Regulation of *SHOOT MERISTEMLESS* genes via an upstream-conserved noncoding sequence coordinates leaf development

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The indeterminate shoot apical meristem of plants is characterized by the expression of the Class 1 *KNOTTED1-LIKE HOMEOBOX* (*KNOX1*) genes. *KNOX1* genes have been implicated in the acquisition and/or maintenance of meristematic fate. One of the earliest indicators of a switch in fate from indeterminate meristem to determinate leaf primordium is the down-regulation of *KNOX1* genes orthologous to *SHOOT MERISTEMLESS* (*STM*) in *Arabidopsis* (hereafter called *STM* genes) in the initiating primordia. In simple leafed plants, this down-regulation persists during leaf formation. In compound leafed plants, however, *KNOX1* gene expression is reestablished later in the developing primordia, creating an indeterminate environment for leaflet formation. Despite this knowledge, most aspects of how *STM* gene expression is regulated remain largely unknown. Here, we identify two evolutionarily conserved noncoding sequences within the 5′ upstream region of *STM* genes in both simple and compound leafed species across monocots and dicots. We show that one of these elements is involved in the regulation of the persistent repression and/or the reestablishment of *STM* expression in the developing leaves but is not involved in the initial down-regulation in the initiating primordia. We also show evidence that this regulation is developmentally significant for leaf formation in the pathway involving *ASYMMETRIC LEAVES1/2* (*AS1/2*) gene expression; these genes are known to function in leaf development. Together, these findings reveal a regulatory point of leaf development mediated through a conserved, noncoding sequence in *STM* genes.

evolution | KNOX1 | shoot apical meristem

The shoot apical meristem (SAM) of plants is an indeterminate structure and the source of stem cells from which all aerial organs are derived. Indeterminacy during development is regulated by a suite of genes that function in the SAM. The process of organ initiation begins when cells in the incipient organ primordium change identity from indeterminate to determinate. The indeterminate SAM is characterized by the expression of the Class 1 *KNOTTED1-LIKE HOMEOBOX* (*KNOX1*) genes. *KNOX1* genes have been implicated in the acquisition and/or maintenance of meristematic fate (1, 2). One of the earliest indicators of a switch in fate from indeterminate meristem to determinate leaf primordium is the down-regulation of *KNOX1* genes orthologous to *SHOOT MERISTEMLESS* (*STM*) in *Arabidopsis* (hereafter called *STM* genes) in the incipient primordia (1).

The SAM can give rise to two different leaf forms, simple or compound (3), and *KNOX1* genes are down-regulated in the incipient primordia in both compound and simple leafed species. In simple leafed plants, this down-regulation persists during leaf formation (4–7), and overexpression of these genes is sufficient to cause leaf lobing and ectopic meristem formation (8–11). However, *KNOX1* expression is reestablished later in the developing primordia of compound leafed plants (12–15). Additionally, overexpression of *KNOX1* genes in transgenic tomato plants or spontaneous tomato mutants results in leaves with increased numbers of leaflets (13, 14, 16). Therefore, it has been concluded that *KNOX1* genes are involved in compound leaf formation by establishing an indeterminate environment within developing primordia.

In *Arabidopsis thaliana*, loss-of-function of the *STM* gene results in loss of the SAM in agreement with its role in meristem acquisition/maintenance (1). *STM* represses *ASYMMETRIC LEAVES1* (*AS1*) (17–20), and *AS1* in turn represses *KNAT1* (also known as *BREVIPEDICELLUS*), another *Arabidopsis KNOX1* gene (17, 19–26). Some upstream factors are reported to regulate *KNAT1* expression (27–30), whereas upstream regulation of *STM* genes is still largely obscure. Thus, two questions still remain relating to how the expression of *STM* genes might be regulated: (*i*) how their expression is initially excluded from the incipient primordia and (*ii*) how their expression is repressed persistently in developing leaves of simple leafed species and reestablished in compound leafed species.

Control of gene expression requires cis-acting regulatory nucleotide sequences. Conserved noncoding sequences (CNSs) that have been maintained across orthologs in distantly related taxa can be candidates for such regulatory elements. Because CNS function is implied by evolutionary conservation, CNSs have attracted a lot of attention in various organisms (31–35) and have also been identified in plants (36–38). Some examples of CNSs include sequences present in introns where they function to repress ectopic expression of the maize *knotted1* (*kn1*) gene (36). In this report, we identify two CNSs within the 5′ upstream region of *STM* genes in monocots and dicots. We show that one of the elements has a significant role in leaf development, representing a regulatory point of leaf formation processes.

Results and Discussion

Identification of CNSs in 5′ Upstream Regions of *STM* Orthologous Genes. We identified two CNSs in the 5′ upstream regions of *STM* genes by comparing gene sequences from various species, including both simple (asparagus, yucca, grape, poplar, cotton, arabidopsis, tobacco, and snapdragon) and compound (palm, acacia, tomato, and ash) leafed species across monocots and dicots. These identified CNSs were named the RB-box and the K-box. Their positions relative to the translation initiation codon are shown in Fig. 1*A*. The core RB-box (≈23 nucleotides) is

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Abbreviations: SAM, shoot apical meristem; CNSs, conserved noncoding sequences; GUS, β-glucuronidase; RLM, RNA ligase-mediated.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. EF494254 (*Asparagus officinali STM*), EF494255 (*Yucca aloifolia STM*), EF494256 (*Gleditsia triacanthos STM*), EF494257 (*Fraxinus angustifolia STM*), and EF612706 (*Washingtonia robusta STM*)].

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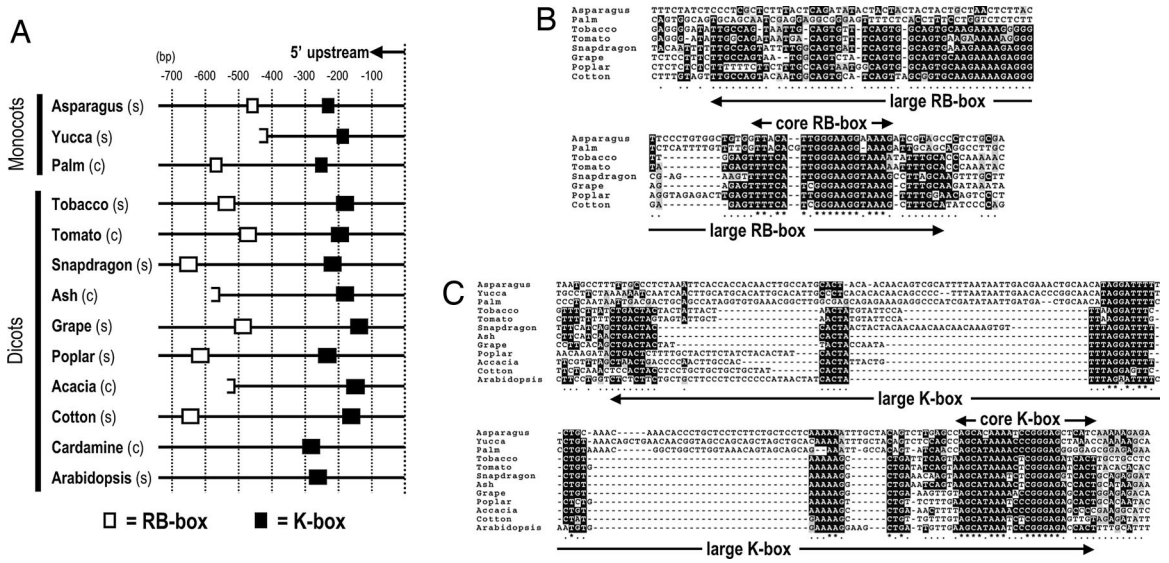

Fig. 1. Two CNSs in the 5′ upstream regions of *STM* orthologous genes. (*A*) The positions of RB-boxes (white boxes) and K-boxes (black boxes) in the 5′ upstream region from the translation start codon are illustrated. s and c indicate simple and compound leafed species, respectively. Halved white boxes for some RB-boxes show that sequences only downstream of the core RB box are available because the 5′ degenerate primer for the core RB-box was used to obtain upstream sequences. (*B* and *C*) The alignment of sequences of the RB-box and K-box are illustrated, respectively.

conserved in both monocots and dicots, whereas the large RB-box (≈80 nucleotides) is conserved only in dicots (Fig. 1*B*). The positions of the RB-box vary between 415 and 630 nucleotides upstream from the translation initiation codon (Fig. 1*A*). Among all of the species that we checked, the RB-box was absent only in *Arabidopsis* and the recently reported *STM* gene of *Cardamine hirsuta* (39). It remains to be seen if the RB-box is absent only in *STM* genes of the clade including *Arabidopsis* and *Cardamine* or in the entire Brassicaceae family. The core K-box (≈23 nucleotides) is conserved well among both monocots and dicots, whereas the large K-box (≈90 nucleotides) is conserved especially in dicots (Fig. 1*C*). The K-box is located in the region between 105 and 271 nucleotides upstream from the translation initiation codon (Fig. 1*A*).

In the Poaceae, including rice and maize, the *kn1* ortholog is known to participate in the maintenance of the SAM (2). Two recently published papers regarding monocot *STM* genes (40, 41) and also our unpublished analysis strongly suggest that the *STM* ortholog was lost in the Poaceae. The Poaceae *kn1* gene is related to *KNAT1*. The *STM* function appears to be taken over by the *kn1* in the Poaceae. Here, we report on our functional analysis of the tobacco (Solanaceae) K-box and RB-box and the *Arabidopsis* (Brassicaceae) K-box.

K-Box Represses *NTH15* Expression Outside SAM in Tobacco. To investigate how the CNSs are involved in the regulation of the tobacco *NTH15* gene (tobacco ortholog of *Arabidopsis STM*), we carried out β-glucuronidase (GUS) reporter fusion experiments. When the GUS reporter gene was fused to the 5′ upstream region from the translation start codon of the tobacco *NTH15* gene and transformed into tobacco plants (NTH15p-GUS), GUS expression was detected only in the SAM (Fig. 2*A*). When the large K-box (81 bp) or the large RB-Box (68 bp) was removed from the reporter construct (ΔK-GUS and ΔRB-GUS, respectively), we observed a dramatic expansion of GUS expression in the ΔK-GUS plants but not in the ΔRB-GUS plants (Fig. 2 *B* and *C*). GUS expression was detected not only in the SAM but also in the basal region of the ΔK-GUS leaves (Fig. 2*B*), and transverse sections of leaves showed GUS expression in the midvein (Fig. 2*E*). These results suggest that the K-box has a role in the repression of *STM* gene expression outside of the SAM. Thus, in this study we focused on elucidating the function of the K-box.

K-Box Functions as a DNA Element. To determine whether the K-box is an element of the promoter or a part of the 5′ UTR of the mRNA, we analyzed the transcription initiation sites of the endogenous *NTH15* gene by 5′ RNA ligase-mediated RACE (RLM-RACE), which is 5′ RACE-specific for capped mRNA. We detected two major PCR products in this experiment (Fig. 2*F*). Sequence analysis showed that one product started at the 5′ end of the core K-box and that the other started downstream of the K-box (Fig. 2*H*). To confirm this, we also performed 5′ RLM-RACE to determine the transcription initiation sites of the GUS reporter transgenes in the NTH15p-GUS and ΔK-GUS plants. We detected one major amplification product from both transgenes (Fig. 2*G*), and sequence analysis indicated that transcription started at the same position downstream of the K-box in both cases. This position was identical to the shorter endogenous *NTH15* mRNA lacking a K-box (Fig. 2*H*). NTH15p-GUS and ΔK-GUS plants showed different GUS expression patterns despite the same transcription initiation site downstream of the K-box (Fig. 2 *A* and *B*). These results indicate that the presence of the K-box in the 5′ UTR of the mRNA is not required for SAM-specific expression of the *NTH15* gene. Therefore, we conclude that the K-box likely functions as a DNA element in the promoter region.

K-Box Represses *STM* Expression in Developing Leaves but Not in Initiating Leaf Primordia of *Arabidopsis*. We next carried out GUS reporter experiments in *Arabidopsis* to confirm whether K-box function is conserved in this species. When the GUS reporter was fused to the 5′ region of the *Arabidopsis STM* gene upstream of the translation start codon and then transformed into *Arabidopsis* (STMp–GUS), GUS expression was detected only in the SAM (Fig. 3*A*). However, when the core K-box (25 bp) was removed from the reporter construct (ΔK-GUS), GUS expression was detected not only in the SAM but also in the midvein of the leaf petioles (Fig. 3*B*). We also determined the transcriptional start sites of the endogenous *STM* gene and GUS reporter transgenes

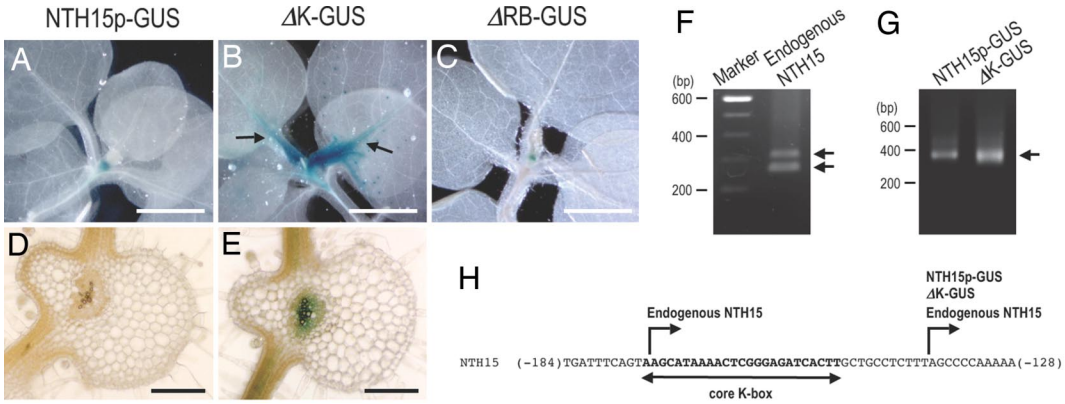

Fig. 2. GUS expression from the *NTH15* upstream region in tobacco and the determination of the transcription initiation sites. (*A–C*) GUS expression in transgenic tobacco seedlings harboring NTH15p–GUS, ΔK-GUS, and ΔRB-GUS transgenes were detected, respectively. Arrows indicate ectopic expression of GUS proteins in leaves. (*D* and *E*) GUS expression in transverse sections of leaves of transgenic tobacco harboring NTH15p–GUS and ΔK-GUS transgenes are shown, respectively. (*F* and *G*) Agarose gel electrophoresis of nested PCR products from the RLM-RACE procedure for endogenous *NTH15* gene and GUS reporter transgenes, respectively. Molecular size markers (base pairs) are shown on the left. Arrows on the right mark the major PCR products. (*H*) Sequence around the core K-box of the *NTH15* gene is illustrated, and arrows above the sequence indicate the identified transcription start sites. Horizontal arrow below the sequence indicates the core K-box. (Scale bars: *A–C*, 2 mm; *D* and *E*, 0.4 mm.)

and confirmed that the K-box is upstream of the identified transcriptional start sites (Fig. 3 *C–E*). Thus, the K-box appears to function as a DNA element in the promoter of both *Arabidopsis* and tobacco to restrict *STM* expression to the SAM.

KNOX1 genes are known to be expressed in the SAM but not in initiating leaf primordia. This repression in leaf primordia is persistent during leaf development in simple leafed species such as *Arabidopsis*. To observe *STM* expression regulation in the shoot apex, we analyzed the GUS expression in STMp–GUS and ΔK-GUS *Arabidopsis* plants by using thin paraffin sections. STMp–GUS plants showed strong GUS expression in the SAM, but this expression was remarkably eliminated from the new leaf primordia (Fig. 4 *A* and *C*, arrowheads). We also noticed a weak trace of GUS expression in the base of leaf primordia of STMp–GUS plants (Fig. 4*C*) compared with the expression patterns of the endogenous *STM* gene reported in ref. 42. Similar observations were reported when a STMp–GUS reporter transgene was used (43). It is likely that this is a characteristic of the *STM* promoter fragment used. Thus, the *STM* upstream region used in these experiments had the elements necessary for down-regulation of *STM* expression in new primordia. This down-regulation was also observed in the ΔK-GUS plants (Fig. 4 *B* and *D*, arrowheads). However, GUS expression was reestablished in older developing leaves (Fig. 4 *B* and *D*, asterisks). These results indicate that the K-box mediates the persistent repression of *STM* expression in developing leaves but not the initial down-regulation in leaf primordia.

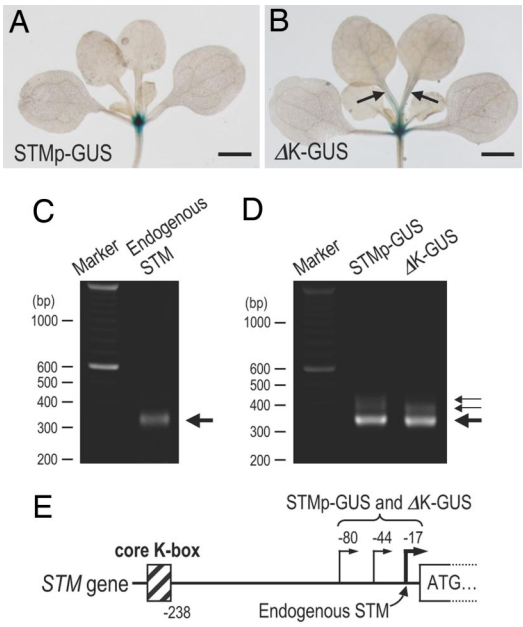

Fig. 3. GUS expression from the *STM* upstream region in *Arabidopsis* and the determination of the transcription initiation sites. (*A* and *B*) GUS expression in transgenic *Arabidopsis* seedlings harboring STMp–GUS and ΔK-GUS transgenes were detected, respectively. Arrows indicate ectopic expression of GUS proteins in leaf petioles. (*C* and *D*) Agarose gel electrophoresis of nested PCR products from the RLM-RACE procedure for endogenous *STM* gene and GUS reporter transgenes, respectively. Molecular size markers (base pairs) are indicated on the left. Thick arrows and thin arrows on the right mark the major and minor PCR products, respectively. (*E*) The identified transcription start sites are illustrated. (Scale bars: 1.5 mm.)

K-box-Mediated *STM* Expression Regulates Leaf Development. To analyze the developmental role of the K-box, a translational fusion between GFP and STM driven by the 5′ upstream region of the *STM* gene with or without the K-box (STMp–STM or ΔK-STM, respectively) was introduced into *Arabidopsis*. The STMp–STM transgene rescued the shoot meristem defect of *stm* mutant seedlings [see supporting information (SI) Fig. 7 and SI Table 1] and did not show an ostensible phenotypic alteration in the wild-type background (Fig. 5 *A*, *B*, *E*, and *F*). ΔK-STM plants, on the other hand, showed an altered plant phenotype with rounded leaves and short petioles, resembling *as1* mutant phenotypes (Fig. 5 *C*, *D*, *G*, and *H*). ΔK-STM plants also harbor leaf-lobing phenotypes. The degree of leaf lobing varies among individuals (for example, compare Figs. 5*G* and 6*D*) as is also the case in *as1-1* mutants grown under controlled conditions. This transgene also rescued the phenotype of *stm* mutant seedlings (SI Fig. 7 and SI Table 1), and the rescued seedlings showed *as1* mutant-like phenotypes (SI Fig. 7*I*). These results indicate that the K-box might have a role in the control of leaf formation in the pathway related to *AS1*, a known participant in leaf development (17), and that the K-box is not required for the acquisition and/or maintenance of SAM fate by the *STM* gene.

AS1 is known to function downstream of the *STM* gene together with *AS2* (17–21, 24). RT-PCR experiments in the ΔK-STM plants showed a severe reduction of the expression of

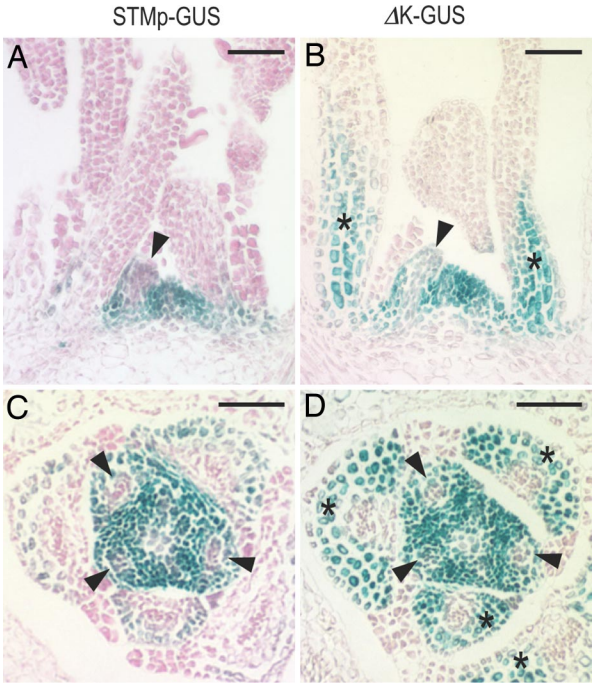

Fig. 4. Paraffin sections of GUS-stained *Arabidopsis* SAM tissues. GUS expression in longitudinal (*A* and *B*) and transverse (*C* and *D*) sections of SAM of transgenic *Arabidopsis* seedlings harboring STMp–GUS (*A* and *C*) and ΔK-GUS (*B* and *D*) transgenes. Arrowheads indicate down-regulation of GUS expression in young leaf primordia. Asterisks indicate the reestablishment of GUS expression in the developing leaf primordia. (Scale bars: 50 μm.)

AS1 and *AS2* mRNA compared with wild-type and STMp–STM plants (Fig. 6*A*). To further confirm the involvement of the K-box in the *AS1* pathway, we also analyzed the expression of *KNAT1* mRNA in leaves of STMp–STM and ΔK-STM plants because *KNAT1* is reported to be down-regulated by *AS1/2* (17, 18, 22, 24). We did not detect *KNAT1* expression in wild-type leaves, but the expression was present in *as1* leaves (Fig. 6*B*). Similarly, *KNAT1* expression was not detected in STMp–STM leaves but was seen in ΔK-STM leaves (Fig. 6*B*). Also, when we compared the GUS expression patterns in leaves of KNAT1p–

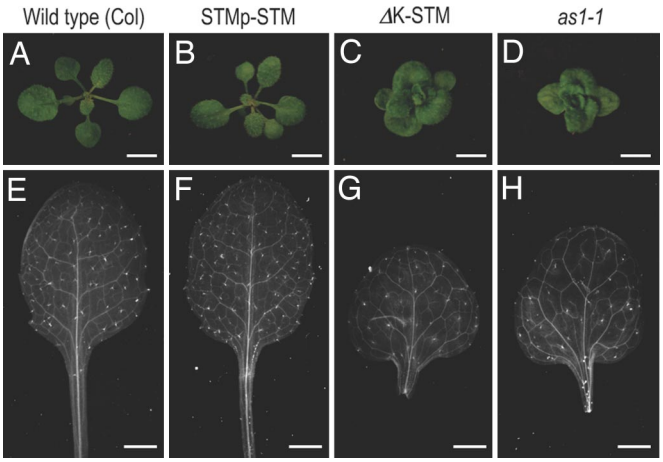

Fig. 5. Transgenic *Arabidopsis* plants expressing the STM protein from the STM upstream region with or without the K-box. Vegetative rosettes (*A–D*) and cleared rosette leaves (*E–H*) of wild-type (Col) (*A* and *E*), STMp–STM (*B* and *F*), and ΔK-STM (*C* and *G*) and *as1-1* (*D* and *H*) *Arabidopsis* plants are shown. (Scale bars: *A–D*, 5 mm; *E–H*, 2 mm.)

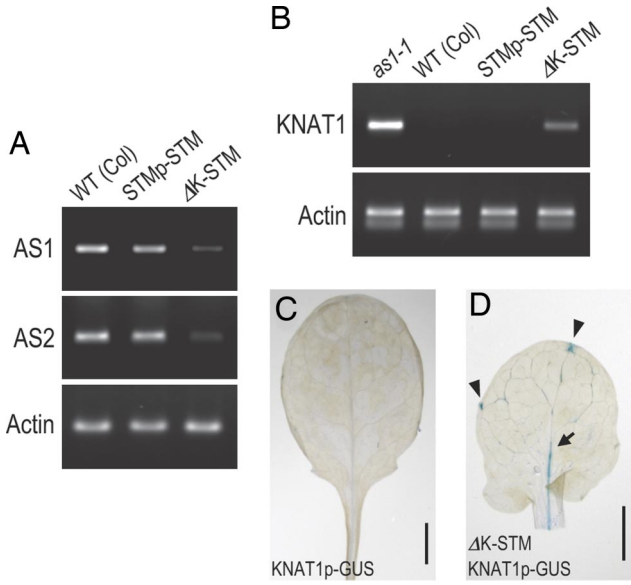

Fig. 6. Involvement of STM regulation by K-box in the AS1 pathway during leaf development. (*A* and *B*) Products by RT-PCR for AS1, AS2, and ACT2 mRNA in tissues containing the SAM and leaf primordia of *Arabidopsis* plants (*A*) and KNAT1 and ACT2 mRNA in leaves of *Arabidopsis* plants (*B*) are shown. (*C* and *D*) GUS expression in transgenic *Arabidopsis* seedlings harboring KNAT1p–GUS with or without ΔK-STM, respectively. Arrow and arrowheads indicate GUS expression in the leaf midvein and leaf hydathodes, respectively. (Scale bars: 2 mm.)

GUS plants with or without the ΔK-STM transgene (Fig. 6 *C* and *D*), *as1*-mutant-like leaves induced by the ΔK-STM transgene showed ectopic GUS expression in leaf midveins and hydathodes. These observations are consistent with the previous reports showing GUS expression patterns from the KNAT1p–GUS reporter gene in *as1* and *as2* mutant plants (22, 25, 27, 28). Thus, the control of *STM* expression via the K-box may be significant for leaf development in the pathway involving *AS1/2* gene expression.

Function of K-box in *STM* Genes Suggests Regulatory Points in Leaf Development. The regulation of leaf development via *STM* orthologous *KNOX1* genes can be partitioned into two steps. The first step is the initial down-regulation of *STM* expression in the incipient primordia, and the second step is either the persistent repression of expression in the developing leaves of simple leafed species or the reestablishment of expression in compound leafed species. In this study, we identify two CNSs within the 5′ upstream region of *STM* genes and show that one of them, K-box, plays a significant role in regulation of *STM* expression. The K-box functions in the persistent repression of *STM* expression in developing leaves of simple leafed species (Figs. 2 and 3). On the other hand, the initial down-regulation of *STM* expression in the incipient primordia is likely controlled in a K-box-independent manner. Current studies suggest that auxin is a key factor responsible for the initial down-regulation of *KNOX1* genes (44–47). Our results show the initial down-regulation in both STMp–GUS and ΔK-GUS plants (Fig. 4), indicating that the signaling pathway triggered by auxin is eventually connected to the *STM* upstream region but outside of the K-box. Future investigation is required to reveal the relationship between auxin and unidentified elements of the *STM* upstream region responsible for the initial down-regulation of *STM* expression.

Simple leafed plants such as *Arabidopsis* show persistent repression of *KNOX1* expression after the initial down-regulation of the expression, whereas in compound leafed spe-

cies, *KNOX1* expression is reestablished later in developing leaf primordia. Our results show that, in developing leaves of ΔK-GUS plants, *STM* promoter activity is reestablished (Figs. 2–4). K-box sequences are conserved among both simple and compound leafed species (Fig. 1). This conservation suggests that understanding upstream regulation of the K-box might be crucial to our understanding of the difference in *STM* expression between both types of species. This regulation may involve transacting factors that work through the K-box, although database searches have not yielded any recognizable binding sites of transcription factors in the K-box. Furthermore, a recent report (39) showed that changes of unknown cis-elements in the *STM* promoter are responsible for altered *STM* expression patterns between compound leafed *Cardamine* and simple leafed *Arabidopsis*, but our sequence analysis showed that the K-box is conserved between these two species. It is possible that the K-box cooperates with other unknown cis-elements of the *STM* promoter to control *STM* expression differently between simple and compound leafed species.

Our findings indicate that the regulation of *STM* gene expression is divided into two steps controlled by distinct mechanisms: a K-box-independent mechanism in the SAM and a K-box-dependent one in the developing leaves. This regulation of *STM* suggests a regulatory point in leaf development that may have been involved in elaborating a variety of leaf shapes in nature.

Materials and Methods

Sequences of *STM* Orthologous *KNOX1* Upstream Region from Various Plant Species. Upstream sequences of *STM* genes of grape (*Vitis vinifera*), cotton (*Gossypium raimondii*), snapdragon (*Antirrhinum majus*), and cardamine (*Cardamine hirsuta*) were obtained from the public database (GenBank accession nos. AM458687, CO081267, AY072735.1, and DQ526380, respectively). The genomic sequences of *STM* genes of *Arabidopsis* and poplar were found in the sequenced genome information. The upstream sequence of the tobacco *NTH15* gene was provided by M. Matsuoka (Nagoya University, Chikusa, Japan) (48). The genomic sequence of the tomato *LeT6* gene was reported in ref. 13. After the identification of the RB-boxes among the above sequences, degenerate primer sets corresponding to the core RB-box and protein-coding sequences were used to amplify genomic fragments of asparagus, yucca, acacia, and ash. Further 5′ extension of the obtained sequences was carried out by thermal asymmetric interlaced PCR. The GenBank database accession numbers of the sequences of palm (*Washingtonia robusta*), asparagus (*Asparagus officinalis*), yucca (*Yucca aloifolia*), acacia (*Gleditsia triacanthos*), and ash (*Fraxinus angustifolia*) are EF612706, EF494254, EF494255, EF494256, and EF494257, respectively.

Plasmid Construction and Plant Transformation. The partial deletion (68 bp, from −516 to −584, relative to the translation initiation codon for the large RB-box and 81 bp, from −164 to −245, for the large K-box) of the 2.5-kb *NTH15* promoter (48) was carried out by PCR. Then, the original fragment and the deleted fragments were cloned into the pCR8/GW/TOPO vector (Invitrogen, Carlsbad, CA). These constructs served as the entry vectors to transfer promoter fragments into the pKGWFS7 vector containing a GFP–GUS reporter gene (49). This construct was made via the GATEWAY system by using the LR reaction (Invitrogen). The 3.3-kb *STM* promoter region of the binary vector harboring the GUS reporter gene (50) was replaced with the deleted version (deletion of the 25-bp core K-box, from −238 to −263, relative to the translation initiation codon) after modification by PCR. STM-coding sequences, GFP6-coding sequences, and cauliflower mosaic virus 35S transcriptional terminator sequences were amplified by PCR by using an *Arabidopsis* cDNA library reverse-transcribed from the total RNA, pK2GW7 vector (49) and the pMDC204 vector (51), respectively. They were cloned into the pCR8/GW/TOPO vector together with the 3.3-kb *STM* promoter region or with the modified version. These expression cassettes were transferred to the pMDC123 binary vector (51) using the LR reaction. The constructs described above were introduced into Agrobacterium GV3101 for *Arabidopsis* transformation or EHA105 for tobacco transformation by electroporation. *Arabidopsis* (Col) and *Nicotiana tabacum* (cv. Samsun NN) were transformed by the floral dip method (52) and the tissue culture method, respectively.

Genotyping. Detection of the *stm-1* allele was performed as described in ref. 53 with some modifications. The primer set used for the dCAPS PCR marker was TTTATTAGATTAAATGATAACATTTAAGTCGATATGAACAATGAATTTGTAGATGCA and GTATAAGGGAAGAGAGTTACCGAAG. The 248-bp PCR product was digested into 198-bp and 50-bp fragments with NsiI when amplified from the *stm-1* allele but not when amplified from the wild-type allele (SI Fig. 7 *B* and *C*). One primer spanned an intron–exon boundary to prevent the interference by the *STM* cDNA contained in the transgene used in this study.

Determination of Transcription Initiation Site. Total RNA was isolated from the shoot apex of plants with an RNeasy Plant Mini kit (Qiagen, Valencia, CA). Determination of the transcription initiation sites was performed by RNA ligase-mediated 5′ rapid amplification of cDNA ends (5′ RLM-RACE) by using a GeneRacer kit (Invitrogen). Briefly, total RNA was dephosphorylated by using calf intestinal phosphatase to eliminate the 5′ phosphates from the truncated (noncapped) mRNA without affecting the 5′ capped mRNA, and the RNA was then treated with tobacco acid pyrophosphatase to remove the 5′ cap structure. GeneRacer RNA Oligo was ligated to the 5′ end of the decapped mRNA by a T4 RNA ligase. cDNA synthesis was performed with SuperScript III reverse transcriptase and with the gene-specific primers for the coding regions (AGGAAGTAAGAGTGAGAGC for the endogenous *NTH15*, AAGTCGTGCTGCTTCATG for the NTH15p(ΔK)–GUS transgene, TGAGGATGAGCCATGATC for the endogenous *STM* gene, and CAAACGGTGATACGTACAC for the STMp(ΔK)–GUS transgene). PCR was performed using the primer sets of the GeneRacer 5′ Primer and the gene-specific primers (GACAAGAGACGAGGGTAGTGAGGATGA for the endogenous *NTH15*, CTGAAGCACTGCACGCCGTAGGT for the NTH15p(ΔK)–GUS transgene, GATTCCTCCTGCAACGATTTCGTTATG for the endogenous *STM* gene, and CTGATGCTCCATCACTTCCTGATTATTGAC for the STMp(ΔK)–GUS transgene). Nested PCR was carried out using the primer sets of the GeneRacer 5′ Nested Primer and the gene-specific primers [GAGGACATAGGGCTGCATTTCCACT for the endogenous *NTH15*, TTCAGGGTCAGCTTGCCGTAGGT for the NTH15p(ΔK)–GUS transgene, CATTAGAGAATAGGCAGGAGCACAAG for the endogenous *STM* gene, and CGTAATGAGTGACCGCATCGAAAC for the STMp(ΔK)–GUS transgene]. The amplified products were gel-purified with the Qiaquick Gel Extraction kit (Qiagen), were cloned into the pCR4Blunt–TOPO vector (Invitrogen), and then were sequenced.

GUS Assay and Histology. Detection of GUS expression was performed with 5-bromo-4-chloro-3-indolyl-β-D-glucuronidase (X-Gluc; Rose Scientific, Edmonton, AB, Canada). Tissues were vacuumed in 50 mM sodium phosphate buffer, pH 7.0 (1% Triton X-100/1% DMSO/10 mM EDTA/0.5 mM potassium ferrocyanide/0.5 mM potassium ferricyanide/1 mM X-Gluc) and then incubated at 37°C. The stained samples were washed twice with 70% ethanol, fixed in 50% ethanol/5% acetic acid/3.7% formaldehyde, and cleared with 70% ethanol. Tissues were mounted in 50% glycerol,

and microscopy was conducted. When necessary, tissues were sectioned with a vibratome (Leica, Deerfield, IL) before observation. Histological analysis followed by paraffin sectioning was performed as described in ref. 54 with some modifications. Briefly, tissues were gently fixed in 90% acetone, washed with water, vacuumed in 50 mM sodium phosphate buffer, pH 7.0 (0.2% Triton X-100Z/10 mM EDTA/10 mM potassium ferrocyanide/10 mM potassium ferricyanide/1 mM X-Gluc), and incubated at 37°C. The stained tissues were fixed in 50% ethanol/5% acetic acid/3.7% formaldehyde, treated with Eosin-Y, and then embedded in Paraplast Plus (McCormick Scientific, St. Louis, MO). Paraffin sections (7 μm) were microtomed (Leica) and were observed after deparaffinization. Observation of leaf vein patterns was performed after incubation in the clearing agent chloral hydrate.

Primer Sets for RT-PCR Analysis. The following primer sets were used for RT-PCR: *ACT2*, TGCTGGATTCTGGTGATG and TTCCAGCAGCTTCCATTC; *AS1*, GGAAGTTGCTCTTGAGTTTG and CAGCAATCTTCTTCCACTTG; *AS2*, CAAAGAAAGGCTTCTTTAATTTACTC and CTGACGAAGCTGATGTTG; and *KNAT1*, GAAGATCGGGAACTCAAG and TCCATCACCCATGTAATGAC. All targeted regions contain exon–exon junctions to avoid the amplification derived from contamination of genomic DNA.

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