTEGUMENTA (ANT) is expressed in leaf primordia, along with other tissues (Elliott et al., 1996; Long and Barton, 2000). Plants expressing ProANT:STM have a similar phenotype to ProAG:STM plants (Lenhard et al., 2002). This would suggest that the expression of STM at the site of presumptive leaf primordia initiation can still produce rudiments of lateral organs, although with the suppression of cell differentiation. Leaf initiation at P0 is characterized by the down-regulation of STM (Long and Barton, 2000) and the accumulation of auxin (Reinhardt et al., 2000; Benková et al., 2003), indicating that these processes can be functionally connected. PIN-FORMED1 (PIN1) and PINOID (PID) regulate polar auxin transport, and in double mutant pin1-3 pid-2 embryos, the expression of STM is expanded to the cotyledon area, resulting in growth inhibition of cotyledon primordia, indicating that both PIN1 and PID repress STM (Furutani et al., 2004).

CUC1 and CUC2 are required for the establishment of organ boundaries in the SAM (Aida et al., 1997, 1999). In the cuc1 cuc2 double mutant, the expression of STM is abolished, indicating that both CUC1 and CUC2 could act as initiators of STM transcription (Aida et al., 1999). Ectopic expression of CUC1 (Pro-35S:CUC1) activates the expression of STM, producing adventitious shoots at the sinuses of cotyledons. These shoots are not formed in the stm mutant background (Hibara et al., 2003). However, it is not known whether CUC1 and CUC2 directly or indirectly regulate STM. Also, single mutants for PINHEAD (PNH) and the related gene ARGONAUTE1 (AGO1) have defective apical meristems, although the expression of STM is detected; however, in the double mutant pnh ago1, the expression of STM is absent (Lynn et al., 1999).

Long-term repression of STM in leaves appears to be mediated by the action of POLYCOMB-REPRESSIVE COMPLEX2 (PRC2) and PRC1-like. CURLY LEAF (CLF) and SWINGER (SWN) are members of PRC2. CLF directly binds the promoter of STM, and clf sun double mutants ectopically express STM in leaves (Schubert et al., 2006). Mutations in members of PRC1-like also misexpress KNOXI genes (Xu and Shen, 2008). It has been shown that the leaf adaxial determinants AS1 and AS2 interact with members of the PRC2 to mediate long-term repression of the KNOXI genes KNAT1, KNAT2, and PTS/KNATM but not STM (Lodha et al., 2013). The histone deacetylase HDA6 participates in a complex with AS1 to repress KNAT1, KNAT2, and PTS/KNATM (Luo et al., 2012). Class II TCP factors bind to the promoters of KNAT1 and KNAT2 and interact with AS2 to exert a repressive function (Li et al., 2012). We determined that class I TCP factors can bind the STM promoter through the K-box region (Aguilar-Martínez and Sinha, 2013). As regulation of STM appears to not be mediated by the AS1-AS2 complex, it will be interesting to determine which TCP-interacting factors other than AS1 and AS2 act in the regulation of STM.

Leaf abaxial polarity determinant YABBY (YAB) factors FILAMENTOUS FLOWER (FIL) and YAB3 have a role in the regulation of the KNOXI genes STM, KNAT1, and KNAT2, as fil yab3 double mutants derepress the expression of these genes in leaves, generating ectopic meristems (Kumaran et al., 2002). YAB factors interact with the transcriptional corepressors LEUNIG (LUG) and LEUNIG_HOMOLOG (LUH) as well as with the LUG/LUH transcriptional adaptor factors SEUSS (SEU) and SEUSS-LIKE (SLK; Stahle et al., 2009). In mutants lug−/+ luh−/−, the expression pattern of STM is expanded (Stahle et al., 2009), while in mutants seu silk2, the expression levels of STM are greatly reduced (Bao et al., 2010). In turn, LUG interacts with HDA19 and the MEDATOR components MEDI4 and Cyclin-Dependent Kinase8 (Gonzalez et al., 2007). Moreover, the mutant med14 shows an irregular expression pattern of STM in the SAM (Aturan et al., 2002). BASIC PENTACYSTEINE factors bind to a promoter region in STM right upstream of the ATG as well as other promoter regions in several other HOMEOBOX genes (Simonini and Kater, 2014). In rice, OSH1 is positively autoregulated mainly through CNSs in introns (Tsuda et al., 2011). A similar mechanism can operate in Arabidopsis STM, although CNSs similar in structure to the RB-box and K-box are not described in introns of the STM-like genes from the species analyzed in this study. Thus, a number of factors have a role in STM regulation, yet a comprehensive framework of the control of STM gene regulation at the transcriptional level remains to be determined.
Role of the ELK Domain in Posttranslational Regulation of STM

The targeted mutation STM-S272A could affect the interaction with STM-binding proteins and hence correct nuclear localization and function. The ELK domain has been suggested to mediate protein-protein interactions (Mushegian and Koonin, 1996; Sakamoto et al., 1999). Nagasaki et al. (2001) showed that the ELK domain is not essential for homodimer formation in rice KNOX factors. The interaction of STM with BELL factors is mediated by the MEINOX domain (Bellaoui et al., 2001; Smith et al., 2002; Bhatt et al., 2004). It will be interesting to determine if the activity of STM-S272A and its associated phenotypic is independent of the interaction with BELL factors in vitro and in vivo. Several members of the OVATE family of factors (AtOFPs) interact with KNOX and BELL factors, regulating their subcellular localization (Hackbusch et al., 2005). BLH1, BLH7, and KNAT1 interact with AtOFP1 (Hackbusch et al., 2005), BLH1 and KNAT3 with AtOFP5 (Pagnussat et al., 2007), and KNAT7 with OPF1 and OPF4 (Li et al., 2011a). The interaction of KNAT17 is mediated by the HD (Li et al., 2011a). However, STM shows no interaction with AtOFPs (Hackbusch et al., 2005).

Experiments with the overexpression of the rice KNOX protein OSH15 carrying a deletion of the ELK domain showed a deletion of the ELK domain produced a novel phenotype in transgenic rice plants, suggesting that this domain could have a defined function, such as interaction with a specific factor (Nagasaki et al., 2001). The Ser residue used for the targeted mutation in this study lies in a putative 14-3-3-interacting binding region (http://scansite.mit.edu). Other regions of the STM protein also are putative binding sites for 14-3-3 factors. The role of these interactions is under study. While the roles of the STM conserved domains KNOX1, KNOX2, HD, and ELK have been studied more extensively, the proposed function of the GSE domain in regulating protein degradation (Vollbrett et al., 1991; Nagasaki et al., 2001) has not yet been determined.

In conclusion, while many aspects concerning the control of STM transcriptional regulation and correct protein localization and stability are still unknown, our findings have revealed regions and factors important for the correct regulation of STM, a transcription factor with important roles in plant development.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Allele fyt-1 (SAIL_292_C04) in Arabidopsis (Arabidopsis thaliana) was obtained from the Syngenta Arabidopsis Insertion Library (Sessions et al., 2002). Alleles fyt-1 (SALK_076709) and fyt-1 (SALK_048606C) were obtained from the Salk Institute Genetic Analysis Laboratory (Alonso et al., 2003) through the Arabidopsis Biological Resource Center. The mutant alleles were in the Columbia-0 (Col-0) ecotype background. Growth conditions were as described by Aguilar-Martinez and Sinha (2013).

Phylogenetic Analysis and Protein Sequence Analysis

For the alignment of the RB-box, we used the promoter regions of Arabidopsis STM (At1g62360.1) and the putative orthologs in Arabidopsis lyrata gene 338146; Capella rubella gene CarVa01022235m; Brassica rapa gene Bra2072650; Thellungiella halophila gene Thb1l003520m; Carica papaya gene eum.TU.supercontig152_57; Populus trichocarpa gene Potri.011G011100; grape (Vitis vinifera) gene GSVIVG0100481101; tomato (Solanum lycopersicum) gene LeT6 (Solyc02g081120; Jansen et al., 1998); Medicago truncatula gene MtkNOKO1 (Medtr5g085860; Koltai et al., 2001); soybean (Glycine max) gene Glyma9g01000; bean (Phaseolus vulgaris) gene Pvu006G145600; and Ricinus communis gene 28842.000028. All sequences were obtained from Phytozone (http://www.phytozone.net/); Goodstein et al., 2012). For cotton (Gossypium raimondii), we used gene Cc010G183800 (GenBank accession no. CC0081267); for Antirrhinum majus, we used GenBank accession number AY0722351; for tobacco (Nicotiana tabacum), we used gene NTH5 (N. tabacum homeobox15; Tamaoki et al., 1997); for pea (Pisum sativum), we used gene HOPI (Hymexox of P. sativum1; GenBank accession no. AF003071; Giles et al., 1998); for Cardamum kirsuta, we used gene OSSM (Hay and Tsiantis, 2006); and for asparagus (Asparagus officinalis), we used GenBank accession number EF649254 (Uchiha et al., 2007).

The sequence alignments were made using MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/) and Chaos+Dialign (http://dialign.gobics.de/chaos-dialign-submission; Brudno et al., 2004). The alignment was edited using MEGA5 software (http://www.megasoftware.net/; Tamura et al., 2011). The analysis of CNSs in the STM promoter of Brassicaceae species upstream of the RB-box was made with nVISTA (http://genome.lbl.gov/vista/index.shtml; Frazer et al., 2004). BIP-like genes were Bra0006386 in B. rapa, Thb1001028742.m.g in T. halophilus, CarVa01022235m in C. rubella, and 489772 in A. lyrata. The protein domains of FRF and RFS factors were analyzed with InterPro (http://www.ebi.ac.uk/interpro/; Hunter et al., 2012) and drawn using Domain Graph, version 2.01 (http://dog.biociao.org/index.php; Ren et al., 2009). For the analysis of BIP-like proteins, BLAST searches were made against the genomes of tomato (International Tomato Annotation Group 2.3 release of the sequenced tomato genome; http://solgenomics.net/organism/Solanum_lycopersicum/geneome), M. truncatula (http://Medicago truncatula Genome Project; http://www.jcvi.org/cgi-bin/medicago/overview.cgi), and grape and rice (Oryza sativa), both at Phytozone (http://www.phytozone.net/). The alignments were made with TranslaterX (Abaschel et al., 2010; http://translatorx.co.uk/) using MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/); for alignment, see Supplemental Data Set S2. We used the conserved FAR1 DNA-binding domain of the FRF-like proteins to generate a neighbor-joining tree and a parsimony tree with 1,000 bootstrap replications. The alignments were done on the translated amino acid sequence. Only unambiguously alignable portions of the sequence that were present in all sequences were used for the tree. The region used was columns 821 to 873 and 895 to 933; all other positions were excluded. Two rice sequences from the alignment were deleted because, even though they were FAR sequences, the alignment algorithm could not align them unambiguously to deduce the relationships to the other sequences. The phylogenetic trees were generated with PAUP* 4.0 (Swofford, 2003; parsed with PAUP* 4.0 beta 10; Swofford, 2003) and with FigTree version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). Sequences of factors LeT6 (AAC49917.1) in tomato, NTH15 (BAA25546.1) in tobacco, and STM (AAC49148.1) in Arabidopsis were aligned with ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Construction of Transgenes and Plant Transformation

The constructs with selected internal deletions in the STM promoter were generated using overlapping PCR (Heckman and Pease, 2007). As templates, we used constructs with fusions of the gene uidA with the native STM promoter (ProSTM::GUS, 3,379 bp; Uchida et al., 2007) or the native STM promoter without the K-box (ProSTM::ΔKUGUS; Uchida et al., 2007). The flowering master promoters were ProSTM::GUS and STM::RBR. The internal promoters were, respectively, Llb and Lbc for ProSTM::ΔKUGUS, STMb2 and STMc2 for ProSTM::ΔCRB::GUS, STMb2 and STMc2 for ProSTM::ΔCRB::CKGUS on ProSTM::ΔKUGS as template, STMb1 and STMb2 for ProSTM::ΔLRG:GUS, STMb1 and STMc1 for ProSTM::ΔLR8::CKGUS on ProSTM::ΔKUGUS as template, and STMb1 and STMc1 for ProSTM::ΔLR8::GUS on ProSTM::ΔKUGUS as template. Primers were F1b and F2c for ProSTM::ΔF1GUS, F2b and F3c for ProSTM::ΔF2GUS, F4b and F5c for ProSTM::ΔF4GUS, F6b and F7c for ProSTM::ΔF6GUS, F8b and F9c for ProSTM::ΔF8GUS, and F10b and F11c for ProSTM::ΔF10GUS. For ProSTM::ΔCRB::GUS and ProSTM::ΔCRB::CKGUS, we used primers STMPrBRB1 and STMPrBRB1 on ProSTM::GUS and ProSTM::ΔKUGUS as templates, respectively.
For the RNAi construct for FRF1, a fragment of 200 bp of the coding sequence
was amplified using primers FARRNA (5'-AACGAACGACA-AAAATTCGAGAATCAGAGAACTGA-3') and FARRNar (5'-CTATCACTGCGGTTCT GTTGATGATCT-3') and cloned into the pCR8GW/TOPO TA entry vector (Invitro- 
gren). We used pKGWIVW2GII (Karimi et al., 2002) as a destination vector.

To generate ProJAG:GFP plants, a 2.2-kb fragment 5' of JAG in the COL-4 ecto-
py was isolated using oligonucleotides JAG_5EcoRI and JAG_BHII. The
fragment was left-to-right (LR) cloned into pGWFS7 (Karimi et al., 2002). T3
lines were analyzed. For the generation of ProJAG:GFP-STM plants, we first
isolated fragment GFPm-STM from the construct STMp-STM (Uchida et al.,
2007) using oligonucleotides GF6FBH1 and T35SRBHI. This fragment was
cloned into an entry vector containing the promoter of JAG and LR cloned into the
plasmid pMDC123 with Basta selection in plants (Curts and Grosinklaus,
2003). COL-4 ectopy vectors were transformed with the ProJAG:GFP-STM construct.
The generation of GFP-STM was described previously (Uchida et al., 2007), and
the sequence of the primer was retrieved from the Web tool at http://signal.salk.

expression cassette in pMDC123 was reported previously (Uchida et al., 2007), and
this fragment was left-to-right (LR) cloned into pkGWFS7 (Karimi et al., 2002). T3
was generated using primers GF6FBHI and T35SRBHI. This fragment
and LBa1 for the insertions. The genotype of the allele
3-AT at 15 mM was suf-

quences. The primer sequences were found in Supplemental Table S2.

sequence was ampli-
ted using primers RBRIF and STMpRBSacIR to obtain the pHisi-RB plasmid. The

Characterization of the Alleles
Allele flf1-1 was genotyped using the combination NS264_S26Rp and NS264_S26lp for the wild type and the combinations NS264_S26lp and LBISAIL as well as NS264_S26lp and LBISAIL for the insertions. To genotype the allele flf1-2, we used the combination NS288_S31lp and NS289_S31Rp for the wild-type copy and the combinations NS288_S31lp and LBk1 as well as NS289_S31Rp and LBk1 for the insertions. The genotype of the allele flf1-1 was made using primers S6lp and S6Rp for the wild type and STMp and LBk1 for the insertion. The sequences of the primers were retrieved from the Web tool at http://signal.salk.
edu/tdaprinnters.2.html, and the positions of the insertions were determined by
sequencing. Primer sequences can be found in Supplemental Table S2.

GUS Histochemical Assays
We used 8-d-old plants of T3 transgenic lines. The staining was for 5 h as
described by Aguilar-Martinez and Sinha (2013).

Yeast One-Hybrid Assay
The yeast one-hybrid assay was made according to the MATCHMAKER
On Hybrid System (Clontech) and to Kooiker et al. (2005). A total of 226 bp
(-2,133 to -1,907 bp) of the Arabidopsis STM promoter including the RB-box
was introduced into the EcoRI and SacI sites of the pHISi plasmid using oli-
gonucleotides RBRIIE and STMPRBsSacIR to obtain the pHISi-RB plasmid. The
plasmid was sequenced and introduced upstream of the HIS3 reporter gene of
the Y187 yeast strain (MATα) using small-scale transformation according to the
Yeast Protocols Handbook (Clontech). In order to assess background due
to the weak expression, 3-amino-triazole (AT, Sigma A-8565) was used as a
competitive inhibitor of the HIS3 protein. The reporter strain was titrated on synthetic defined/-His plates with varying amounts of 3-AT (0-60 ms)
to determine the optimal concentration of 3-AT for background suppression.
3-AT at 15 ms was sufficient for background suppression in the yeast strains.
We mated with the yeast strain AH-109 (MATa) containing a normalized li-
brane of cDNA from Arabidopsis in the plasmid pGADT7 (kindly provided by
Simona Masiero). The growing colonies were plated again to confirm the in-
teractions. The pGADT7 plasmids were isolated and sequenced with primer
V7 (5'-CTATTCATGATGATAGAATCCC-3').

EMSA
EMSA was performed as described by Aguilar-Martinez and Sinha (2013).
Briefly, the coding sequence of FRF1 was cloned and used to generate a

Microscopy and Chemical Treatment
For the analysis of ProJAG:GFP-STM and ProSTM:GFP-STM transgenic
plants, an LSM 710 laser scanning microscope (Carl Zeiss) was used. Chlo-
rophyll autofluorescence collected at wavelengths 647 to 721 nm and GFP
fluorescence collected at wavelengths 495 to 528 nm were excited using a combi-
ation of excitation from 488- and 633-nm lasers. In addition, the range from 416 to 728 nm was collected to increase the visibility of nonfluorescing
features. Light and fluorescence microscopy on vibratome sections of ProJAG:
GFP-STM plants was also employed according to Koening et al. (2009) using
bandpass filters at 525 nm (GFP1) and 500 nm (GFP2).

Detection of GFP-tagged proteins was performed by agroinfiltration according to a previous report (Voinnet et al., 2003). Briefly, A. tumefaciens
strains harboring pMD32/GFP-STM or pMD32/GFP-STM-S227A were infiltrated into the abaxial air spaces of Nicotiana benthamiana plants together with
the A. tumefaciens C58C1 strain expressing p19, a silencing suppressor
encoded by Tomato bushy stunt virus. After 2 d, GFP fluorescence was observed using a TCS SP2 confocal microscope (Leica). When required, leaves were treated with 20 mM LMB (Sigma) 2 h before observation as reported previously
(Igarashi et al., 2001; Ishida et al., 2004).

The Arabidopsis Information Resource (http://www.arabidopsis.org/) locus identifiers for the FRF genes are as follows: FRF1 (At3g9470), FRF2
(At1g07500), FRF3 (At4g12850) and FRF4 (At2g34320). FRF-like proteins are
Soly06g732002.1, Soly06g732100.2, Soly06g057980.2, and Soly06g057870.1
in tomato; Medtrg1g0989201 and Medtrg1g098880.1 in contig_103701_3.1
and contig_49211_1.1, respectively, in M. truncatula; GSVIVT01010765001,
GSVIVT01010762001, GSVIVT0109099001, GSVIVT01010761001, GSVIVT01010760001,
GSVIVT01010759001, GSVIVT0102950001, GSVIVT0103842001, GSVIVT0106422001,
and GSVIVT01012799001 in grape; and LOC_Os11g230401.1, LOC_Os09g08601.1,
LOC_Os11g190301.1, LOC_Os11g209641.1, LOC_Os51g182601.1, LOC_Os05g008601.1,
and LOC_Os05g394901.1 in rice.

Supplemental Data
The following supplemental materials are available.

Supplemental Figure S1. Expression pattern of GUS in ProSTM::ΔLK:GUS,
ProSTM::ΔRB:GUS, and ProSTM::ΔRB::GUS plants.

Supplemental Figure S2. FRF1 binds the RB-box.

Supplemental Figure S3. Phylogenetic tree of FRF factors.

Supplemental Figure S4. Expression pattern of FRF genes.

Supplemental Figure S5. Sequence conservation on introns of KNOX1 genes in Brassicaceae spp.

Supplemental Figure S6. GUS expression pattern in ProJAG::GUS plants.

Supplemental Figure S7. Leaf phenotype in plants expressing STM under the control of the JAG promoter, and GFP distribution under the control of the
endogenous STM promoter or the JAG promoter.

Supplemental Figure S8. Sequence alignment of STM ortholog factors.

Supplemental Figure S9. Effect of LMB treatment on STM cellular distribution.

Supplemental Table S1. Sequenced clones from the yeast one-hybrid assay.

Supplemental Table S2. List of primers used in this study.

Supplemental Data Set S1. Sequence alignment of the FRF and FRS factors.

Supplemental Data Set S2. Sequence alignment of FRF-like factors in
eudicot species.
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LITERATURE CITED


New Insights on the Regulation of STM Gene Function
Disruption of 14-3-3 proteins


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