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# *KNAT6*: An *Arabidopsis* Homeobox Gene Involved in Meristem Activity and Organ Separation [W]

**Enric Belles-Boix,[1] Olivier Hamant,[1,2] Sarah Melissa Witiak,[3] Halima Morin, Jan Traas,[2] and Véronique Pautot[4]**

Laboratoire de Biologie Cellulaire, Institut Jean-Pierre Bourgin, Institut National de la Recherche Agronomique, 78026 Versailles Cedex, France

**The homeobox gene family plays a crucial role during the development of multicellular organisms. The *KNOTTED*-like genes from *Arabidopsis thaliana* (*KNAT6* and *KNAT2*) are close relatives of the meristematic genes *SHOOT MERISTEMLESS* (*STM*) and *BREVIPEDICELLUS*, but their function is not currently known. To investigate their role, we identified null alleles of *KNAT6* and *KNAT2*. We demonstrate that *KNAT6* contributes redundantly with *STM* to the maintenance of the shoot apical meristem (SAM) and organ separation. Consistent with this role, the expression domain of *KNAT6* in the SAM marks the boundaries between the SAM and cotyledons. The lack of meristematic activity in the *knat6 stm-2* double mutant and the fusion of cotyledons were linked to the modulation of *CUP-SHAPED COTYLEDON* (*CUC*) activity. During embryogenesis, *KNAT6* is expressed later than *STM* and *CUC*. In agreement with this fact, *CUC1* and *CUC2* were redundantly required for *KNAT6* expression. These data provide the basis for a model in which *KNAT6* contributes to SAM maintenance and boundary establishment in the embryo via the *STM*/*CUC* pathway. *KNAT2*, although the closest related member of the family to *KNAT6*, did not have such a function.**

## INTRODUCTION

In plants, the *KNOTTED*-like homeobox (*KNOX*) genes play crucial roles in both embryonic and postembryonic development (Takada and Tasaka, 2002; Hake et al., 2004). In particular, they are required for the initiation and maintenance of the shoot apical meristem (SAM), a group of dividing cells that controls organ production throughout the plant life cycle. Class I of the *KNOTTED*-like from *Arabidopsis thaliana* (*KNAT*) family consists of *SHOOT MERISTEMLESS* (*STM*), *KNAT1* or *BREVIPEDICELLUS* (*BP*), *KNAT6*, and *KNAT2* (Hake et al., 2004). Although *STM* is required for the formation and maintenance of the SAM (Clark et al., 1996; Endrizzi et al., 1996; Long et al., 1996), *BP* regulates internode development (Douglas et al., 2002; Venglat et al., 2002; Smith and Hake, 2003) and contributes with *STM* to SAM maintenance (Byrne et al., 2002). In addition to its role in the SAM, *STM* interacts with the *CUP-SHAPED COTYLEDON* (*CUC*) genes to separate the organs (Takada et al., 2001). During embryogenesis, the *CUC* gene family plays a crucial role in the establishment of the SAM and in organ separation. The *CUC1* and *CUC2* genes are redundantly required for cotyledon separation and SAM formation via the activation of the *STM* gene (Aida et al., 1999; Takada et al., 2001). Further studies demonstrate that the action of *CUC1* is mediated by *STM*: the induction of adventitious SAMs on cotyledons in *CUC1* overexpressor lines depends on STM activity (Hibara et al., 2003). In turn, *STM* is required for the correct expression patterns of *CUC2* and to a lesser extent of *CUC1* during later stages of embryogenesis (Aida et al., 1999; Takada et al., 2001). The *KNAT2* and *KNAT6* genes are expressed in the SAM and are the most closely related members of the *KNAT* family (Dockx et al., 1995; Semiarti et al., 2001). Using an inducible *KNAT2* overexpressor line, we previously showed a link between *KNOX* genes and carpel development (Pautot et al., 2001). A *knat2* mutant allele has been reported; however, its development was normal (Byrne et al., 2002). Previous studies using RNA interference lines that downregulate *KNAT6* suggested a putative role for *KNAT6* in lateral root initiation but did not reveal an obvious role in the shoot (Dean et al., 2004). To investigate the role of *KNAT6* and *KNAT2* in the SAM, we identified null alleles with T-DNA insertions in each gene and examined their interactions with *STM*. Here, we show that *KNAT6* but not *KNAT2* contributes, redundantly with *STM*, to SAM maintenance and to the establishment of the boundaries via the *STM*/*CUC* pathway.

## RESULTS

### *KNAT6* Is Expressed in the SAM Boundaries

To determine the expression pattern of *KNAT6*, we generated a *pKNAT6-GUS* (for β-glucuronidase) transcriptional fusion. During

[1] These authors contributed equally to this work.

[2] Current address: Laboratoire Reproduction et Développement des Plantes, Ecole Normale Supérieure Lyon, 46 Allée d'Italie, 69364 Lyon Cedex 07, France.

[3] Current address: 122 Chemical Ecology Lab, Orchard Road, University Park, PA 16802.

[4] To whom correspondence should be addressed. E-mail pautot@versailles.inra.fr; fax 33-01-30-83-30-99.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Véronique Pautot (pautot@versailles.inra.fr).

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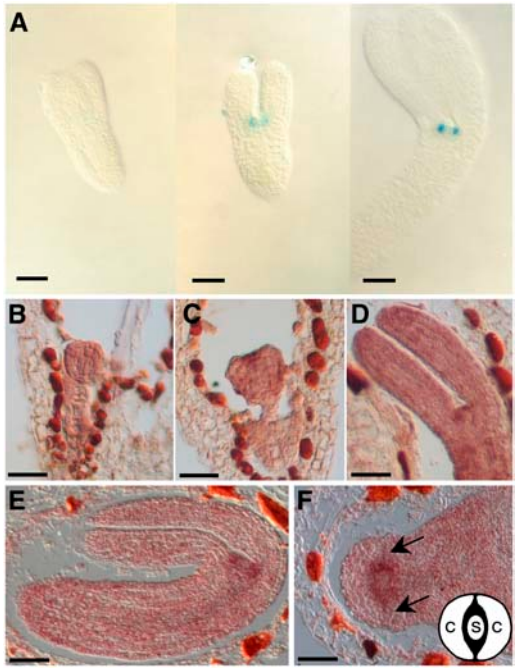


**Figure 1.** *KNAT6* Is Expressed in Boundaries.

**(A)** Expression of *pKNAT6-GUS* was detected at the torpedo stage. The embryos were cleared.
**(B)** Longitudinal section through a globular embryo. The *KNAT6* mRNA was not detected.
**(C)** Longitudinal section through a triangular embryo showing *KNAT6* mRNA accumulation in the presumptive SAM.
**(D)** and **(E)** Longitudinal sections through late-torpedo **(D)** and mature **(E)** embryos showing *KNAT6* mRNA accumulation in the boundaries between the SAM and the cotyledons.
**(F)** Transverse section through a mature embryo showing *KNAT6* mRNA accumulation in a region surrounding the SAM. This region corresponds to the boundaries between the SAM (S) and the cotyledons (C) and the boundaries between the cotyledons.
Bars = 50 μm.

embryogenesis, *pKNAT6-GUS* expression was not detected before the early torpedo stage. In torpedo embryos, expression was restricted to the boundaries between the presumptive SAM and the cotyledons (Figure 1A). To confirm *pKNAT6-GUS* expression, we used in situ hybridization. The *KNAT6* mRNA was not detected at the globular stage (Figure 1B). It was first detected in the triangular stage embryo, where it appeared in the presumptive SAM (Figure 1C). At the torpedo stage, its expression was detected between the cotyledons and the meristem (Figure 1D). A transverse section of the mature embryo indicated that *KNAT6* was expressed between the cotyledons and the meristem and between the cotyledons (Figure 1F). In 8-d-old seedlings, *pKNAT6-GUS* was expressed in the stipules and marked the boundaries between the SAM and the emerging primordia (see Supplemental Figure 1A online). The expression pattern of *KNAT6* is more restricted than that of *KNAT2*, which is expressed in the rib zone of the meristem (Pautot et al., 2001; see Supplemental Figure 2 online).

### Characterization of *knat6* and *knat2*

To analyze *KNAT6* function, two *Arabidopsis* lines (Columbia [Col-0] ecotype) with T-DNA insertions located in introns 3 and 2 of the *KNAT6* gene were characterized. These insertions were named *knat6-1* and *knat6-2*, respectively. A T-DNA insertion line in exon 3 of the *KNAT2* gene, named *knat2-5*, was selected for further analysis. These lines were backcrossed twice to the Col-0 ecotype, and mutant homozygous lines were obtained. To monitor the *KNAT6* and *KNAT2* transcripts, RNA was isolated from wild-type, *knat6-1*, *knat6-2*, and *knat2-5* 8-d-old seedlings. RT-PCR analysis showed that T-DNA insertions led to null *knat6* and *knat2* alleles (Figures 2A and 2B). The development of *knat6-1*, *knat6-2*, and *knat2-5* homozygous single lines was normal. The analysis of RNA interference lines has suggested that the down-regulation of *KNAT6* affects lateral root initiation (Dean et al., 2004). Even though we confirmed that *KNAT6* is expressed at the site of the lateral root (see Supplemental Figures 1B and 1C online), the development of both null alleles *knat6-1* and *knat6-2* was normal. To test a potential redundancy with other *KNAT* genes, we generated the *knat6-1 knat2-5* and *knat6-2 knat2-5* double mutants. Surprisingly, these double mutants did not show any defect in SAM function (data not shown). This finding indicates that *KNAT2* and *KNAT6* do not share a redundant

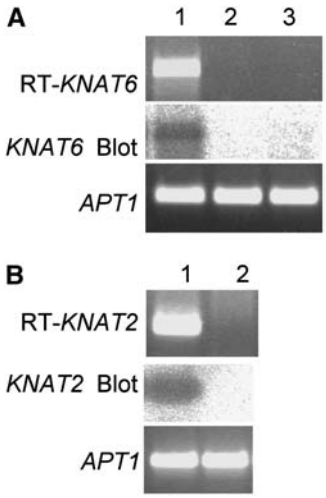


**Figure 2.** Characterization of the Loss-of-Function *knat6* and *knat2* Mutant Alleles.

**(A)** RT-PCR analysis using *KNAT6* gene-specific primers. After 38 cycles, the PCR products were transferred to a nylon membrane and hybridized with a $^{33}$P-labeled *KNAT6* probe. Amplification occurred in wild-type seedlings (lane 1) but not in *knat6-1* and *knat6-2* alleles (lanes 2 and 3, respectively), indicating that both alleles are null. Control amplification of *APT1* indicates that equal amounts of cDNA were present in each sample.
**(B)** RT-PCR analysis using *KNAT2* gene-specific primers. After 40 cycles, the PCR products were transferred to a nylon membrane and hybridized with a $^{33}$P-labeled *KNAT2* probe. Amplification occurred in wild-type seedlings (lane 1) but not in the *knat2-5* allele (lane 2), indicating that the *knat2-5* allele is null. Control amplification of *APT1* indicates that equal amounts of cDNA were present in each sample.

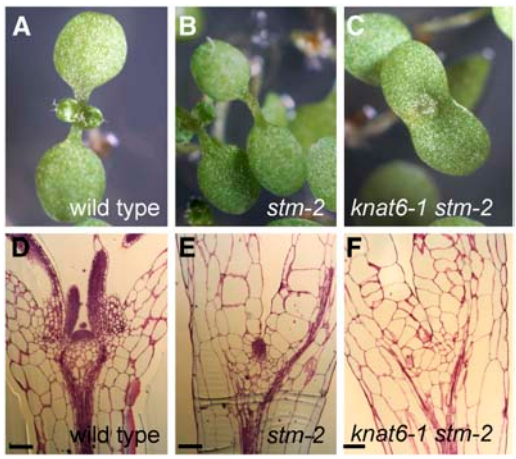


**Figure 3.** Phenotype of the *knat6 stm* Double Mutant.

All seedlings shown are 8 d old. Bars = 100 μm.
**(A)** Wild-type seedling. The SAM gives rise to leaf primordia.
**(B)** *stm-2* seedling.
**(C)** *knat6-1 stm-2* seedling showing a fusion that extends to the blade of the cotyledons.
**(D)** Longitudinal section through a wild-type vegetative meristem.
**(E)** Longitudinal section through a *stm-2* seedling. A primordium is visible between the cotyledons.
**(F)** Longitudinal section through a *knat6-1 stm-2* seedling. The cells are large and vacuolated.

function with each other or that other *KNAT* genes can compensate for the lack of *KNAT6* and *KNAT2* expression.

### *KNAT6*, but Not *KNAT2*, Contributes with *STM* to SAM Maintenance

The role of *BP* in the SAM was revealed in the intermediate *stm-2* background, showing a redundancy between *BP* and *STM* (Byrne et al., 2002). To determine whether that is also the case for *KNAT6*, we generated the *knat6-1 stm-2* double mutant. The *stm-2* allele displays a pool of nonorganized meristematic cells and retains the ability to produce vegetative shoots (Clark et al., 1996). Although the *stm-2* mutants could produce primordia (Figure 3E), double *knat6-1 stm-2* mutants could not and lacked a SAM (Figure 3F). In addition, *knat6-1 stm-2* seedlings exhibited a fusion of cotyledons that extended to the lamina (Figure 3C). In *stm* mutants, the fusion is restricted to the petiole of the cotyledons, even in the strong alleles (Clark et al., 1996; Long et al., 1996). The *knat6-1 stm-2* mutants did not develop further, in contrast with both weak and strong *stm* mutants, which retain a residual organogenic potential (Clark et al., 1996; Long et al., 1996). The same phenotype was observed with the second *knat6* allele (*knat6-2 stm-2*) or with a strong *stm* allele (*knat6-1 stm-5*) (data not shown). Thus, the inactivation of the *KNAT6* gene entirely abolished the residual meristematic activity of the *stm* mutants. By contrast, mutations in *KNAT2* showed no such interaction with *STM* and confirmed previous data (Byrne et al., 2002) (see Supplemental Figure 3 online). We also examined the triple mutant *knat6-1 stm-2 knat2-5*. The phenotype of these seedlings was identical to that of the double mutant *knat6-1 stm-2*, showing that *KNAT2* does not contribute to the phenotype conferred by *knat6-1 stm-2* (see Supplemental Figure 3 online).

### *STM* and *KNAT6* Are Required for the Maintenance of *CUC3* Expression

The fusion of the cotyledons observed in the *knat6-1 stm-2* double mutant strongly suggests an interaction between *KNAT6*, *STM*, and the *CUC* genes. Previous data showed that the expression patterns of *CUC2* and, to a lesser extent, *CUC1* were altered in a strong *stm* mutant. In addition, a mutation in *CUC1* or *CUC2* enhanced the fusion phenotype of both *stm-2* and *stm-1* alleles, showing a synergistic interaction between the *CUC* and *STM* pathways (Aida et al., 1999). More recently, *CUC3* has been shown to have a greater contribution to the separation of the cotyledons than *CUC1* and *CUC2* (Vroemen et al., 2003). Therefore, we used the *WET368 GUS* line that mimics the *CUC3* gene expression pattern to examine the expression of a boundary marker in the *knat6 stm-2* double mutant (Vroemen et al., 2003). In wild-type seedlings, the expression of *WET368 GUS* is restricted to the boundaries of the cotyledon margins and the boundaries between the SAM and the cotyledons (Vroemen et al., 2003). The *knat6-1/knat6-1 stm-2*/+ line was crossed to the *WET368 GUS* homozygous line. F2 plants homozygous for the *knat6-1* mutation and for the *WET368 GUS* marker and heterozygous for the *stm-2* mutation were selected (see Methods). GUS assays were performed with siliques from these plants in parallel with the *WET368 GUS* control line. Embryos that displayed GUS activity corresponded to *knat6-1/knat6-1 stm-2*/+ and *knat6-1/knat6-1* +/+ genotypes, indicating that the expression

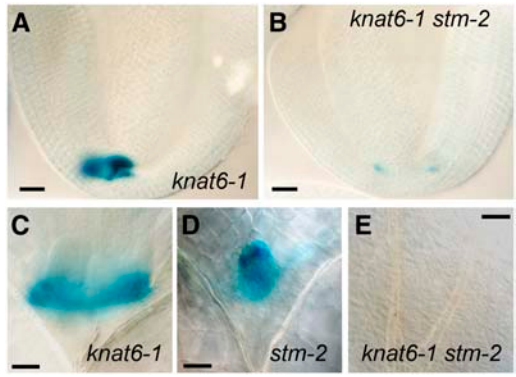


**Figure 4.** *WET368 GUS* Expression in *knat6-1* Single Mutant and *knat6-1 stm-2* Double Mutant.

**(A)** *knat6-1/knat6-1* mature embryo showing GUS activity in the SAM boundaries. This activity was identical in the wild-type embryo.
**(B)** *knat6-1/knat6-1 stm-2/stm-2* mature embryo showing a dramatic reduction of GUS activity.
**(C)** *knat6-1/knat6-1* 8-d-old seedling showing GUS activity in the meristem. Seedlings were cleared.
**(D)** *stm-2/stm-2* 8-d-old seedling exhibiting GUS activity.
**(E)** *knat6-1/knat6-1 stm-2/stm-2* 8-d-old seedling exhibiting no GUS activity.
Bars = 50 μm.

of *WET368 GUS* was not altered in the *knat6-1* single mutant (Figure 4A). Embryos that displayed weak GUS activity corresponded to *knat6-1/knat6-1 stm-2/stm-2* double mutants (Figure 4B). The F3 progeny were further analyzed. Among 485 seedlings, 351 (72.4%) showed a wild-type phenotype and typical WET368 GUS activity (Figure 4C) and 134 (27.6%) showed the double mutant phenotype. Among the double mutant seedlings, 117 (24.1%) did not show GUS activity (Figure 4E) and 17 (3.5%) displayed weak local residual activity. The WET368 GUS activity was still detected in the *stm-2* single mutants (Figure 4D) and in a strong *stm-1* allele even in the absence of a proliferating SAM (Vroemen et al., 2003). Thus, the WET368 GUS activity was not maintained in the absence of both *STM* and *KNAT6*.

### The Phenotype Conferred by *knat6-1 stm-2* Is Slightly Enhanced in a *cuc* Background

Because we observed residual *CUC3* expression in *knat6-1 stm-2* embryos, we wondered whether the complete absence of *CUC3* expression would aggravate the phenotype conferred by *knat6-1 stm-2*. We thus constructed and examined the triple mutant *knat6-1 stm-2 cuc3-2* (see Methods). The wild-type plants and the majority of the *cuc1-1*, *cuc2*, or *cuc3-2* homozygous single mutants have separate cotyledons (Aida et al., 1999; Vroemen et al., 2003). By contrast, double mutant combinations of *CUC1*, *CUC2*, or *CUC3* lead to cup-shaped seedlings (Aida et al., 1999; Vroemen et al., 2003). The majority (90%) of the *knat6-1 stm-2 cuc3-2* triple mutants exhibited a stronger cotyledon fusion than the *knat6-1 stm-2* double mutant (Figures 5I, 5L, 5M, and 5P). This finding reflects the contribution of the *CUC3* residual activity present in the *knat6-1 stm-2* embryo. The stronger fusion phenotype in the *cuc1-1 cuc2* double mutant than in the *knat6 stm-2* background suggests that the inactivation of both *KNAT6* and *STM* is also not sufficient to completely repress *CUC1* and *CUC2* (Figures 5B, 5F, 5I, and 5M). To determine whether the complete absence of *CUC1* or *CUC2* expression would aggravate the phenotype conferred by *knat6-1 stm-2*, we examined the *knat6-1 stm-2 cuc1-1* and *knat6-1 stm-2 cuc2* triple mutants. As shown in Figures 5J to 5L, the fusion observed in the absence of *CUC2* was less severe than that observed in the absence of *CUC1* or *CUC3*. In addition, only 15% of the *knat6-1 stm-2 cuc2* and 21% of the *knat6-1 stm-2 cuc1-1* seedlings showed a stronger fusion than that of the *knat6-1 stm-2* double mutant. This could suggest that the inactivation of both *KNAT6* and *STM* has a stronger impact on the expression of *CUC2* and *CUC1* than on *CUC3*. Together, these results suggest that *KNAT6* and *STM* are corequired for the maintenance of *CUC* gene expression during late embryogenesis.

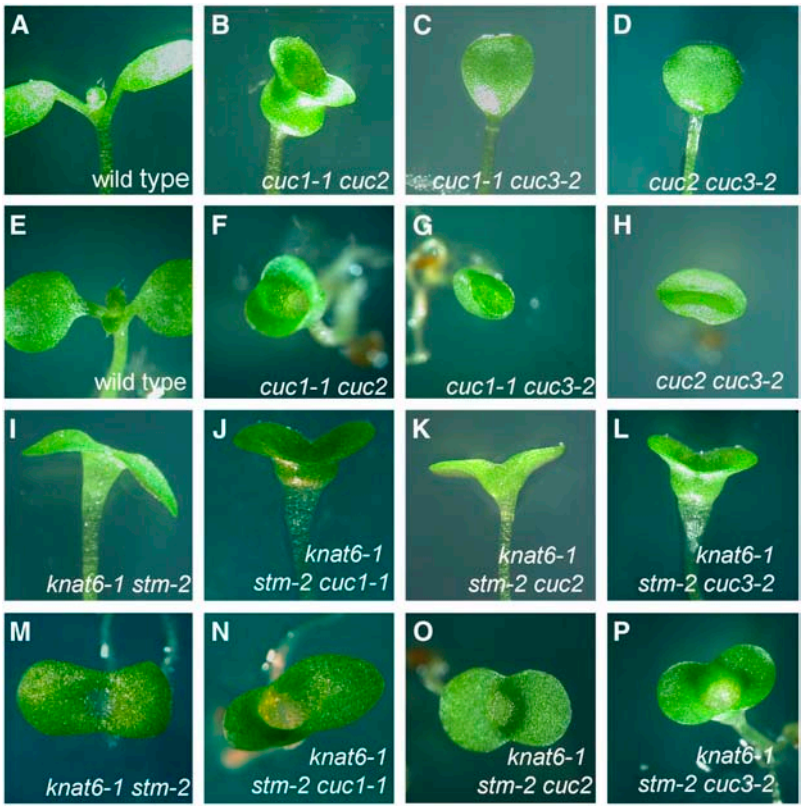


**Figure 5.** Triple Mutants of *knat6-1 stm-2 cuc1-1*, *knat6-1 stm-2 cuc2*, and *knat6-1 stm-2 cuc3-2*.

Eight-day-old seedlings of the wild type (**[A]** and **[E]**), *cuc1-1 cuc2* (**[B]** and **[F]**), *cuc1-1 cuc3-2* (**[C]** and **[G]**), *cuc2 cuc3-2* (**[D]** and **[H]**), *knat6-1 stm-2* (**[I]** and **[M]**), *knat6-1 stm-2 cuc1-1* (**[J]** and **[N]**), *knat6-1 stm-2 cuc2* (**[K]** and **[O]**), and *knat6-1 stm-2 cuc3-2* (**[L]** and **[P]**).

### Activation of *KNAT6* Is *CUC* Dependent

We have shown that *CUC3* expression was altered in the *knat6 stm-2* background. We wondered next whether, conversely, *KNAT6* expression was impaired in a *cuc* background. To this purpose, the homozygous *pKNAT6-GUS* line was crossed to a line homozygous for the *cuc1-1* mutation and heterozygous for the *cuc2* mutation. F2 plants homozygous for the *pKNAT6-GUS* construct, homozygous for the *cuc1-1* mutation, and heterozygous for the *cuc2* mutation were selected. In the F3 progeny, among 195 seedlings, 149 (76.4%) showed a wild-type phenotype and 46 (23.6%) showed a cup-shaped phenotype. The cup-shaped seedlings corresponded to *cuc1-1/cuc1-1 cuc2/cuc2* double mutants. The wild-type plants exhibited GUS activity similar to that of the control, whereas the cup-shaped seedlings did not exhibit GUS activity (Figures 6D and 6E). F2 plants homozygous for the *pKNAT6-GUS* construct, heterozygous for the *cuc1-1* mutation, and homozygous the *cuc2* mutation from the progeny that segregated the cup-shaped phenotype were selected by PCR. In the F3 progeny, among 250 seedlings, 168 (67.2%) showed a wild-type phenotype, 17 (6.8%) showed a heart-shaped phenotype, and 65 (26.0%) showed a cup-shaped phenotype. The low percentage of the heart-shaped seedlings corresponded to the *cuc1/+ cuc2/cuc2* genotype (Aida et al., 1997), and the cup-shaped seedlings corresponded to the *cuc1-1 cuc2* double mutant genotype. The wild type exhibited GUS activity in the meristem, whereas the cup-shaped seedlings did not show GUS activity (Figures 6D and 6E). During embryogenesis, KNAT6-GUS activity was never detected in *cuc1-1 cuc2* double mutant embryos except in the tip of the cotyledons (Figure 6B). Collectively, the analysis of these two F3 progeny indicates that *CUC1* and *CUC2* are redundantly required to activate the *KNAT6* gene during embryogenesis. These data are consistent with the fact that *KNAT6* is expressed after the *CUC* genes during embryogenesis.

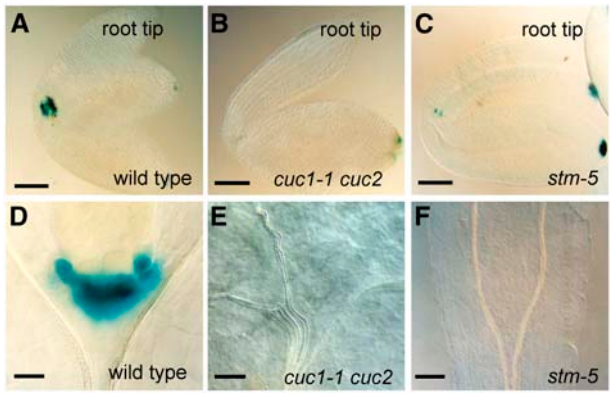


**Figure 6.** *pKNAT6-GUS* Expression in Mutant Backgrounds.

**(A)** Wild-type mature embryo showing GUS activity in the SAM boundaries and in the tips of cotyledons. The embryo was cleared.
**(B)** *cuc1-1/cuc1-1 cuc2/cuc2* double mutant mature embryo showing no GUS activity in the SAM. The expression in the cotyledon tips is maintained.
**(C)** *stm-5/stm-5* mature embryo showing a reduction of GUS activity in the SAM. This activity is reduced to two faint spots. The expression in the cotyledon tips is maintained.
**(D)** Wild-type 8-d-old cleared seedling showing GUS activity in the meristem.
**(E)** *cuc1-1/cuc1-1 cuc2/cuc2* seedling showing no GUS activity.
**(F)** *stm-5/stm-5* seedling exhibiting no GUS activity.
Bars = 50 μm.

### Maintenance of *KNAT6* Expression Is *STM* Dependent

We next examined *KNAT6* expression in a strong *stm* mutant. For this purpose, the *pKNAT6-GUS* homozygous line was crossed to a line that was heterozygous for the *stm-5* strong allele. F2 plants homozygous for the *pKNAT6-GUS* construct and heterozygous for the *stm-5* mutation were selected. GUS assays were performed on siliques from these F2 plants. Although the expression pattern was normal in the wild-type and heterozygous embryos, the homozygous *stm-5* embryos showed a reduction of GUS activity in the SAM (Figure 6C). GUS activity was still detected at the tips of the cotyledons. We next examined *pKNAT6-GUS* expression in 8-d-old seedlings in the F3 progeny. All of the seedlings with a wild-type phenotype exhibited GUS activity similar to that of the control (Figure 6D), whereas the majority of the *stm-5* seedlings did not show GUS activity (Figure 6F). The residual GUS activity seen in *stm-5* embryos was not further maintained during development. These data suggest that *STM* maintains the activity of *KNAT6* in the SAM.

## DISCUSSION

### *KNAT6*, a Gene Involved in Meristem Maintenance and Boundary Establishment

The absence of meristematic defects in the *knat6* single mutant confirms the major role of *STM* in the SAM. However, our data showed that the inactivation of *KNAT6* entirely abolishes the residual meristematic activity of the *stm-2* weak allele and impairs organ separation, revealing a role for *KNAT6* in the SAM. Interestingly, no such phenotype was observed in *knat2 stm* or in *bp* (*knat1*) *stm* double mutants. The inactivation of *KNAT2* did not enhance the phenotype of the weak *stm* allele or the *knat6 stm-2* double mutant. By contrast, the inactivation of *BP* enhances a weak allele of *stm* without affecting the fusion phenotype of the mutant (Byrne et al., 2002). The specific contribution of *KNAT6* in organ separation is consistent with its expression pattern because it matches the expression domain of the *CUC* genes in late embryogenesis: in mature embryos, the expression of *KNAT6* was detected between the cotyledons and the meristem and between the cotyledons where the fusion takes place in the *knat6 stm* double mutant. Thus, to date, *KNAT6* is with *STM* the only class I *KNAT* gene involved in boundary establishment during embryogenesis.

### Position of *KNAT6* in the *STM*/*CUC* Network

The *KNAT6* mRNA was first detected at the triangular stage. Thus, *KNAT6* is expressed later than *STM* and *CUC* genes. In contrast with other meristematic genes such as *BP* or *CLAVATA3* that are still expressed in the absence of a proliferating

SAM (Brand et al., 2002; Hay et al., 2002), the maintenance of *KNAT6* expression required *STM*. Therefore, *KNAT6* is likely a downstream effector in the *STM* pathway in the embryo. However, the enhanced fusion of the cotyledons and the lack of a SAM in the *knat6 stm* double mutant indicate that the residual activity of KNAT6 in the *stm* embryo prevents the fusion of the cotyledon lamina. This phenotype led us to examine the link between *KNAT6*, *STM*, and the *CUC* genes. Our analysis indicates that the expression of *CUC3* was not altered in the *knat6-1* single mutant. By contrast, *CUC3* expression was reduced in *knat6-1 stm-2* embryos and absent in the double mutant seedlings. Examination of the *knat6-1 stm-2 cuc3-2* triple mutant supported these data: the loss of function of *CUC3* enhanced the fusion of the *knat6-1 stm-2* phenotype. Additionally, the fusions observed in the absence of *CUC1* or *CUC2* were less severe than that observed in the absence of *CUC3*. This finding confirmed the greater contribution of *CUC3* to the separation of cotyledons (Vroemen et al., 2003). We never observed in any of the triple mutants the typical cup-shaped phenotype, suggesting that the expression of the two remaining *CUC* genes was not abolished completely. This is in agreement with the fact that, in the wild type, *KNAT6* is expressed after *STM* and the *CUC* genes. Together, our results show that *KNAT6* function is integrated in a network comprising *STM* and the *CUC* genes to regulate organ separation and to maintain the SAM. *KNAT2*, the closest member to *KNAT6*, did not display such a role.

### The Function of KNAT2 Is Still Unknown

It is possible that the function of *KNAT2* could be revealed in a different background. In previous work, we showed that in the presence of ethylene, *KNAT2* expression is restricted to the boundaries between the SAM and the cotyledons (Hamant et al., 2002). Therefore, it is possible that a redundancy between *KNAT2* and *KNAT6* could be revealed by modulating some effectors of the ethylene transduction pathway. Furthermore, the BP and STM proteins interact with the BELLRINGER (BLR) protein to control inflorescence stem growth (Byrne et al., 2003; Smith and Hake, 2003; Bhatt et al., 2004). Although the loss of function of *KNAT2* alone has no phenotypic effect on the phenotype of the *blr* mutant (Byrne et al., 2003), it is possible that KNAT2 interacts with other KNAT and BELL family members. More recently, a study reported another plant protein family, the OVATE family, that interacts with the TALE KNOX and BELL proteins to control their intracellular localization (Hackbusch et al., 2005). This study indicates potential functional redundancy between the members of these families. Further genetic studies are required to determine whether *KNAT2* contributes to the SAM.

## METHODS

### Plant Material and Growth Conditions

*Arabidopsis thaliana* plants were grown either on soil or in vitro as described by Hamant et al. (2002). The *Arabidopsis* ecotypes Landsberg *erecta* (L*er*), Col-0, and Wassilewkija (Ws) were used in this study. Mutant alleles of *knat6-1*, *knat6-2*, and *knat2-5* were obtained from the Nottingham Arabidopsis Stock Centre (http://nasc.nott.ac.uk). Mutant alleles of *stm-2* (weak allele, L*er* ecotype) and *stm-5* (strong allele, L*er* ecotype) were provided by Thomas Laux. The *cuc1-1* (L*er*) and *cuc2* (L*er*) mutant alleles were provided by Mitsuhiro Aida (Aida et al., 1997; Takada et al., 2001). The *cuc3-2* (Ws) mutant allele and the WET368 GUS line (L*er*) that expresses the *CUC3* gene were provided by Casper W. Vroemen (Vroemen et al., 2003). The translational *KNAT2-GUS* fusion has been described (Dockx et al., 1995).

### Insertion Mutant Screen and RT-PCR Analysis

Two T-DNA insertion lines in *KNAT6* (At1g23380) were identified using the signal T-DNA express *Arabidopsis* gene-mapping tool (http://signal.salk.edu/cgibin/T-DNAexpress) (Alonso et al., 2003). To check the T-DNA insertion positions, genomic DNA from mutant plants was amplified with the left border Lba1 T-DNA primer (5′-TGGTTCACGTAGTGGGCCATCG-3′) and the *KNAT6* specific primers *KNAT6-03* (5′-GAAGATAAACCCTAGCTACAAG-3′) for N547931 and *KNAT6-40* (5′-ACAATTTCCATTCGGCCGGTGATT-3′) for N554482. PCR products were sequenced using dye terminator cycle sequencing (Applied Biosystems). The insertion line N547931, with a T-DNA insertion at position +1740 from the ATG (intron 3), was named *knat6-1*. The insertion line N554482, with a T-DNA insertion at position +647 from the ATG (intron 2), was named *knat6-2*. Nottingham Arabidopsis Stock Centre line N59837, which has a T-DNA insertion in *KNAT2* (At1g70510), was named *knat2-5.* PCR amplification of the flanking sequences using the Lba1 T-DNA primer and either the forward *KNAT2* specific primer *4K1* (5′-CGCTTCTCATCCTTTGTATC-3′) or the reverse *KNAT2* specific primer *k11* (5′-GATTCAATACGATACGATCC-3′) showed that the insertion contains two T-DNAs in reverse tandem at position +758 from the ATG (exon 3).

Total RNA was extracted using Tri reagent (Sigma-Aldrich), treated with DNase I (Invitrogen), and purified through RNeasy mini columns (Qiagen). cDNA was synthesized using 2 μg of total RNA and 50 units of SuperScript II RNase $H^-$ reverse transcriptase (Invitrogen) according to the manufacturer's instructions. PCR was performed using the *KNAT6-15* (5′-AGATAAGTCGGTTCTGATGATG-3′) and *KNAT6-18* (5′-GATCCACTAGAATCATCCATCATAGCGAA-3′) specific primers. After 38 cycles, PCR products were subjected to DNA gel blot hybridization using a $^{33}$P-labeled *KNAT6* specific probe. To monitor the *KNAT2* mRNA levels, RT-PCR was performed using the *KNAT2* specific primers *4K1* (5′-CGCTTCTCATCCTTTGTATC-3′) and *6K1* (5′-CCGCTGCTATGTCATCATC-3′). After 40 PCR cycles, PCR products were subjected to DNA gel blot hybridization using a $^{33}$P-labeled *KNAT2* specific probe. Control amplification was performed using the forward (5′-TCCCAGAATCGCTAAGATTGCC-3′) and reverse (5′-CCTTTCCCTTAAGCTCTG-3′) specific primers of APT1 (At3g18780) and 38 cycles.

### Construction of the *pKNAT6-GUS* Fusion

A 3.8-kb genomic fragment containing the *KNAT6* promoter was amplified from the Col-0 ecotype using the high-fidelity Pfu polymerase (Stratagene) and *KNAT6-38F* (5′-CTAGTCTAGACTAGTATAAGGCTTGATTATGAACAACAAAGAC-3′) and *KNAT6-39R* (5′-CTAGTCTAGACTAGTCCATCCATAAGTATAAGATCTCCGGTAA-3′) primers. Each primer contained a *Xba*I restriction site allowing the PCR product to be cloned. The promoter was sequenced and subcloned upstream of the GUS coding sequence in the binary vector pBI101-1 (Clontech). The binary vector harboring the *pKNAT6-GUS* transcriptional fusion was introduced into *Agrobacterium tumefaciens* C58. *Arabidopsis* (Ws ecotype) was transformed by floral dip (Clough and Bent, 1998). Transformants were selected on medium containing 50 mg/L kanamycin. Homozygous lines for the *pKNAT6-GUS* construct were selected.

### In Situ Hybridization

In situ hybridizations were performed as described previously (Vernoux et al., 2000). The *KNAT6* antisense probe was generated using the

pDONOR201 vector that contains the *KNAT6* full-length cDNA as a template and using *KNAT6-34b* (5′-GGGAGCTCATGTACAATTTCCATTC-3′) and *KNAT6-04T7* (5′-TGTAATACGACTCACTATAGGGCTCATTCCTC-GGTAAAGAA-3′), which incorporates a binding site for T7 polymerase, as primers. A PCR product that did not contain the homeobox gave the same expression pattern. Hybridization was performed at 43°C. In situ hybridization of *KNAT2* mRNA was described previously (Pautot et al., 2001).

### Plant Genetics

To construct the *knat6-1 stm-2* double mutants, plants homozygous for *knat6-1* were crossed to plants heterozygous for *stm-2*. A novel phenotype segregated in the F2 progeny in the expected 1:16 ratio. The same phenotype was observed when using other allelic combinations: the second allele of *knat6* (*knat6-2 stm-2*) or a null allele of *stm* (*knat6-1 stm-5*). F2 plants homozygous for *knat6-1* and heterozygous for *stm-2* were selected by PCR analysis. To follow the *stm-2* mutation, we generated CAPS primers. The *stm-2* and wild-type L*er* alleles were sequenced. The sequence of the *stm-2* allele revealed a point mutation (G to A) at position +2053 from ATG (+906 from ATG in the cDNA). This conversion generates a new *Bcc*I restriction site and introduces a premature stop codon. This truncated protein has 252 amino acids instead of the 383 amino acids of the wild-type protein. Thus, the *stm-2* genotype can be followed by PCR amplification and enzymatic restriction. Genomic DNA was amplified with the *STM* specific primers *STM1* (5′-GAGACAGCAATTGA-TAGGAACAAT-3′) and *STMR1* (5′-ATGGTGGAGGAGATGTGATCC-3′). The PCR program used was as follows: denaturation at 95°C for 3 min; 38 cycles of priming at 93°C for 20 s and annealing at 58°C for 20 s; and extension at 72°C for 40 s. The amplification produces a fragment of 1077 bp (Col-0) or 1110 bp (L*er* and *stm-2*). The PCR mixture was desalted during 20 min with Millipore dialysis membranes and then digested at 37°C for 3 h with 5 units of the restriction endonuclease *Bcc*I. Digestion of the wild-type Col-0 *STM* allele generates two fragments of 327 and 752 bp. Digestion of the wild-type L*er STM* allele generates two fragments of 330 and 780 bp. Digestion of the mutant *stm-2* allele generates three fragments of 211, 330, and 569 bp. The double mutant was confirmed by PCR. In the F3 progeny from a selfed *knat6-1/knat6-1 stm-2*/+ plant, a total of 303 seedlings were scored: 221 (73%) were wild type and 82 (27%) were double mutants. This ratio corresponds to the expected 3:1 ratio.

To generate the *knat6-1 stm-2 cuc1-1* triple mutants, plants homozygous for *knat6-1* and heterozygous for *stm-2* were crossed to plants homozygous for *cuc1-1*. F2 plants homozygous for *knat6-1* and *cuc1* and heterozygous for *stm-2* were selected by PCR analysis (as described above and in Takada et al., 2001). In the F3 progeny from a selfed *knat6-1/knat6-1 cuc1-1/cuc1-1 stm-2*/+ plant, 239 seedlings were scored: 77.2% of seedlings showed the wild-type phenotype and 22.8% showed a mutant phenotype. Among the mutant seedlings, 79% showed a typical double mutant phenotype and 21% exhibited an enhanced cotyledon fusion.

To generate the *knat6-1 stm-2 cuc2* triple mutants, plants homozygous for *knat6-1* and heterozygous for *stm-2* were crossed to plants homozygous for *cuc2*. F2 plants homozygous for *knat6-1* and *cuc2* and heterozygous for *stm-2* were selected by PCR analysis (as described above and in Takada et al., 2001). In the F3 progeny from a selfed *knat6-1/knat6-1 cuc2/cuc2 stm-2*/+ plant, 172 seedlings were scored: 76.7% of seedlings showed the wild-type phenotype and 23.3% showed a mutant phenotype. Among the mutant seedlings, 85% showed a typical double mutant phenotype and 15% exhibited a slight enhanced cotyledon fusion.

To generate the *knat6-1 stm-2 cuc3-2* triple mutants, plants homozygous for *knat6-1* and heterozygous for *stm-2* were crossed to plants homozygous for *cuc3-2*. F2 plants homozygous for *knat6-1* and *cuc3-2* and heterozygous for *stm-2* were selected by PCR analysis. To detect the *cuc3-2* allele, the TAG5 T-DNA primer (5′-CTACAAATTGCCTTTTCTTA-TCGAC-3′) and the *CUC3* specific primer (5′-ATGATGCTTGCGGTGGA-AGA-3′) were used. In the F3 progeny from a selfed *knat6-1/knat6-1 cuc3-2/cuc3-2 stm-2*/+ plant, 254 seedlings were scored: 73.6% of seedlings showed the wild-type phenotype and 26.4% showed a mutant phenotype. Among the mutant seedlings, 10% showed a typical double mutant phenotype and 90% exhibited an enhanced cotyledon fusion.

To examine the expression of *CUC3* in the *knat6-1* and *knat6-1 stm-2* mutants, the homozygous *WET368 GUS* line was crossed to the line homozygous for the *knat6-1* mutation and heterozygous for the *stm-2* allele. The *WET368 GUS* line is a hypomorph allele of *CUC3*, because the *CUC3* mRNA level is reduced to 20% of the wild-type level (Vroemen et al., 2003). When we examined the F2 progeny, the *knat6-1 stm-2* double mutant seedlings never exhibited GUS activity. This finding shows that *WET368 GUS* is downregulated in the *knat6-1 stm-2* double mutant even in the heterozygous seedlings for the *WET368 GUS* marker. By contrast, GUS activity was still detected in the *stm-2* single mutants. F2 plants were analyzed by PCR to select plants homozygous for *knat6-1* and heterozygous for *stm-2*. The F3 progeny of these F2 plants were examined for GUS activity to select plants homozygous for the WET368 GUS marker.

To examine the expression of *KNAT6* in *cuc1* and *cuc2* mutants, the homozygous *pKNAT6-GUS* line was crossed to a line homozygous for the *cuc1-1* mutation and heterozygous for the *cuc2* mutation. Two types of F1 progeny were obtained: those that segregated the cup-shaped phenotype in F2 and those that did not. F2 plants were analyzed by PCR to genotype the *cuc1-1* and *cuc2* mutations. An F2 plant homozygous for the *cuc1* mutation and heterozygous for the *cuc2* mutation and one heterozygous for the *cuc1* mutation and homozygous for the *cuc2* mutation were selected. The F3 progeny of these F2 plants were examined for GUS activity to select plants homozygous for the *pKNAT6-GUS* construct.

To examine the expression of *KNAT6* in a strong *stm* mutant, the *pKNAT6-GUS* homozygous line was crossed to a line that was heterozygous for the *stm-5* allele. F2 plants homozygous for the *pKNAT6-GUS* construct and heterozygous for the *stm-5* mutation were selected based on analysis of the F3 progeny. Among 342 F3 seedlings, 261 (76.3%) displayed the wild-type phenotype and exhibited GUS activity and 81 (23.7%) displayed an *stm* phenotype. Of these 81 *stm* seedlings, 78 showed no GUS activity and 3 exhibited very weak KNAT6-GUS activity in the apical part.

### Histological Analysis

Tissue clearing, GUS staining, and Nomarski microscopy were performed as described previously (Pautot et al., 2001).

### Accession Numbers

The Arabidopsis Genome Initiative numbers for *KNAT2* and *KNAT6* are At1g70510 and At1g23380, respectively.

### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure 1.** *pKNAT6-GUS* Expression.

**Supplemental Figure 2.** *KNAT2* Expression in Embryo.

**Supplemental Figure 3.** Phenotypes of the *knat2-5 stm-2* and *knat2-5 knat6-1 stm-2* Mutants.

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