PCP
PLANT & CELL PHYSIOLOGY

Regular Paper

Interactions of *CUP-SHAPED COTYLEDON* and *SPATULA* Genes Control Carpel Margin Development in *Arabidopsis thaliana*

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(Received March 20, 2012; Accepted April 9, 2012)

A characteristic feature of flowering plants is the fusion of carpels, which results in the formation of an enclosed gynoecium. In *Arabidopsis thaliana*, the gynoecium is formed by the fusion of two carpels along their margins, which also act as a meristematic site for the formation of internal structures such as ovules, the septum and transmitting tract. How gene interactions coordinate the fusion and differentiation of the marginal structures during gynoecium development is largely unknown. It was previously shown that the *SPATULA* (*SPT*) gene is required for carpel fusion, whereas overexpression of the *CUP-SHAPED COTYLEDON* genes *CUC1* and *CUC2* prevents it. Here we provide evidence that *SPT* promotes carpel fusion in the apical gynoecium partly through the negative regulation of *CUC1* and *CUC2* expression. In *spt*, transcripts of both *CUC* genes accumulated ectopically, and addition of *cuc1* and *cuc2* mutations to *spt* suppressed the split phenotype of carpels specifically along their lateral margins. In the basal gynoecium, on the other hand, all three genes promoted the formation of margin-derived structures, as revealed by the synergistic interactions of *spt* with each of the *cuc* mutations. Our results suggest that differential interactions among *SPT*, *CUC1* and *CUC2* direct the formation of domain-specific structures of the Arabidopsis gynoecium.

Keywords: *Arabidopsis thaliana* • Carpel margin • Congenital fusion • Gynoecium • Post-genital fusion.

Abbreviations: bHLH, basic helix–loop–helix; CUC1, CUP-SHAPED COTYLEDON1; CUC2, CUP-SHAPED COTYLEDON2; GUS, β-glucronidase; MIR164, microRNA164; NAC, NAM, ATAF1/2 and CUC2; SEM, scanning electron microscopy; SPT, SPATULA.

Introduction

The gynoecium is the female reproductive organ of flowering plants. Along the apical–basal axis, it typically develops three distinct structures called the stigma, style and ovary, which have specialized functions for successful pollination, seed maturation and seed dispersal. An important characteristic of the gynoecium is its enclosed form, which provides a cavity for ovules that develop inside. In many species, this enclosure is formed by fusion of the carpels, each of which is a developmental unit that is homologous to the leaf, and protects the ovules from external stresses and undesirable pollinations (Ferrandiz et al. 2010).

In gynoecium development, the carpel margins are particularly important as a site for fusion and for formation of internal tissues and organs (Okada et al. 1989, Smyth et al. 1990, Sessions 1997, Ferrandiz et al. 1999, Alvarez and Smyth 2002). Carpel fusion can occur in two ways: when primordia are initiated as a united structure early in their inception, the fusion is referred to as congenital, whereas a fusion that occurs after discrete primordia are formed is termed post-genital (Verbeke 1992). In *Arabidopsis thaliana*, the lateral margins of the two carpel primordia are fused congenitally to form a continuous ovary wall, whereas their apical margins fuse post-genitally to form a solid style capped by stigmatic papillae (**Fig. 1A**; Sessions and Zambryski 1995, Bowman et al. 1999). The lateral margins of the carpels contain a proliferative tissue called the medial ridge (Sessions 1997), which gives rise to internal structures such as the ovules, septum and transmitting tract, all of which are important for reproductive competence (Bowman et al. 1999). How these developmental processes in the carpel margins are controlled is therefore a key question to understand gynoecium development.

Molecular genetic studies have identified a number of genes that are required for carpel fusion (Sessions and Zambryski 1995, Roe at al. 1997, Liu et al. 2000, Alvarez and Smyth 2002, Balanza et al. 2006). Importantly, most mutants that are defective in carpel fusion also display defects in the formation of marginal structures, suggesting a close link between the two processes. Among these, mutations in *SPATULA* (*SPT*), which encodes a basic helix–loop–helix (bHLH) transcription factor, cause a split carpel phenotype in the apical part of the gynoecium. In addition, the medial ridges of the *spt* mutant contain fewer cells than those of the wild type, resulting in partial

Plant Cell Physiol. 53(6): 1134–1143 (2012) doi:10.1093/pcp/pcs057, available online at www.pcp.oxfordjournals.org

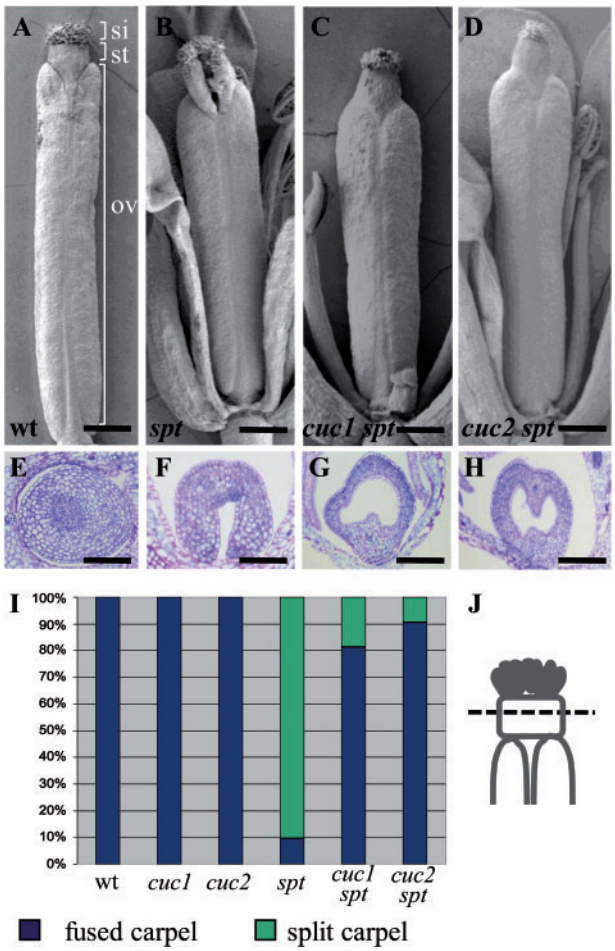

Fig. 1 Carpel fusion phenotype of *cuc1 spt* and *cuc2 spt*. (A–D) Scanning electron micrographs of wild-type (A), *spt* (B), *cuc1 spt* (C) and *cuc2 spt* (D) of stage 14 gynoecia. Medial views. (A) Wild type. (B) *spt* displays a split carpel from the top to the upper ovary. (C and D) *cuc1 spt* (C) and *cuc2 spt* (D) double mutants with fused carpels at the top. (E–H) Transverse sections showing the fusion phenotypes at the central region of the style. No fusion defect occurs inside the style of wild-type gynoecia (E), whereas the central region of the style is hollow in *spt* (F), *cuc1 spt* (G) and *cuc2 spt* (H). (I) Percentages of carpel fusion phenotypes. The first eight flowers on the main inflorescence of four plants were scored. (J) Schematic diagram of the apical gynoecium. Dotted lines indicate approximate positions of the sections. Bars in A–D = 350 μm; E–H = 50 μm. wt, wild type.

defects in margin-derived structures (Alvarez and Smyth 1999, Alvarez and Smyth 2002). Expression of *SPT* occurs in all these regions from early in their development (Heisler et al. 2001), suggesting that *SPT* acts continuously to regulate carpel margin formation.

On the other hand, the *CUP-SHAPED COTYLEDON* genes *CUC1* and *CUC2*, which encode a paralogous pair of NAC transcription factors, represent another class of genes that affect carpel fusion. These genes are negatively regulated by the MIR164 family of microRNAs, and disruption of this regulation results in overaccumulation of *CUC1* and *CUC2* mRNAs and strong carpel fusion defects (Nikovics et al. 2006, Sieber et al. 2007, Larue et al. 2009). *CUC1* and *CUC2* are also redundantly involved in the development of marginal structures; single mutations in these genes have no major effect, whereas double mutations result in the loss of the septum and ovules (Ishida et al. 2000). Besides their roles in carpel margin development, *CUC1* and *CUC2* promote congenital separation of several lateral shoot organs such as cotyledons, leaves and floral organs (Aida et al. 1997, Hibara et al. 2006), consistent with their ability to prevent carpel fusion when overexpressed.

Although a number of regulatory factors have been identified, how these factors interact to coordinate distinct developmental processes in the carpel margins remains elusive. Here, we provide evidence that the *cuc1* and *cuc2* mutations partially suppress the split carpel phenotype of *spt*. Moreover, expression of *CUC1* and *CUC2* is down-regulated by *SPT* in the apical gynoecium, and this negative regulation is important to ensure congenital carpel fusion. We also show that the function of *SPT* in facilitating septum and ovule development overlaps that of *CUC1* and *CUC2*, and the three genes may act coordinately to promote the formation of these marginal structures. Our results demonstrate that the effect of *SPT* on *CUC1* and *CUC2* expression differs between the apical and basal regions of the gynoecium primordia, and hence suggest that interactions among the three genes coordinate the proper development of region-specific structures of the Arabidopsis gynoecium.

Results

cuc1 and *cuc2* mutations rescue the congenital carpel fusion defect of *spt*

In contrast to the wild-type showing complete carpel fusions with a solid style (**Fig. 1A, I**), approximately 90% of the *spt* gynoecia displayed the split carpel phenotype under our growth conditions (**Fig. 1B, I**; see also Alvarez and Smyth 1999). Typically, the split part ranged from the top of the style to the uppermost part of the ovary.

In the wild-type gynoecial primordium, the upward growth in the medial region initially dominated that in the lateral region (stage 8; **Fig. 2A**) and then became even (stage 11; **Fig. 2E**), yielding a flat rim of apical tissues. The adaxial wall of the gynoecial cylinder initiates the medial ridges, which grew inward and fused post-genitally (**Fig. 2A**), resulting in the solid style (**Fig. 1E**). In the strong allele *spt-2* (hereafter called *spt*), in contrast, the medial regions of the gynoecial rim showed retarded growth in the apical direction (arrow in **Fig. 2B**; Alvarez and Smyth 2002) and subsequently formed a cleft (arrow in **Fig. 2F**), manifesting the congenital fusion defect. In addition, the inward growth of the medial ridges decreased and failed to fill up the central hollow of the *spt* style (**Figs. 1F**, **2B**). These results indicate that *SPT* promotes the growth of the apical medial domain in two ways: upward growth in the rim

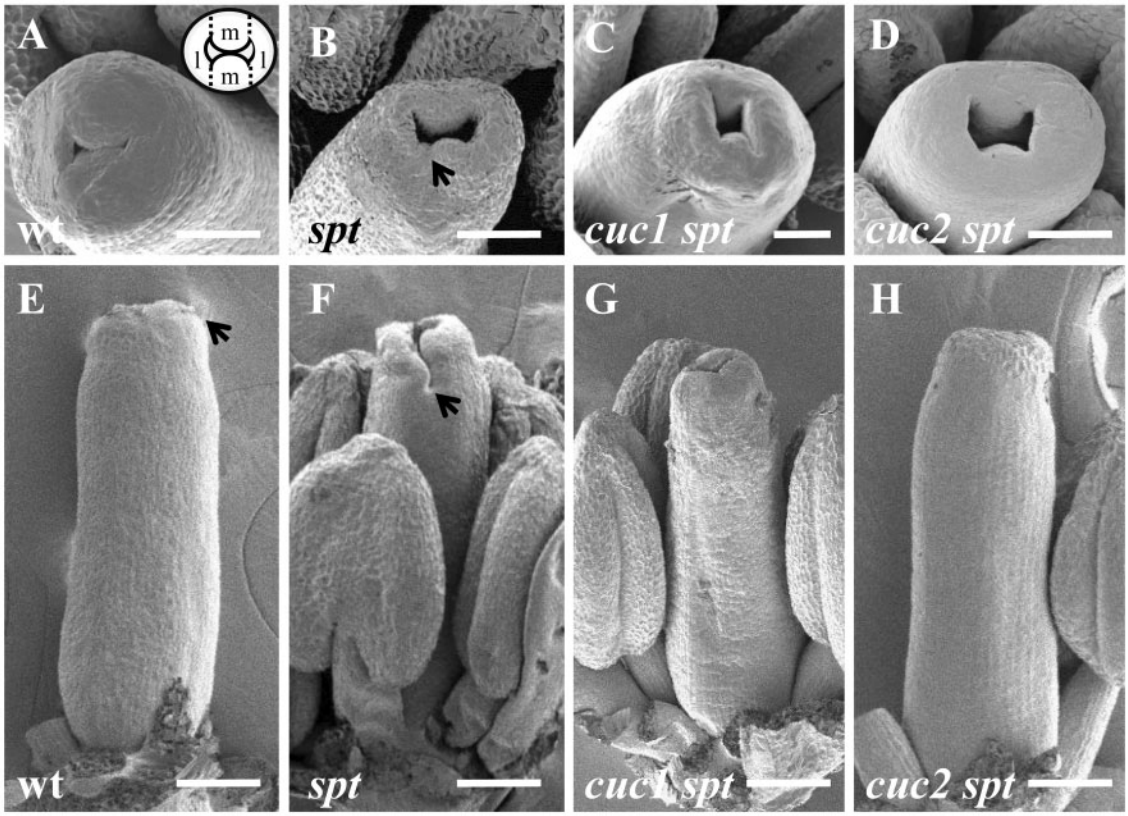

Fig. 2 Early gynoecium development in wild-type, *spt*, *cuc1 spt* and *cuc2 spt*. (A–D) Stage 8 gynoecia viewed from above. (E–H) Lateral view of stage 11 gynoecia. (A) In the wild type, upward growth of the medial positions is more advanced than laterally, and ingrowths of the medial ridges meet at the center of the apical gynoecial tube. The inset diagram shows medial (m) and lateral (l) domains of the gynoecial tube. (B) In *spt*, a part of the medial region shows retarded apical growth (arrow). (C and D) In *cuc1 spt* (C) and *cuc2 spt* (D), apical growth of the gynoecial tube occurs evenly, but the inner medial surfaces fail to make contact due to reduced growth of the medial ridges. (E) In the wild type, the gynoecium closes at the upper end and begins to produce stigmatic papillae (arrow). (F) In *spt*, a central cleft deepens in the medial region (arrow). (G and H) In *cuc1 spt* (G) and *cuc2 spt* (H), the gynoecium continues to grow without any cleft. Bar in A, B, C, D = 50 μm; E–H = 150 μm. wt, wild type.

facilitates congenital carpel fusion and inward growth of the medial ridges fills up the central cavity.

In contrast to *spt*, neither of the strong alleles *cuc1-1* and *cuc2-1* (hereafter called *cuc1* and *cuc2*, respectively) displayed the split carpel phenotype (**Fig. 1I**), and even a *cuc1 cuc2* double mutant possessed a normal style (Ishida et al. 2000). To understand the relationship among *SPT*, *CUC1* and *CUC2* genes, we generated *cuc1 spt* and *cuc2 spt* double mutants. The split carpel phenotype was rescued in the majority of *cuc1 spt* and *cuc2 spt* gynoecia, which resembled the wild-type gynoecium externally (**Fig. 1A, C, D, I**), whereas in the remainder the carpels were split as in *spt*. Histological sections showed that the lateral margins of the carpels were fused but that the central region remained hollow (**Fig. 1G, H**), indicating that *cuc1* and *cuc2* single mutations suppressed the *spt* defect only partially. No recognizable phenotypic interaction was observed in other organs such as cotyledons, leaves and floral organs, other than carpels, where the *CUC1*, *CUC2* and *SPT* genes are known to function (Aida et al. 1997, Hibara et al. 2006, Ichihashi et al. 2010).

We next analyzed the early gynoecium development of *cuc1 spt* and *cuc2 spt*. In these double mutants, the gynoecial rim grew evenly upward (**Fig. 2C, D**) and no cleft formation occurred (**Fig. 2G, H**). On the other hand, the inward growth of the medial ridges remained severely reduced (**Fig. 2C, D**), leaving a central hollow similar to that observed in the *spt* single mutant (**Fig. 2B**). Taken together, these results indicate that *cuc1* and *cuc2* mutations specifically rescue the congenital carpel fusion defect in the apical part of the *spt* gynoecium.

SPT negatively regulates *CUC1* and *CUC2* expression in the apical part of the gynoecium

To examine the effect of the *spt* mutation on *CUC1* and *CUC2* expression, we performed in situ hybridization. In wild-type gynoecial primordia from stage 8 to 10, *CUC1* transcripts were detected on the adaxial side of the medial region, extending most of the way along the apical–basal axis, but were absent from or only weakly detected in the uppermost part

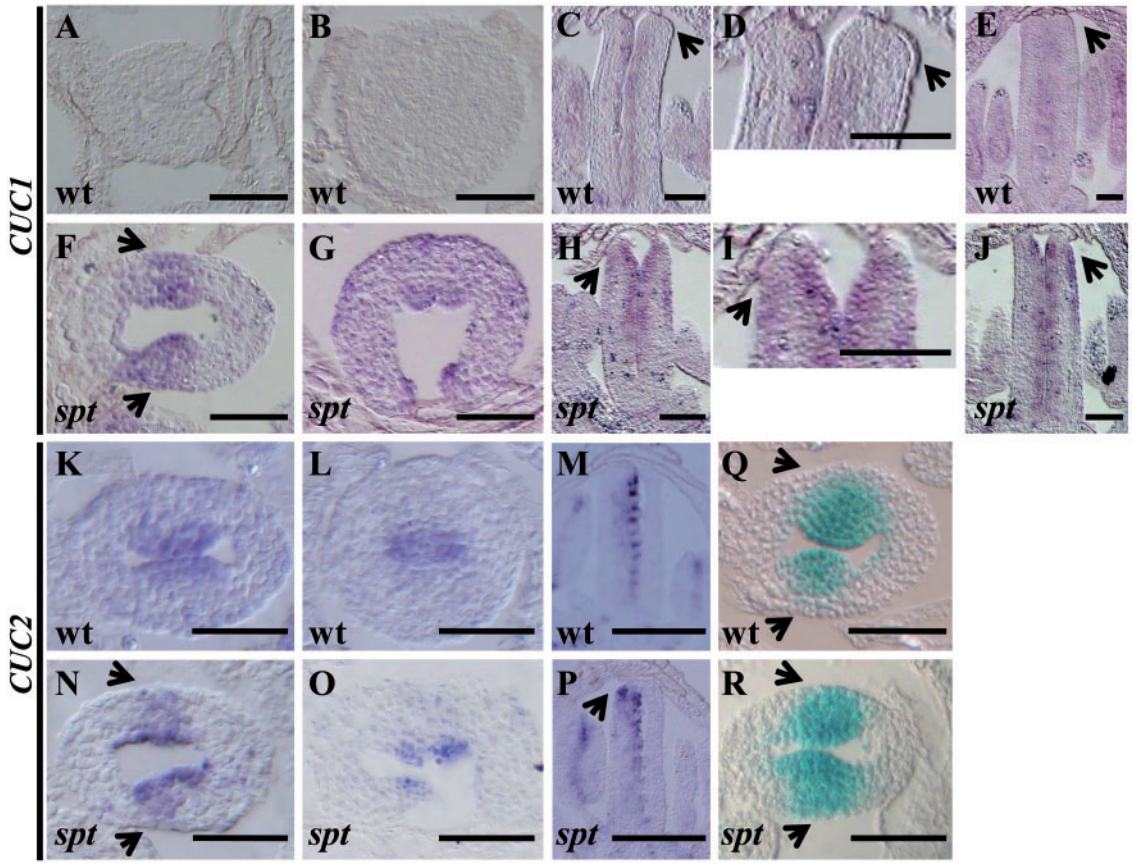

Fig. 3 *CUC1* and *CUC2* expression in the apical region of wild-type and *spt* gynoecia. All transverse sections represent the apical part of the gynoecium. Probes detect (A–J) *CUC1* transcripts, (K–P) *CUC2* transcripts and (Q–R) *ProCUC2::GUS* localization patterns. (A–E) *CUC1* expression in the wild-type gynoecium. Transverse sections at (A) stage 8 and (B) stage 10. Longitudinal sections at (C) stage 8, (D) close-up views of the apical gynoecium of C, and (E) stage 10; arrows indicate the absence of *CUC1* expression at the top. (F–J) *CUC1* expression in *spt* gynoecium at (F) stage 8 and (G) stage 10 (transverse sections). Longitudinal sections at (H) stage 8, (I) close-up views of the apical gynoecium of H, and (J) stage 10; arrows indicate ectopic expression. (K–M) *CUC2* expression in the wild type at (K) stage 8, (L) stage 10 (transverse sections) and (M) stage 10 (longitudinal section). (N–P) *CUC2* expression in *spt* at (N) stage 8, (O) stage 10 (transverse sections) and (P) stage 10 (longitudinal section); arrows indicate the ectopic expression. (Q–R) *ProCUC2::GUS* expression in the wild type at (Q) stage 8; note that GUS activity is not detected on the abaxial side (arrows). (R) *ProCUC2::GUS* activity is detected ectopically on the abaxial side at stage 8 (arrows). Bars, A–J, K, L, N, O, Q and R = 50 μm; M and P = 130 μm; wt, wild type.

(**Fig. 3A–E**; **Supplementary Fig. S1**). In *spt*, in contrast, we detected *CUC1* mRNA in the uppermost part of the medial region at stage 8 (**Fig. 3F, H, I**). Expression was not restricted to the adaxial side, but extended to the abaxial side. Ectopic *CUC1* mRNA accumulation continued in the adaxial and abaxial surfaces of the upper medial region (**Fig. 3J**), and was also detected at the split parts (**Fig. 3G**).

We also tested *CUC2* expression in the wild-type and *spt* backgrounds. In the wild type at stage 8 and 10, *CUC2* was expressed in the medial region of the gynoecium, from the base to the uppermost part (**Fig. 3K, L, M**), restricted to the adaxial side. In *spt*, in contrast, *CUC2* expression extended to the abaxial side of the apical medial region at stage 8 (**Fig. 3N**). At stage 10, the signal was also frequently detected at the split ends (**Fig. 3O**) or at the abaxial side (**Fig. 3P**). Taken together, these results show that *CUC1* and *CUC2* mRNAs are absent or restricted to small areas at the top of the early gynoecium, where the style and stigma will later arise, while the *spt* mutation causes the expression of each gene to spread throughout the medial region.

The expression of *CUC1* and *CUC2* is regulated transcriptionally by *cis*-regulatory elements in their promoters and post-transcriptionally by the microRNA miR164 (Laufs et al. 2004, Mallory et al. 2004, Sieber et al. 2007). We therefore tested whether the *spt* mutation affected the promoter activity of *CUC2*. To this end, we used transgenic plants harboring the 3.2 kb *CUC2* promoter fused to the *uidA* gene encoding β-glucuronidase (GUS) (*ProCUC2::GUS*; Nikovics et al. 2006). In wild-type apical gynoecium at stages 8, intense GUS activity was detected on the adaxial side of the medial domain but was absent from the abaxial side (**Fig. 3Q**). In *spt*, in contrast, *ProCUC2::GUS* expression extended fully from the adaxial to

the abaxial region of the apical medial region at stage 8 (Fig. 3R). The patterns of GUS activity in the wild type, and how they changed in *spt*, are in accordance with those of *CUC2* mRNA (Fig. 3K, N). These observations suggest that the *spt* mutation affects transcriptional regulation of *CUC2* in the apical part of the gynoecium, leading to ectopic abaxial accumulation of its mRNA.

Development of carpel margin-derived organs is strongly affected in *cuc1 spt* and *cuc2 spt* double mutants

Shortly after the initiation of the gynoecial tube, the lateral margins of carpels form two meristematic tissues called the medial ridges, which grow inward and fuse with each other to form the septum, and ovules are formed from placentae that arise near their boundary with the valves (Sessions 1997, Bowman et al. 1999). *CUC1*, *CUC2* and *SPT* reportedly play important roles in the development of these carpel margin-derived structures (Alvarez and Smyth 1999, Ishida et al. 2000). To elucidate the relationship among the three genes, we first examined the septum phenotype of *cuc1 spt* and *cuc2 spt* double mutants and compared it with those of the wild type and of *spt* and *cuc1 cuc2* mutants. In the wild type and each single mutant of *cuc1* and *cuc2*, most gynoecia had an intact septum (Fig. 4A, F, G, P), whereas ~85% of *spt* gynoecia showed a mild septum defect in which the unfused region covered less than half of the entire ovary length (Fig. 4B, H, I, P). The *cuc1 cuc2* double mutant showed a severe septum defect throughout the ovary (Fig. 4E, N, O).

We next examined the septum phenotype of *cuc1 spt* and *cuc2 spt* double mutants. In comparison with *spt*, the *cuc1 spt* and *cuc2 spt* double mutant displayed an enhanced septum phenotype. About 40% of *cuc1 spt* gynoecia exhibited a strong septum defect in which the unfused region covered more than half of the total length of the ovary (Fig. 4C, J, K, P) whereas the remaining ~60% showed a milder defect. In *cuc2 spt*, ~90% of the gynoecia displayed a strong septum defect (Fig. 4D, L, M, P) and only ~10% were mildly defective. Together, these results demonstrate a synergistic interaction of *spt* with single mutants of *cuc1* and *cuc2* in septum formation.

The *spt* gynoecium forms slightly fewer ovules than the wild type, and only a small fraction of these ovules develop into seeds, whereas neither *cuc1* nor *cuc2* single mutation affects ovule number (Ishida et al. 2000). We then examined whether the *cuc* mutations also enhance ovule and seed formation in *spt* (Table 1). The average number of ovules at anthesis in *cuc1 spt* and *cuc2 spt* double mutants was reduced compared with that in the *spt* single mutant. A similar phenotypic enhancement of *spt* by *cuc1* or *cuc2* was also observed for seed set. This appears to result from the severe reduction in the extent of septum development in the double mutants, which could further inhibit correct pollen tube growth and hence reduce the efficiency of fertilization. These findings suggest that the *CUC1*, *CUC2* and *SPT* genes are required for ovule development and mature seed set.

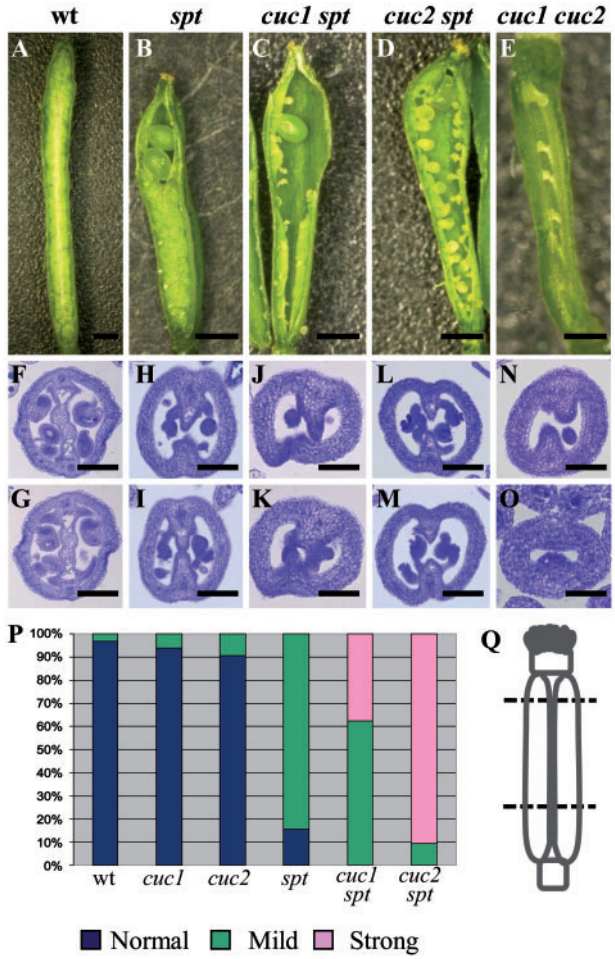

Fig. 4 Septum development in the wild type, *spt*, *cuc1 spt*, *cuc2 spt* and *cuc1 cuc2*. (A–E) Lateral views of mature siliques at stage 17 with one valve removed. Developing seeds in the front side are also removed. (F–O) Transverse sections (see Q) showing the septum phenotype in apical (F, H, J, L, N) and basal (G, I, K, M, O) parts of the ovary of the stage 14 gynoecium. Sections are stained with toluidine blue. (A) Wild-type silique has a fused and intact septum. (B) In *spt*, the upper part of the septum is not fused. No fusion occurred between the septa across the apical–basal axis of the ovary in (C) *cuc1 spt*, (D) *cuc2 spt* or (E) *cuc1 cuc2*. Transverse sections show no septum defect in apical (F) and basal (G) parts of the wild-type ovary. In *spt*, septum development is defective in the apical part (H), but normal in the basal part (I), of the ovary. Severe septum defects were observed throughout the ovary in *cuc1 spt* (J, K), *cuc2 spt* (L, M) and *cuc1 cuc2* (N, O). (P) Percentage of septum defects. The first eight flowers on the main inflorescence of four plants were scored. (Q) Schematic diagram of the mature gynoecium. Dotted lines (upper, apical; lower, basal) indicate approximate positions of the section plane in F–O. Bar in A–E = 250 μm and F–O = 50 μm. wt, wild type.

SPT is not required for *CUC1* or *CUC2* expression within the developing ovary

To investigate the relationship among *CUC1*, *CUC2* and *SPT* in the formation of marginal structures, we examined the effect of the *spt* mutation on *CUC1* and *CUC2* expression

Table 1 Numbers of ovules and seeds per gynoecium in the wild type, *spt*, *cuc1 spt* and *cuc2 spt*

Genotype	No. of ovules	No. of seeds	Percentage seed set
Wild type	55.66 ± 0.83	53.75 ± 0.94	96.57
spt-2	48.38 ± 0.61**	7.44 ± 0.54**	15.38
cuc1-1 spt-2	36.44 ± 0.59**	2.53 ± 0.28**	6.94
cuc2-1 spt-2	34.31 ± 0.49**	1.73 ± 0.15**	5.04

The first eight flowers on the main flowering shoot of four plants were scored for each genotype. Values are means, and errors are standard error of the mean ($n = 32$).
**Differences between each mutant and the wild type are significant at $P < 0.01$.

in the basal region of developing gynoecium primordium, the part giving rise to the ovary. In the wild-type gynoecium at stage 8, both *CUC1* and *CUC2* are expressed on the adaxial side of the medial region, where the septum will form (**Fig. 5A, G**; Ishida et al. 2000, Takada et al. 2001). Subsequently, *CUC1* and *CUC2* transcripts are detected at the base of the ovule primordia and in the fused region of the two medial ridges (**Fig. 5B, H**). In later development, *CUC1* and *CUC2* are expressed at the boundary between the nucellus and chalaza in the ovule, and in presumptive transmitting tract cells in the developing septum (**Fig. 5C, I**). In the *spt* mutant, expression of *CUC1* and *CUC2* was very similar to that in the wild type throughout these stages (**Fig. 5D–F, J–L**), except that *CUC1* transcripts showed relatively weak expression in presumptive transmitting tract cells in the septum (**Fig. 5F**). Our analysis indicates that *CUC1*, *CUC2* and *SPT* together play a critical role in margin-derived organ development in the gynoecium.

CUC1 and *CUC2* affect expression of *SPT* specifically in the basal region of developing gynoecium

To examine the possibility that *CUC* genes affect *SPT* expression, we carried out in situ hybridization in the wild type and in the *cuc1 cuc2* double mutant, using the *SPT* probe. In the apical part of the stage 8 gynoecium, *SPT* expression was detected in the apical medial domain of the wild type, where it extended from the adaxial to the abaxial side (**Fig. 6A, B**). On the other hand, within the basal region, *SPT* is expressed in an internal region of the medial ridge and the septum, including differentiating transmitting tract cells, at stage 8 and also later at stage 10 (**Fig. 6C, D**; Heisler et al. 2001). In contrast, in the *cuc1 cuc2* double mutant, whereas *SPT* transcript accumulation was observed in the apical medial domain, as for the wild type, *SPT* expression was missing lower down within the ovary throughout these stages (**Fig. 6E, F**). Taken together, these results show that *CUC1* and *CUC2* are redundantly required for *SPT* expression in the medial ridge of the ovary.

Discussion

SPT controls carpel fusion by repressing *CUC* gene expression

One of the important functions of *SPT* is to promote carpel closure (Alvarez and Smyth 1999, Alvarez and Smyth 2002), although how *SPT* executes this function has remained largely unknown. We showed here that the split carpel phenotype of *spt* was partially suppressed by each of the *cuc1* and *cuc2* mutations, resulting in the formation of a tubular style that is open only at the top. This partial recovery indicates that the *SPT*-dependent carpel closure involves at least two genetically separable processes: fusion along the lateral margins, and fusion at the center. The defect of *spt* in the former process can be traced back to the retarded apical growth and subsequent cleft formation at the medial domain (Alvarez and Smyth 2002; **Fig. 2B, F**), indicating that the process involves congenital fusion. On the other hand, the latter defect is due to the reduced inward growth of the medial ridge, causing a failure of surface contact that leads to subsequent post-genital fusion (**Fig. 2B, F**). In *cuc1 spt* and *cuc2 spt* double mutants, the retardation of apical growth was largely suppressed and no cleft was observed, whereas the inward growth of the medial ridge remained the same as that of *spt* (**Fig. 2B, C, D**), strongly indicating that *cuc1* and *cuc2* mutations specifically suppressed the congenital fusion defect of *spt*.

The suppression of the *spt* phenotype by *cuc1* and *cuc2* indicates that the defect of *spt* in congenital fusion is dependent on the activities of *CUC1* and *CUC2*, and our expression data are consistent with this notion. In the wild-type apical region, *SPT* expression is detected throughout the medial domain (Heisler et al. 2001; **Fig. 6A**). In the same domain, *CUC1* is not expressed and *CUC2* expression is restricted to the adaxial domain. In *spt*, on the other hand, both of these genes are ectopically expressed throughout the medial domain. Taken together, these results indicate that *SPT* negatively regulates the expression of *CUC1* and *CUC2* in the apical region of the gynoecial primordium, and that this repression is essential for complete congenital fusion of the carpels along their lateral margins (**Fig. 7**, orange arrows). On the other hand, no detectable contribution of *CUC1* and *CUC2* to the medial ridge growth and subsequent post-genital fusion in the centrally apical gynoecium of *spt* was found, indicating that only *SPT* is involved in post-genital fusion to form a solid style (**Fig. 7**, green arrows).

It has been demonstrated that *CUC1* and *CUC2* are under post-transcriptional control by the microRNA miR164, and this negative regulation is important for a number of developmental processes including carpel fusion (Nikovics et al. 2006, Sieber et al. 2007, Larue et al. 2009). On the other hand, our analysis using the *GUS* reporter showed that the control of *CUC2* expression by *SPT* involves transcriptional regulation through the *CUC2* promoter, uncovering an additional level of gene interactions that are essential for carpel closure.

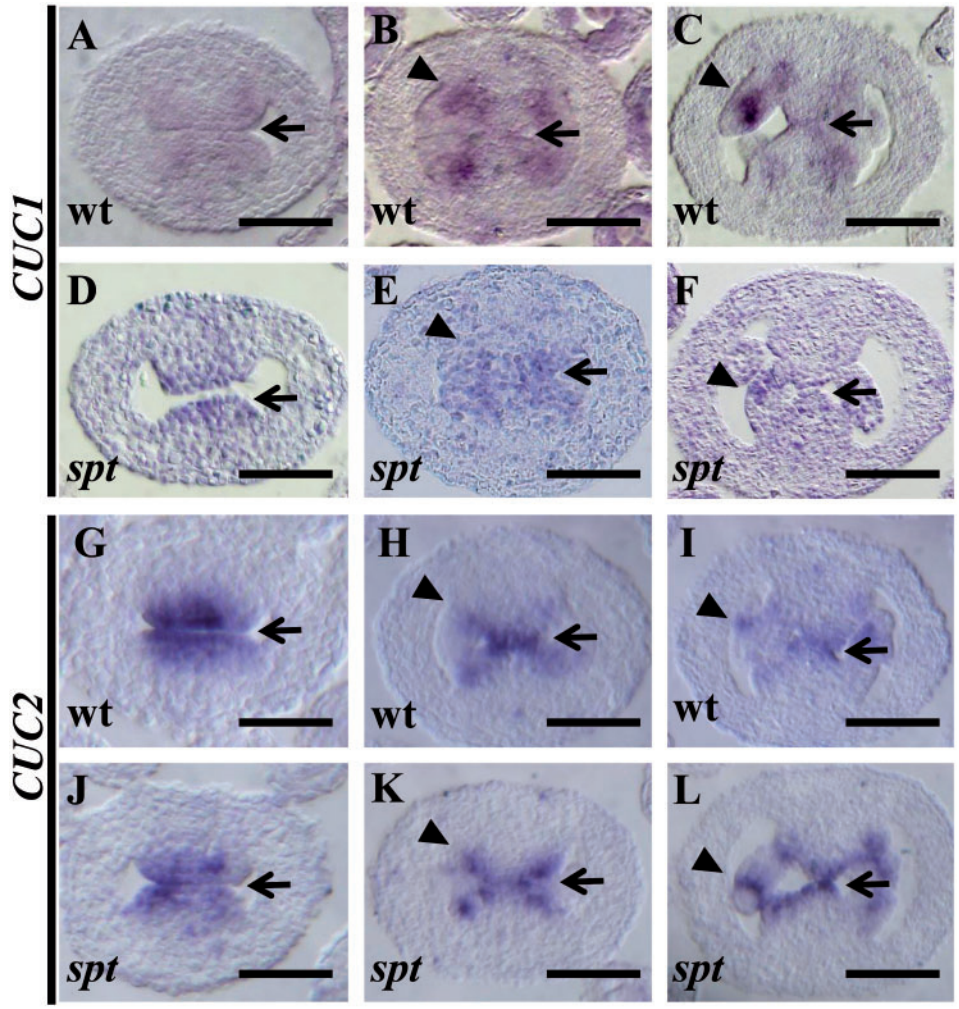

Fig. 5 *CUC1* and *CUC2* expression in the basal region of wild-type and *spt* gynoecium. All images are transverse sections from the developing ovary. Probes detect (A–F) *CUC1* and (G–L) *CUC2*. (A–C) *CUC1* expression in wild-type stage 8 (A), stage 9 (B) and stage 11 (C) gynoecium. (D–F) *CUC1* expression in *spt* stage 8 (D), stage 9 (E) and stage 11 (F) gynoecium. (G–I) *CUC2* expression in wild-type stage 8 (G), stage 9 (H) and stage 11 (I) gynoecium. (J–L) *CUC2* expression in *spt* stage 8 (J), stage 9 (K) and stage 11 (L) gynoecium. (A–L) Arrows indicate expression at the medial ridges and developing septum; arrowheads indicate expression in the ovule primordia. Bar, A–L = 50 μm. wt, wild type.

How does *SPT* affect *CUC2* promoter activity? Since the SPT protein is suggested to act as a transcriptional activator (Groszmann et al. 2008), it is unlikely that negative regulation of *CUC2* transcription by SPT is direct. Rather, SPT may control *CUC2* transcription by activating a negative regulator(s) of *CUC2* such as auxin or *ASYMMETRIC LEAVES1*, which are known to suppress *CUC2* expression during leaf and cotyledon development (Koyama et al. 2010). In this regard, it should be noted that *SPT* function has been linked to auxin. First, the split carpel phenotype of the *spt* mutant is suppressed by application of an auxin transport inhibitor, which causes auxin to accumulate in the apical gynoecium (Nemhauser et al. 2000). Secondly, expression of a constitutively active form of SPT induces ectopic expression of *STYLISH2*, which can in turn activate the expression of auxin biosynthetic genes (Groszmann et al. 2008, Eklund et al. 2010). Thirdly, auxin response is greatly weakened at the apex of *spt* mutant gynoecia, and SPT jointly regulates genes involved in auxin transport (Girin et al. 2011). These results are consistent with the possibility that *SPT* negatively affects the *CUC2* promoter by enhancing auxin accumulation in the apical gynoecium.

CUC1, *CUC2* and *SPT* are essential for carpel margin-derived organ development

Previous studies have shown that *SPT* as well as *CUC1* and *CUC2* are required for ovule and septum formation in the ovary (Alvarez and Smyth 1999, Ishida et al. 2000, Alvarez and Smyth 2002), and these findings are consistent with the synergistic interactions of *spt* with *cuc1* and *cuc2* observed here (**Fig. 4**). The *spt* mutant defect in these organs is associated with the reduction of the medial ridge size, suggesting that *SPT*

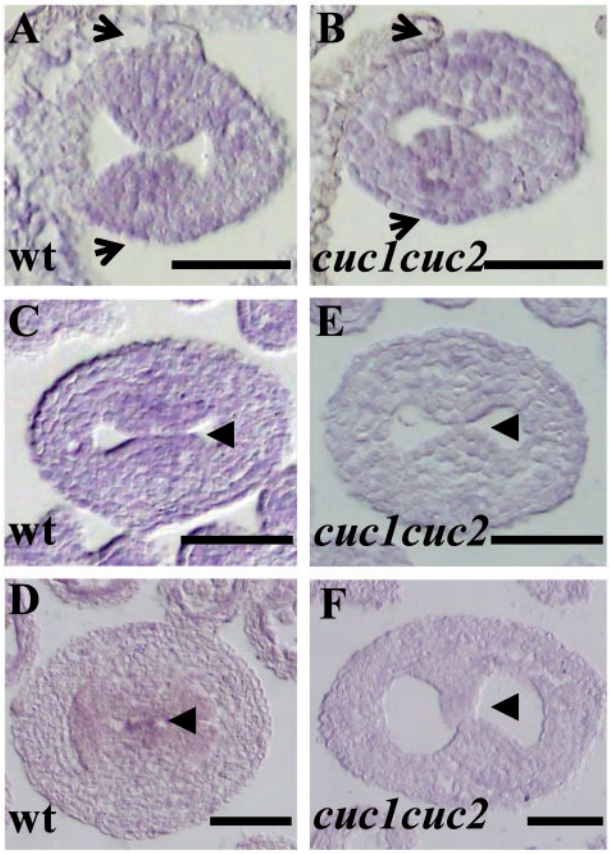

Fig. 6 *SPT* expression in wild-type and *cuc1 cuc2* gynoecium. All images are of transverse sections. Sections from the apical (A, B) and basal (C–F) regions of the gynoecium. (A, B) In the apical region at stage 8, *SPT* expression extends from the adaxial to abaxial sides of the medial domain of the wild type (A, arrows) and *cuc1 cuc2* (B, arrows). In the basal gynoecium of the wild type, *SPT* expression is restricted to the medial ridges on the adaxial side at stage 8 (C, arrowhead) and in the developing septum at stage 10 (D, arrowhead). In the basal region of *cuc1 cuc2*, expression was not detected in the corresponding regions at stage 8 (E, arrowhead) or stage 10 (F, arrowhead). Bar = 50 μm. wt, wild type.

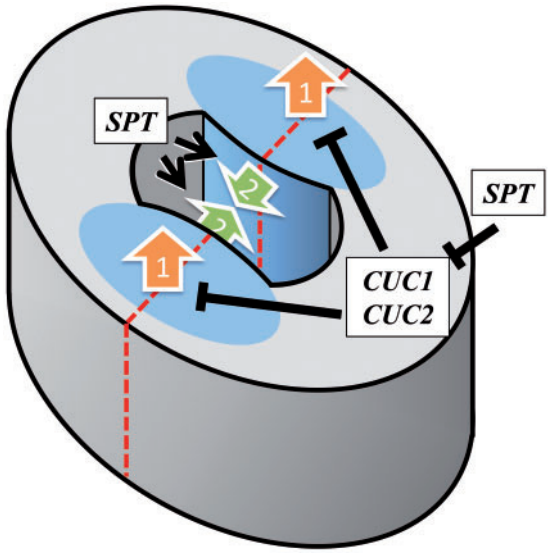

Fig. 7 A model for the control of apical carpel fusion by *SPT*, *CUC1* and *CUC2* in the Arabidopsis gynoecium. The closure of the apical gynoecium in the region of the style and stigma results from the combination of two types of growth activities: upward growth of the gynoecial tube ensures congenital fusion of carpels along their lateral margins (dashed red line), and inward growth of the medial ridges fills up the central hollow of the style and closes the top. *SPT* represses *CUC1* and *CUC2* expression to ensure the upward growth (orange arrows). Inward growth (green arrows) of the apical medial ridges is promoted by *SPT* but does not involve *CUC1* and *CUC2* activities.

and possibly *CUC1* and *CUC2* have roles in regulating cell proliferation in the ridges, which would directly affect septum and ovule development (Alvarez et al. 1999, Ishida et al. 2000, Takada et al. 2001).

What is the relationship among *SPT*, *CUC1* and *CUC2* during ovary formation? The absence of *SPT* transcripts from the basal region of the *cuc1 cuc2* gynoecium primordia (**Fig. 6**) suggests that *CUC1* and *CUC2* act upstream of *SPT* to initiate or maintain its expression. In contrast, both *CUC1* and *CUC2* transcripts are detected in the medial ridges as well as in the margin-derived organs of *spt* (**Fig. 5**), suggesting that the expression of *CUC1* and *CUC2* is not regulated by *SPT*. Alternatively, *SPT* may promote *CUC* gene expression in the ovary but the effect of the *spt* mutation may be compensated for by other redundant factors such as *ALCATRAZ* (*ALC*) and *INDEHISCENT* (*IND*), both of which encode bHLH proteins (Girin et al. 2011, Groszmann et al. 2011).

Our data demonstrate that the effect of *SPT* on *CUC1* and *CUC2* expression differs between the apical and basal regions of the gynoecium primordia. In the apical region, *SPT* suppresses the expression of *CUC1* and *CUC2*, whereas in the basal region it does not affect expression of either gene. It has been reported that *SPT* requires one or more co-activators for its functions in carpel development (Groszmann et al. 2008). It is therefore possible that such additional factors may modulate the position-dependent effect of *SPT* on *CUC* gene expression.

Relation between *CUC/SPT* functions and gynoecium patterning

Our results suggest that *CUC1*, *CUC2* and *SPT* are involved in interpreting regional information and interacting in the differentiation of regional tissue types. The distribution of tissue types observed in *spt* suggests that one role of *SPT* is to promote carpel fusion in the apical region of the gynoecium partly through the repression of *CUC1* and *CUC2*. Within the basal region, on the other hand, all three genes act together to differentiate carpel-margin-derived organs of the ovary. However, none of them appears to be involved in partitioning the gynoecium primordium into distinct regions. For example, despite *CUC* gene expression being restricted to the adaxial side

of the carpel margins, mutations in the *CUC* genes do not affect adaxial–abaxial polarity (Ishida et al. 2000).

Complex genetic networks ensure the maintenance of the meristem–primordia, adaxial–abaxial, medial–lateral and apical–basal dichotomies (Balanza et al. 2006). Many of the genes involved share some functional redundancy (Alvarez and Smyth 1999, Liu et al. 2000, Azhakanandam et al. 2008) and, although the contribution of each gene may be limited, their collective activity is essential for domain-specific organogenesis in gynoecium development. For example, the *CRABS CLAW* gene, which plays a role in abaxial identity establishment in carpels redundantly with other polarity genes (Eshed et al. 1999), also affects carpel fusion in the apex. Combination of the *crc* mutation with *spt* results in complete failure of carpel fusion, indicating that the regulation of carpel fusion involves establishment of abaxial identity (Alvarez and Smyth 1999, Alvarez and Smyth 2002). Therefore, it will be important to study how these patterning mechanisms regulate specific expression patterns of *CUC1*, *CUC2* and *SPT*, and how they modify the region-specific functions of these genes.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana accession Landsberg *erecta* (L*er*) was used as the wild type. The strong alleles *cuc1-1*, *cuc2-1* and *spt-2* were described previously (Aida et al. 1997, Alvarez and Smyth 1999). After crossing parental mutants, all double mutant combinations were identified by PCR-based genotyping in the F_2 and subjected to analysis in the F_3 and F_4. Seeds were surface-sterilized, sown on Murashige Skoog plates, and germinated as previously described (Aida et al. 1997). About 2 weeks after germination, seedlings were transplanted into soil and grown at 23°C under constant white light as previously described (Fukaki et al. 1996).

Microscopy and histology

Close-up images were photographed using a digital microscope (VHX-900, Keyence). For histological sections, Paraplast-embedded flowers or inflorescences embedded in Paraplast Plus (Fischer Scientific) were sectioned with 8 µm thickness and fixed onto slides. Sections were then de-waxed with lemosol, rehydrated through an ethanol series and stained with toluidine blue. Scanning electron microscopy (SEM) was carried out as described previously (Aida et al. 1999).

Reporter gene construct and histochemical staining

The *ProCUC2::GUS* reporter construct (Nikovics et al. 2006) was transformed into the L*er* background. To detect GUS activity, tissues were permeabilized with 90% ice-cold acetone for 15 min on ice, rinsed with water and stained in a staining solution [50 mM sodium phosphate buffer pH 7.0, 10 mM EDTA, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 0.1% Triton X-100 and 0.5 mg ml^{-1} X-Gluc] at 37°C for 12 h. Stained specimens were dehydrated in a graded ethanol series (30, 50, 70, 90 and 100%) for 15 min each to remove Chl, and embedded in Paraplast for sectioning.

In situ hybridization

RNA in situ hybridization was performed according to Takada et al. (2001). Inflorescence apices were collected and fixed shortly after bolting. For antisense probes of *CUC1* and *CUC2*, full-length coding sequences in pBluescript KS+ were used as templates for in vitro transcription. The plasmid used to generate the *SPT* probe was described previously (Heisler et al. 2001). Hybridization was carried out at 45°C. Western blue was used as the substrate for signal detection.

Supplementary data

Supplementary data are available at PCP online.

Funding

This work was supported by the Ministry of Education, Culture, Sports, Science, and Technology [Global COE Program A9 to M.A.U.N., M.T. and M.A., 14036222 to M.T., 23012031 to M.A., 21024008 to M.A.]; Japan Society for the Promotion of Science [23370023 to M.A.]; Takeda Science Foundation [to M.A.]

Acknowledgments

The authors thank Md. Rezaul Karim (National Agriculture and Food Research Organization, Tsukuba, Japan) for instructions regarding in situ hybridization experiments, Seiji Takeda for helpful discussion, Maki Niidome for technical assistance, and Ian Smith (Nara Institute of Science and Technology) for critical reading of the manuscript.

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