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Interactions among Genes Involved in Gynoecium Development in *Arabidopsis thaliana*

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ABSTRACT

The gynoecium is the female reproductive organ of angiosperms. In *Arabidopsis thaliana*, two carpels, developmental units that compose the gynoecium, are fused along their margins to form an enclosed structure. The mature gynoecium is separated into several domains, within which different tissue identities are established during its development. The early gynoecium primordium is tubular shape and consists of two carpel primordia that are congenitally fused along their lateral margins. The apical region of the gynoecium primordia later fuses postgenitally to form a solid style capped with stigmatic papillae, whereas the basal region gives rise to the ovary, which contains ovules and the septum. The ovules and septum are produced from the medial ridge, a meristematic tissue initiated at the lateral margins of the carpel primordia. The aim of this thesis is to elucidate how gene interactions specify the carpel fusion and differentiation of the marginal tissues during gynoecium development in *Arabidopsis thaliana*.

Several studies have shown that the *CUP-SHAPED COTYLEDON* (*CUC1*), *CUC2* and *SPATULA* (*SPT*) genes encoding transcription factors are involved in carpel fusion and the development of the carpel-margin-derived organs. Double mutations in *CUC1* and *CUC2* result in the lack of the carpel margin-derived structures. *CUC1* and *CUC2* also have potential activity to prevent carpel fusions, as ectopic expression of these genes results in partial separation of the gynoecium at the carpel margins. On the other hand, mutations in *SPT* result in mild reduction of the septum and partial split of the carpel margins in the apical part of the gynoecium.

In an attempt to understand the regulatory mechanism among *CUC1*, *CUC2* and *SPT* in gynoecium development, I analyzed genetic interactions among the corresponding mutations. In

the apical part of the *cuc1 spt* and *cuc2 spt* gynoecium, the lateral margins of the carpels were congenitally fused whereas the central region remained hollow due to the failure of postgenital fusion, indicating that *cuc1* and *cuc2* single mutations suppressed the defect of *spt* partially. Furthermore, *CUC1* and *CUC2* were ectopically expressed in the apical part of the early *spt* gynoecium, suggesting that the restriction of *CUC1* and *CUC2* expression by *SPT* is required for the carpel fusion of the wild type gynoecium.

Next, I examined the relationship among *CUC1*, *CUC2* and *SPT* in the development of carpel-margin-derived organs in the ovary. The septum and ovules were severely reduced in *cuc1 spt* and *cuc2 spt* whereas any of the parental mutants only showed mild or no phenotype, showing the synergistic interaction between *spt* and each of the *cuc1* and *cuc2* mutations. In contrast to the apical region, expression of *CUC1* and *CUC2* in the basal region was not altered by the *spt* mutation, suggesting that *CUC1* and *CUC2* are not direct targets of *SPT* during ovary formation. Moreover, *SPT* expression was significantly reduced in the *cuc1 cuc2* double mutant within the basal region whereas expression is preserved in the apical part, indicating that *CUC1* and *CUC2* are specifically required for *SPT* expression within the ovary but not in apical gynoecium.

The present study thus suggests that the negative regulation of *CUC1* and *CUC2* by *SPT*, which specifically occurs in the apical gynoecium, is important for the congenital carpel fusion of the gynoecium. Functions of *SPT* in septum and ovule development overlap that of *CUC1* and *CUC2* and the three genes may act coordinately to promote the formation of carpel-margin-derived organs. Thus, the interactions among *CUC1*, *CUC2* and *SPT* orient the proper development of the domain-specific structures of the gynoecium in *Arabidopsis thaliana*.

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CHAPTER 1

INTRODUCTION

1.1 Gynoecium of angiosperms

The gynoecium is the female reproductive organ of flowering plants or angiosperms. The unit of the gynoecium is carpel. Major defining features that make angiosperms unique in seed plants are carpel fusion. Carpels are fused at their margins to form an enclosed chamber, which provides a cavity to protect the ovules from external stresses, undesirable pollinations and facilitates seed maturation and dispersal (Fig. 1A, B; Ferrandiz et al. 2010). A typical gynoecium develops three distinct structures namely stigma, style and ovary along the apical-basal axis (Fig. 1A, B). The enlarged basal part that houses the ovules is ovary, on which lies the elongated style. The style connects the ovary to the stigma. The stigma is usually at the tip of the style and is the receptive surface for pollen grains. Thus, the gynoecium have specialized functions for successful pollination and fertilization in angiosperms. After fertilization, the ovules develop into seeds and the ovary matures into a fruit.

Most angiosperm gynoecium possesses more than one carpel. A gynoecium with a single carpel is called monocarpous (e.g. avocado). When the gynoecium has more than one carpel and these carpels are free (unfused), it is called apocarpous (e.g. buttercups, strawberry; Fig. 2A, E). When the gynoecium has multiple carpels fused into a single structure, it is called syncarpous (e.g. arabidopsis, tulip; Fig. 2B-D, F-H). More than 80% angiosperm species are syncarpic: their carpels are fused into a single female structure (gynoecium) in the center of flower (Endress 1982; Fig. 1A). Angiosperm flowers consist of a precise pattern of organ arrangement in four concentric whorls. The inner most whorl contains the gynoecium (Endress 1992), which is surrounded by the stamens and then by the petals and sepals (Fig. 1A). Besides other floral parts

gynoecium is the most important for reproductive success in angiosperms.

Basically, syncarpic gynoecium undergoes two types of fusion events during its development (Verbeke 1992). Based on the timing of the fusion each is termed congenital and postgenital. When the carpels are fused from the earliest emergence of the carpel primordia, the fusion is called congenital, whereas the fusion that takes place during development is termed postgenital. A syncarpic gynoecium is developed by congenital, postgenital, or a combination of both fusion events. In the model plant *Arabidopsis thaliana*, a member of *Brassicaceae*, the gynoecium retains both types of fusion events during its development. Little is known how these fusion phenomena are regulated in *Arabidopsis* gynoecium.

1.2 The evolution of syncarpy

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Syncarpy is defined as the congenital fusion of carpels (Carr and Carr 1961) and is regarded as a key innovation in the evolution of angiosperms (Endress 2001). Over apocarpy (free carpels), syncarpy (fused carpels) offers numerous evolutionary advantages such as guidance of pollen tube, increased pollination efficiency, protection of the ovules (Endress 1982, Armbruster et al. 2002) and later contributes to the dissemination of seeds by a wide variety of mechanisms in different species. For all of these reasons, the carpel development was undoubtedly a major factor in the evolutionary success of the angiosperms. The mechanisms of carpel closure and fusion between carpels have yet to be discovered in highly evolved syncarpic species (Armbruster et al. 2002). The molecular control of carpel development has been investigated in several model species, although most thoroughly in *Arabidopsis thaliana*. In the *Arabidopsis thaliana* (syncarpic) gynoecium, numerous mutations generate various degrees of carpel separation, either single or in double mutant combinations. It is not however clear whether these

genes played any role in the evolution of syncarpy in the *Arabidopsis* lineage. Moreover, how organ differentiation processes first evolved in the gynoecium of this model plant is still elusive.

1.3 Gynoecium morphology in *Arabidopsis*

In the *Arabidopsis* gynoecium, two carpels are congenitally fused along their margin. The fusion of the carpels leads to form an enclosed gynoecium. The gynoecium exhibits specific tissue arrangement along the apical-basal, abaxial-adaxial and medial-lateral axes (Fig. 3). Along the apical-basal axis, the mature gynoecium consists of an apical stigma, a short style and a basal ovary (Fig. 3; Bowman et al. 1999, Sessions 1997, Sessions and Zambryski 1995).

Flower development in *Arabidopsis* has been divided into 20 stages (Smyth et al. 1990) and developmental events characterizing stages have been placed within this context. The gynoecial primordium first appears at stage 6 with two fused carpels (Fig. 4A). The lateral margins of the two carpel primordia are fused congenitally and start to rise as a hollow cylinder-like structure at stage 7 (Fig. 4B; Okada et al. 1989, Ferrandiz et al. 1999). The carpel margins contain a proliferative tissue called medial ridge. At stage 8, the two opposing ridges form in the internal medial regions of the cylinder. The medial ridges grow inwardly to meet each other and fuse postgenitally to form the septum (Fig. 4C; Bowman et al. 1999). The septum divides the ovary longitudinally into two compartments. During stage 9, the placental tissues which will give rise to the ovules are developed from the flank of the medial ridges (Fig. 4D). Later at stages 10-11 the apical margins fuse postgenitally to form a solid style that closes the top of the gynoecium, capped by stigmatic papillae (Fig. 3; Sessions and Zambryski 1995, Bowman et al. 1999). At stage 12 gynoecium, central transmitting tract tissues arise, consisting of polysaccharide rich cells. The transmitting tract tissue originates in the stigma and continues through the style and

the septum to facilitate pollen tube growth (Fig. 3). At anthesis stage (stage 13) the gynoecium becomes mature (Fig. 4E). All these medial tissues such as septum and ovules together with the apical style and stigma are collectively termed marginal tissue (Bowman et al. 1999) and are important for reproductive competence. In order to understand the gynoecium development, it is important to know how developmental processes of these organs are controlled.

1.4 Molecular genetic analysis of *Arabidopsis* gynoecium development

Genetic data and gene expression pattern revealed that a number of regulatory factors interact to coordinate distinct developmental processes in gynoecium development. The current understandings about the roles of these factors are reviewed here.

1.4.1 Carpel identity and patterning of the gynoecium

The establishment of carpel identity is specified by the MADS-box gene *AGAMOUS* (*AG*) (Bowman et al. 1989, 1991; Mizukami and Ma 1995). However, *AG* is not unique in providing all carpelloid features and two other highly redundant MADS-box genes *SHATTERPROOF1* (*SHP1*) and *SHP2* also play a pivotal role in this pathway. On the other hand, carpel identity is also specified by two other transcription factors encoded by the bHLH gene *SPATULA* (*SPT*) and the YABBY gene *CRABS CLAW* (*CRC*) (Alvarez and Smyth 1999).

As previously stated, the gynoecium arises as a single primordium and later it is separated into different domains, within which various tissue identities develop (Bowman et al. 1999; Dinneny et al. 2005, Ferrandiz et al. 1999, Ostergard 2009). Many genes like *SPT*, *CRC*, *ETTIN* (*ETT*), *LEUNIG* (*LUG*), and *STYLISH* (*STY*) (Sessions and Zambryski 1995, 1997, Liu et al. 2000, Alvarez and Smyth 2002, Balanza et al. 2006) act as major factors promoting the

proliferation of cells in the apical end of the gynoecium. In addition, the plant hormone auxin plays an important role in establishing the organ patterning along the apical-basal axis of the gynoecium. Mutations in the auxin response factor genes *MP*/*ARF5* and *ETT*/*ARF3* result in apical basal defects (Prezemeck et al.1996, Sessions et al. 1997), indicting auxin as a major morphogen directing patterning in the apical-basal axis of the gynoecium.

Abaxial-adaxial patterning in gynoecium appears to be determined by members of the YABBY and KANADI gene families (Bowman and Smyth 1999, Eshed et al. 1999, 2001; Siegfried et al. 1999). The YABBY gene *CRC* is involved in the regulation of the radial and lateral expansion of the gynoecium (Alvarez and Smyth 1999).

The medial and lateral domains within gynoecium are also specified in early stages of development. The medial domain, which corresponds to the lateral margins of the two carpels, gives rise to the marginal tissues like septum, ovules, while the lateral domain develop into valves. Several genes such as *SHOOT MERISTEMLESS* (*STM*), *CUC1*, *CUC2* and *SPT* (Long et al. 1996, Aida et al. 1997, Heisler et al. 2001) are expressed in the medial domain. The genes *ASYMMETRIC LEAVES1* and *2*, and the HD-ZIP genes JAG and NUB (Siegfrid et al. 1999; Dinney et al. 2005) are involved in lateral domain development. All these pieces of information indicate that a complex genetic network arranges the domain specific tissue differentiation in *Arabidopsis* gynoecium.

1.4.2 Genes involved in carpel margin development in *Arabidopsis*

Molecular genetic studies have identified different transcription factors such as SPT, ANT, CRC, LUG, SEU, CUC1, and CUC2 (Sessions and Zambryski 1995, Ishida et al. 2000, Liu et al. 2000, Alvarez and Smyth 2002, Balanza et al. 2006, Azhakanandam et al. 2008) regulate carpel fusion

as well as development of carpel margin-derived organs in *Arabidopsis*. I mentioned above that two different fusion events occur in gynoecium development, named as congenital and postgenital fusions (Verbeke 1992). In *Arabidopsis*, congenital fusion along the carpel margin ensures the upward growth of the gynoecial tube whereas postgenital fusion occurs between the margin-derived organs that develop inside the gynoecium. Hence, carpel margins are particularly important as a site for organ 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). Importantly, most mutants that are defective in carpel fusion also display defects in the formation of septum and ovule, suggesting a close link between the two processes. Phenotypic characterizations of some of these mutants are reviewed.

The *crc* mutant gynoecia have unfused apical ends toward the top. The *crc* gynoecia are shorter and wider than those of the wild type gynoecia. Sometimes the septum is unfused in the apical region (Alveraz and Smyth 2002). Furthermore, the *spt crc* double mutant shows severer defect in carpel fusion than each of the single mutants, indicating the synergistic interaction between these genes.

Similarly, the apical region of the *lug* gynoecium is composed of four unfused parts. Two of the medial parts are capped by stigmas but other two are unfused valves. The *lug* gynoecium also has defects in the septum fusion (Chen et al. 2000). Another gene, *ANT*, encodes a transcription factor of the *APETALA2*-like family (Elliott et al. 1996) and *ant* mutant gynoecia have minor defects in apical fusion and restriction in stigmatic development. However, in combination with *lug*, the double mutant gynoecia developed two completely unfused valves. This suggested that *LUG* and *ANT* play a role in the fusion events of the gynoecium and in the development of the marginal tissues (Liu et al. 2000).

Mutations in *SPT*, which encodes a 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 defects in margin-derived structures (Alvarez and Smyth 1999, 2002; e.g. Fig. 6B, F). Thus, the important function of *SPT* is to promote the carpel fusion and differentiation of marginal tissues in gynoecium development. Expression of *SPT* also 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, *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 (Fig. 5F), 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). It has been observed that *cuc1 cuc2* double mutant gynoecium do not show split carpel (Ishida et al. 2000; Fig. 5D). *CUC1* and *CUC2* are also redundantly involved in the development of marginal structures; single mutations in these genes have no major effect on septum and ovule development and produce well developed septum and ovules like wild type (Fig. 5B, C) whereas double mutations result in the loss of the septum and ovules (Ishida et al. 2000; Fig. 5E). 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.

The above discussion indicates that many genes share functional redundancy in carpel fusion and their collective activity is essential for normal marginal tissue development. However, the precise mechanism of carpel fusion is yet to be investigated. How the developmental

processes are regulated in carpel margin is therefore a key question to understand gynoecium development.

1.5 Rationales for this study

In the *Arabidopsis* gynoecium, two carpels are fused at their margins to form a chamber in which the ovules develop. The underlying genetic controls that coordinate the fusion and differentiation of the marginal structures during gynoecium development are largely unknown. The main focus of this study is how genetic interactions regulate carpel margin development in the *Arabidopsis* gynoecium. To address these fundamental issues the genes *SPT*, *CUC1,* and *CUC2* have been chosen because of their functions in carpel fusion and organogenesis in the gynoecium. I analyze the interactions among *CUC1*, *CUC2* and *SPT* genes. It has been previously observed that *SPT* promotes the carpel fusion (Alvarez and Smyth 1999, 2002), whereas overexpression of the *CUC1* and *CUC2* (Aida et al. 1997, Hibara et al. 2006) prevents it. However, it is not investigated how *SPT* control the carpel fusion during gynoecium development. To investigate how the position specific tissue arrangements occur in relation to carpel fusion as well as carpel margin-derived organs in the *Arabidopsis* gynoecium, I carried out the following experiments.

Part 1. *SPT*, *CUC1* and *CUC2* gene interaction in carpel fusion of the gynoecium

To understand *SPT* gene function in carpel fusion, I constructed *cuc1 spt* and *cuc2 spt* double mutants and analyzed their fusion phenotype. I found more than 80% of both double mutants showed fused carpel in the apical part of the gynoecium. Thus, the results suggest that *cuc1* and *cuc2* mutations can suppress the congenital fusion defect of *spt* in the apical part.

To examine whether *cuc1* and *cuc2* also rescue the inward growth of medial ridges in the

apical gynoecium of *spt*, I examined histological sections of *cuc1 spt* and *cuc2 spt* to compare them with those of wild type and *spt*. These results indicate that neither *cuc1* nor *cuc2* rescues the inward growth defect of *spt* in the apical gynoecium.

To analyze how *CUC* gene expression is regulated in the *spt* mutant background, I carried out *in situ* hybridization and compared *CUC1* and *CUC2* expression in the wild-type and *spt* at the top of the gynoecium. Ectopic expression of *CUC1* and *CUC2* was detected in the apical part of the *spt* gynoecium, indicating that *SPT* is required for the repression of *CUC1* and *CUC2* in the apical part of the wild type gynoecium.

To investigate how *SPT* regulates *CUC* gene expression, I examined the effect of the *spt* mutation on the promoter activity of *CUC2* by using the *CUC2p*::GUS reporter line. In wild-type gynoecium GUS expression was detected in the adaxial side of the apical gynoecium whereas in *spt* mutant GUS was broadly expressed in the abaxial-adaxial region of this domain. The expression pattern of *CUC2p*::GUS overlaps with the *CUC2* mRNA within apical part of *spt* mutant, suggesting that *spt* mutation affects the transcriptional regulation of *CUC2* in the apical part of gynoecium.

Part 2. Interaction between *CUC1*, *CUC2* and *SPT* in carpel margin derived organ development

cuc1 spt and *cuc2 spt* double mutants showed severe reduction of septum and ovule development in the ovary, implying that *SPT*, *CUC1* and *CUC2* coordinately promote development of carpel margin-derived organs. In situ hybridization analysis showed that *CUC1* and *CUC2* were expressed in the medial ridges of *spt* mutant, indicating that *CUC1* and *CUC2* are not direct targets of *SPT*.

To analyze whether the *CUC* genes affect the *SPT* expression, I performed in situ hybridization of *SPT* in the wild-type and *cuc1cuc2* double mutant backgrounds. In wild type *SPT* expression extended from adaxial to abaxial side of the apical medial domain and in the adaxial medial ridges within the ovary while the expression of *SPT* was significantly reduced in *cuc1cuc2* double mutant within the ovary. In contrast, the SPT expression was preserved in the apical part of the gynoecium, namely style, indicating that *CUC1* and *CUC2* regulate *SPT* expression in the ovary but not apical medial domain. Here, I provide evidence that genetic interactions between *CUC1*, *CUC2* and *SPT* is important for domain specific tissue differentiation in the *Arabidopsis* gynoecium.

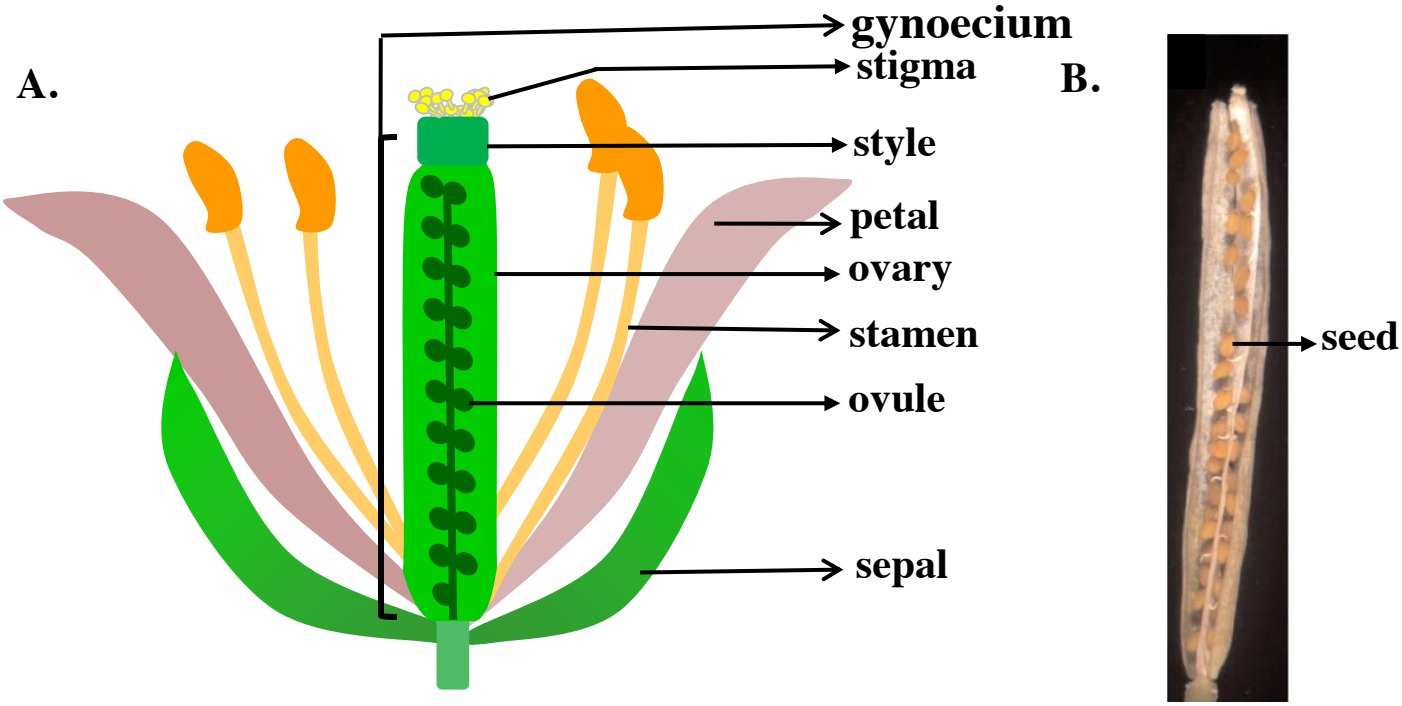

Fig. 1 Morphology of angiosperm gynoecium

(A) Carpel fusion forms the unified compound gynoecium which is positioned at the centre of the flower. In apical basal axis, the gynoecium contains stigma, style and ovary. Enclosed gynoecium protects ovules inside the ovary.

(B) Carpel fusion facilitates seed dispersal. This photo has been adopted from Fruit development in *Arabidopsis* by Adrienne H. K. Roeder and Martin F. Yanofsky. First published on February 22, 2006; doi: 10.1199/tab.0075.

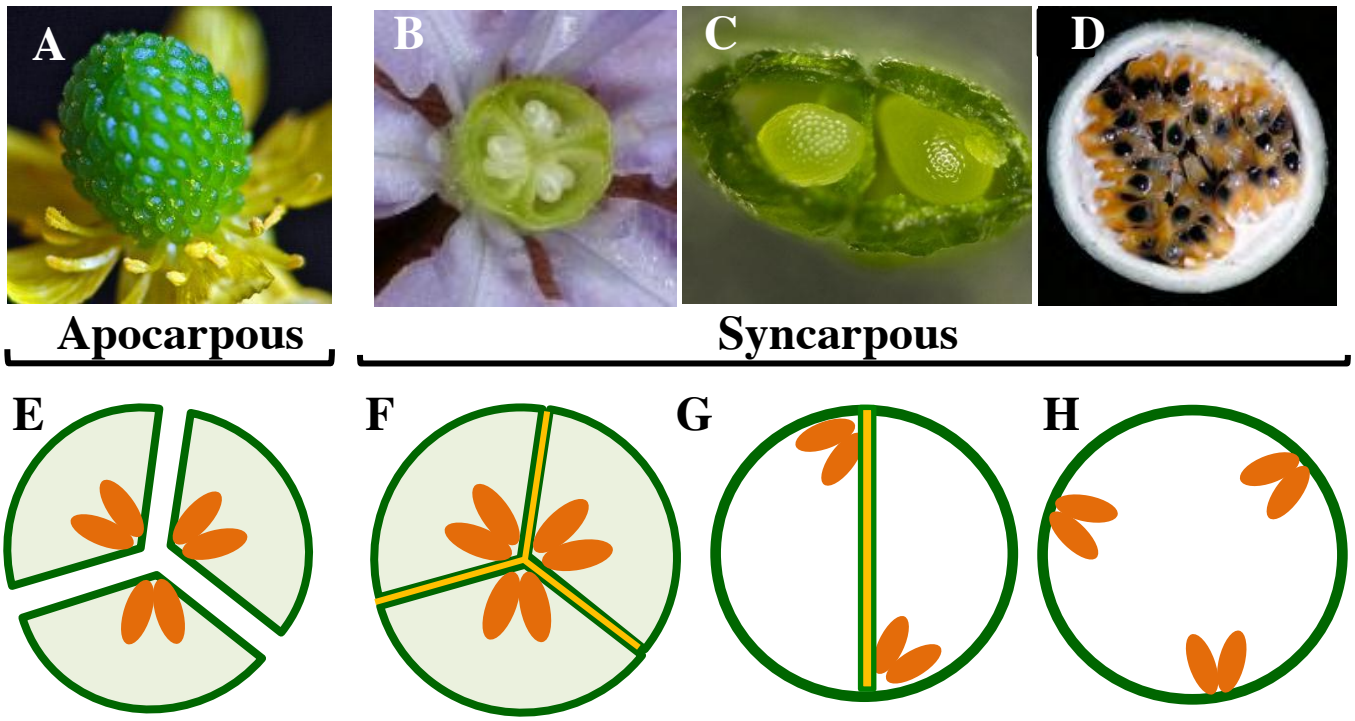

Fig. 2 Types of carpel fusion in angiosperms

(A) Apocarpous gynoecium in *Ranunculus abortivus*. This photo has been adopted from this URL: http://www.microscopy-uk.org.uk/mag/artnov06/bj-Three Buttercups.html

(B-D) Cross sections of syncarpous gynoecium

(B) Three carpels are fused in *Camassia scilloides*. This photo has been adopted from bobklips.com

(C) Two carpels are fused in *Arabidopsis thaliana*

(D) Three carpels are fused in *passiflora edulis* This photo has been adopted from this URL: http://www.bitany.hawaii.edu/)

(E-H) Diagram of carpel fusion in angiosperm gynoecium

(E) Unfused carpels in apocarpous gynoecium

(F- H) Fused carpels in syncarpous gynoecium .

(E-H) ovule (orange), septum (yellow)

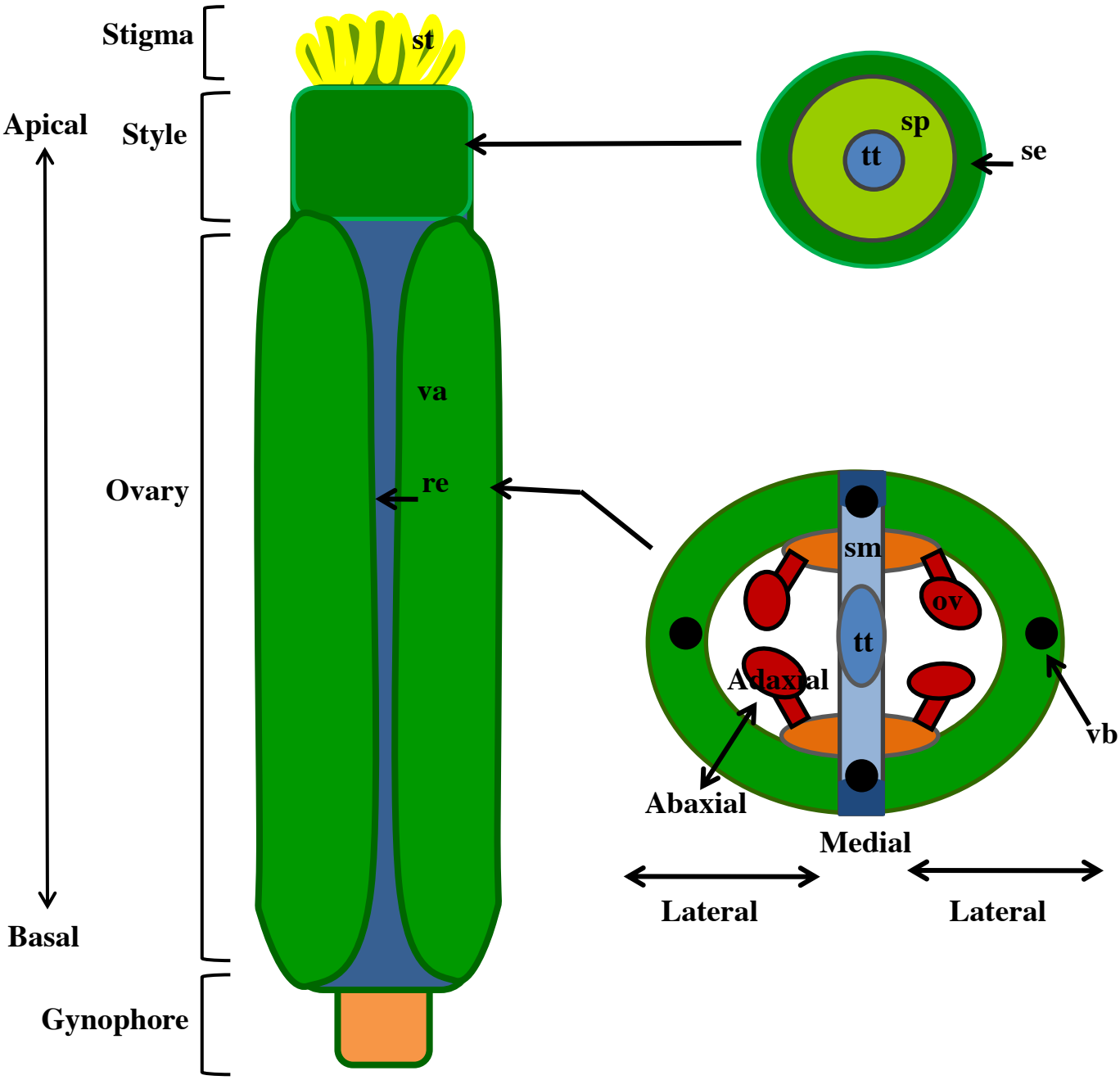

Fig. 3 Major tissue types in *Arabidopsis* gynoecium

st, stigmatic papillae (yellow); se, style epidermis (dark green); sp, style parenchyma (green); tt, transmitting tract (blue); va, valve (light green); re, replum (dark blue); sm, septum (light blue); pl, placenta (orange); ov, ovules and funiculi (dark red); vb, vascular bundle (black).

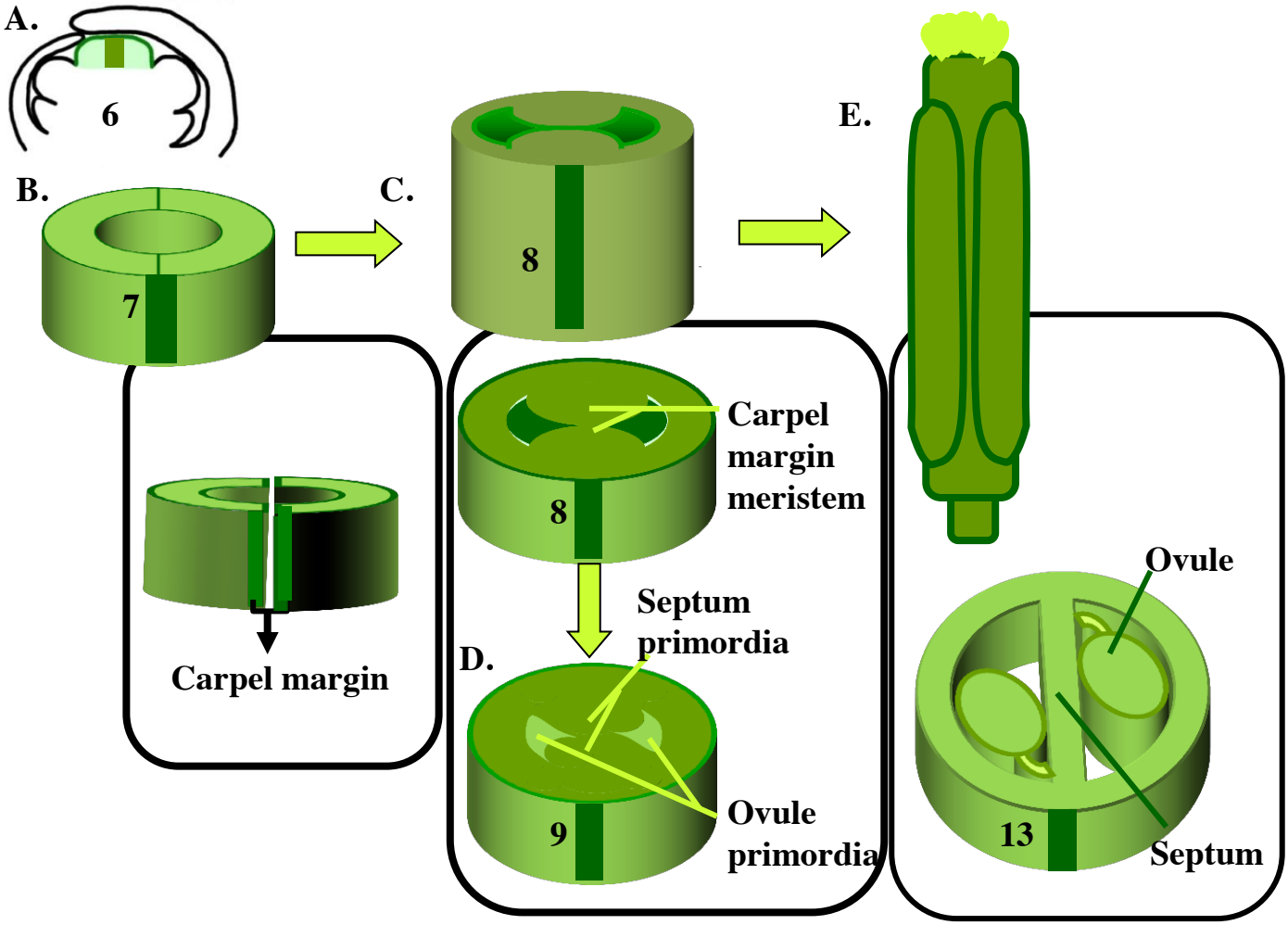

Fig. 4 Developmental stages of *Arabidopsis* gynoecium

(A) Gynoecium primordium is initiated at stage 6 from floral primordium.

(B) The gynoecium is a cylinder like structure consisting of two fused carpels. It is initiated as a single primordium at stage 6 and start to grow as a tube- like structure at stage 7.

(C) At stage 8 medial ridges are initiated at the adaxial side of the carpel margins. (D) The ridges are meristematic tissue and produce septum and ovule primordia (stage 9). The two septum primordia grow inward until they meet in the centre of the gynoecium and fuse postgenitally, forming a single septum. The ovule primordia initiated laterally from the medial ridges.

(E) Stage 13 whole gynoecium and sections shows septum and ovules.

Numbers inside the cartoon indicate the corresponding stage.

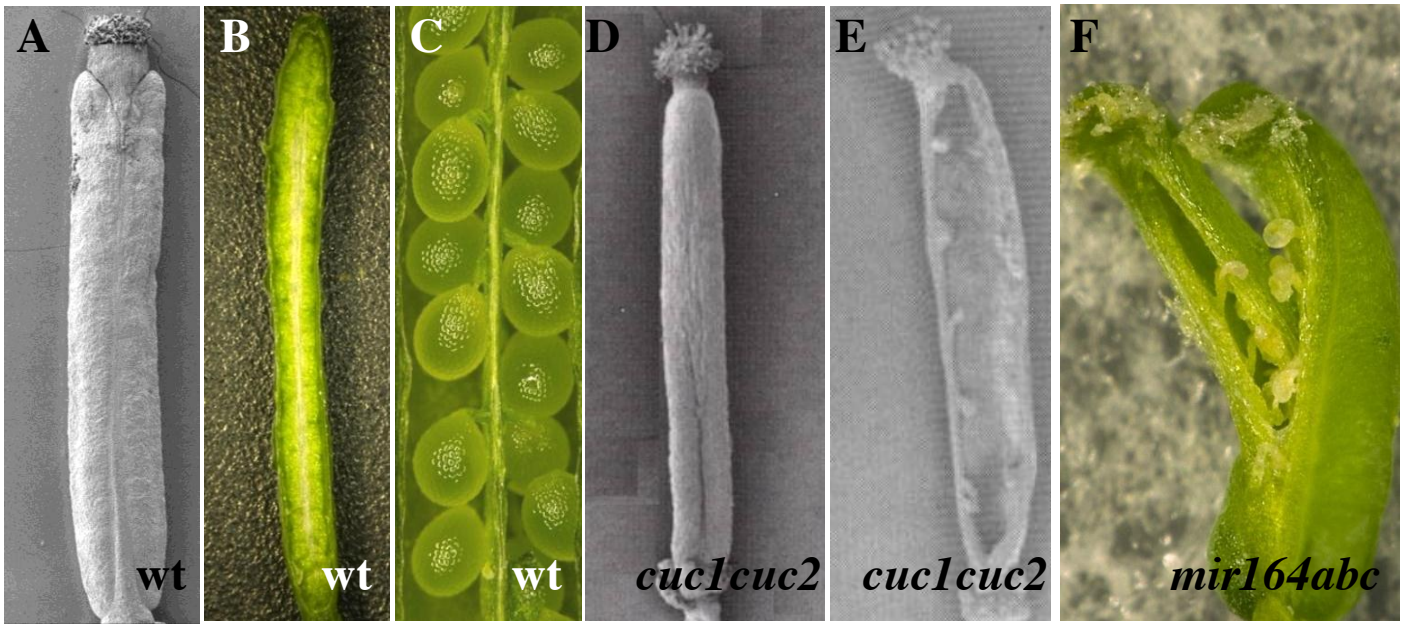

Fig. 5 *CUC1* and *CUC2* are involved in carpel margin-derived organ development and prevent carpel fusion along the margin

(A) Wild type gynoecium consists two fused carpels.

(B) Wild type gynoecium has intact septum.

(C) Wild type gynoecium possess well developed ovules.

(D) *cuc1 cuc2* double mutant gynoecium consists two fused carpels. This photo has been adopted from Ishida et al. 2000. *Plant Cell Physiol*. 41(1): 60-67.

(E) Septum and ovule development are defective in the *cuc1 cuc2* double mutant. This photo has been adopted from Ishida et al. 2000. *Plant Cell Physiol*. 41(1): 60-67.

(F) *mir164abc* triple mutant gynoecium shows unfused carpel along the carpel margin.

wt, wild type.

CHAPTER 2

MATERIALS AND METHODS

2.1 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 Smith 1999). After crossing parental mutants, all double mutant combinations were identified by PCR-based genotyping at the F2 and subjected to analysis at the F3 and F4. Seeds were surface-sterilized, sown on Murashige and Skoog plates, and germinated as previously described (Aida et al.1997). About 2 weeks after germination, seedlings were transplanted onto soil and grown at 23°C under constant white light as previously described (Fukakiet al. 1996).

2.2 Microscopy and histology

Close-up images were photographed using a digital microscope (VHX-900, Keyence). For scanning electron microscopy (SEM), fresh inflorescence apices were fixed in FAA (formaldehyde-acetic acid- alcohol) at 4°C for 2 h and then dehydrated through a graded ethanol series of 70, 85, 95 and 100% (2 times) each for 30 minutes. Samples were then critical-point-dried using liquid carbon dioxide. Individual flower buds were removed from inflorescences and mounted on SEM stubs. To expose gynoecia, the outer organs were dissected using glass needles. The mounted specimens were sputter-coated with gold and palladium (4:1) and examined with a scanning electron microscope S-4700 (Hitachi).

For histological sections, Paraplast-embedded flowers or inflorescences 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. Stained sections were viewed by Nikon ECLIPSE E800 Normaski microscope.

2.3 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 pH7.0, 10mM EDTA, 5 mM$K_3Fe(CN)_6$, 5 mM$K_4Fe(CN)_6$ and 0.1% Triton X-100 and 0.5 mg/ml 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 chlorophyll, and embedded in Paraplast for sectioning. Embedded specimens were sectioned at a thickness of 8 μm with a microtome and viewed after de-waxed under the Nikon ECLIPSE E800 Normaski microscope.

2.4 In situ hybridization

RNA in situ hybridization was performed according to Takada et al. (2001). Inflorescence apices were collected shortly after bolting and fixed. 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.

CHAPTER 3

RESULTS

3.1 Part 1. *SPT*, *CUC1* and *CUC2* gene interaction in carpel fusion of the gynoecium

3.1.1 *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. 6A, I), approximately 90% of the *spt* gynoecia displayed the split carpel phenotype under the lab growth conditions (Fig. 6B, I; 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.7A) and then became even (stage 11; Fig. 7E), yielding a flat rim of apical tissues. The adaxial wall of the gynoecial cylinder initiates the medial ridges, which grew inward and fused postgenitally, resulting in the solid style (Fig. 6E). In the strong allele *spt-2* (hereafter called *spt*), by contrast, the medial regions of the gynoecial rim showed retarded growth in the apical direction (arrow in Fig. 7B; Alvarez and Smyth 2002) and subsequently formed a cleft (arrow in Fig. 7F), 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 (Fig. 6F, 7B). These results indicate that *SPT* promotes the growth of the apical medial domain in two ways: upward growth in the rim facilitates congenital carpel fusion and inward growth of the medial ridges fill 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. 6I) 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, I 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. 6A, 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, indicating that *cuc1* and *cuc2* single mutations suppressed the *spt* defect only partially (Fig. 6G, H).

I next analyzed the early gynoecium development of *cuc1 spt* and *cuc2 spt*. In these double mutants, the gynoecial rim grew upward evenly (Fig. 7C, D) and no cleft formation occurred (Fig. 7G, H). On the other hand, the inward growth of the medial ridges remained severely reduced (Fig. 7C, D), leaving a central hollow similar to that observed in the *spt* single mutant (Fig. 7B). 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.

3.1.2 *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, I 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. 8A-E; Supplementary Fig. S1). In *spt*, by contrast, I detected *CUC1* mRNA in the uppermost part of the medial region at stage 8 (Fig. 8F, H, I). The 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. 8J), and was also detected at the split parts (Fig. 8G).

I 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. 9A, B, C), restricted to the adaxial side. In *spt*, by contrast, *CUC2* expression extended to the abaxial side of the apical medial region at stage 8 (Fig. 9D). At stage 10 and the signal was also frequently detected at the split ends (Fig. 9E) or at the abaxial side (Fig. 9F). Taken together, these results show that in wild type *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 caused 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). I therefore tested whether the *spt* mutation affected the promoter activity of *CUC2*. To this end, I 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. 9G). In *spt*, by contrast, *ProCUC2::GUS* expression extended fully from the central to the peripheral region of the apical medial region at stage 8 (Fig. 9H). The patterns of GUS activity in the wild type, and how they changed in *spt*, are in accordance with those of *CUC2* mRNA (Fig. 9A, D). 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.

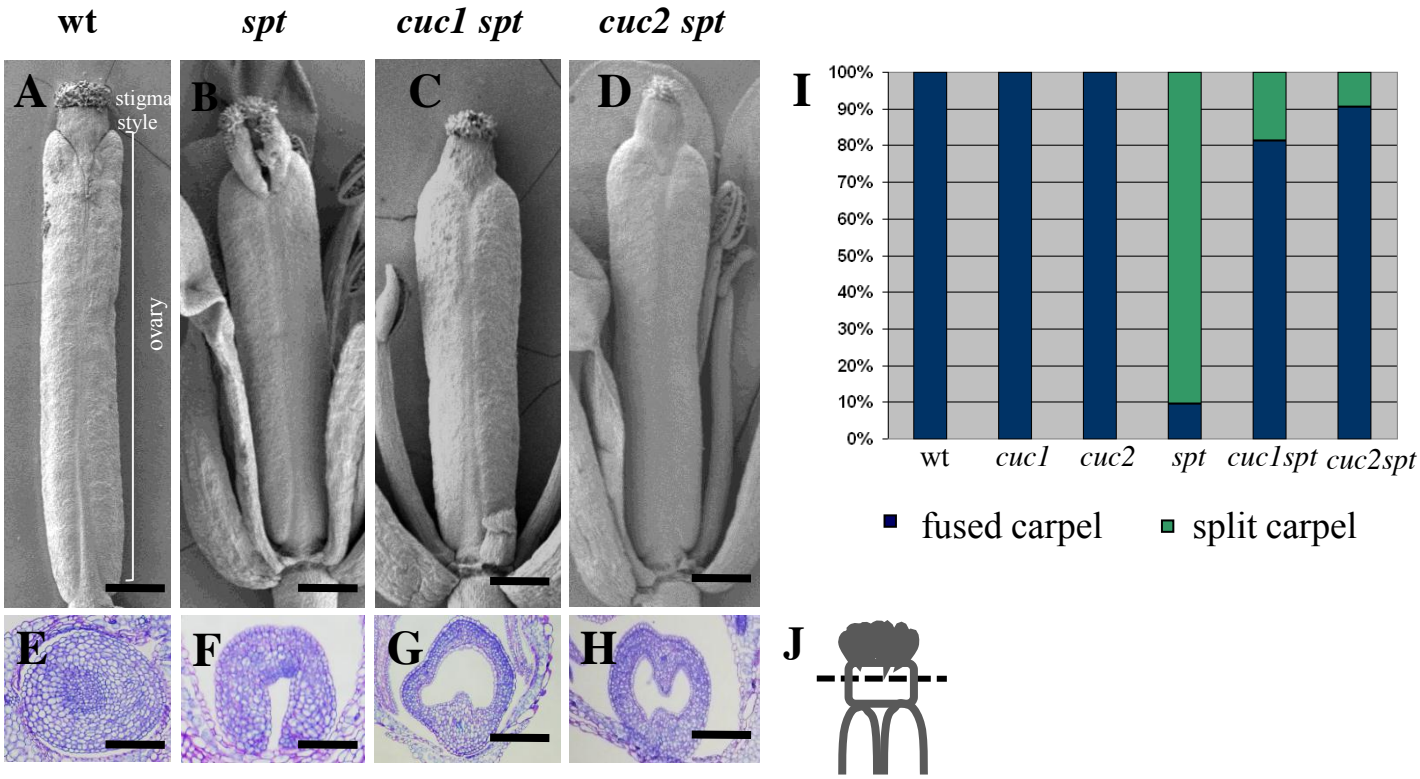

Fig. 6 Carpel fusion phenotype of *cuc1 spt* and *cuc2 spt*

(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 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 8 flowers on the main inflorescence of 4 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.

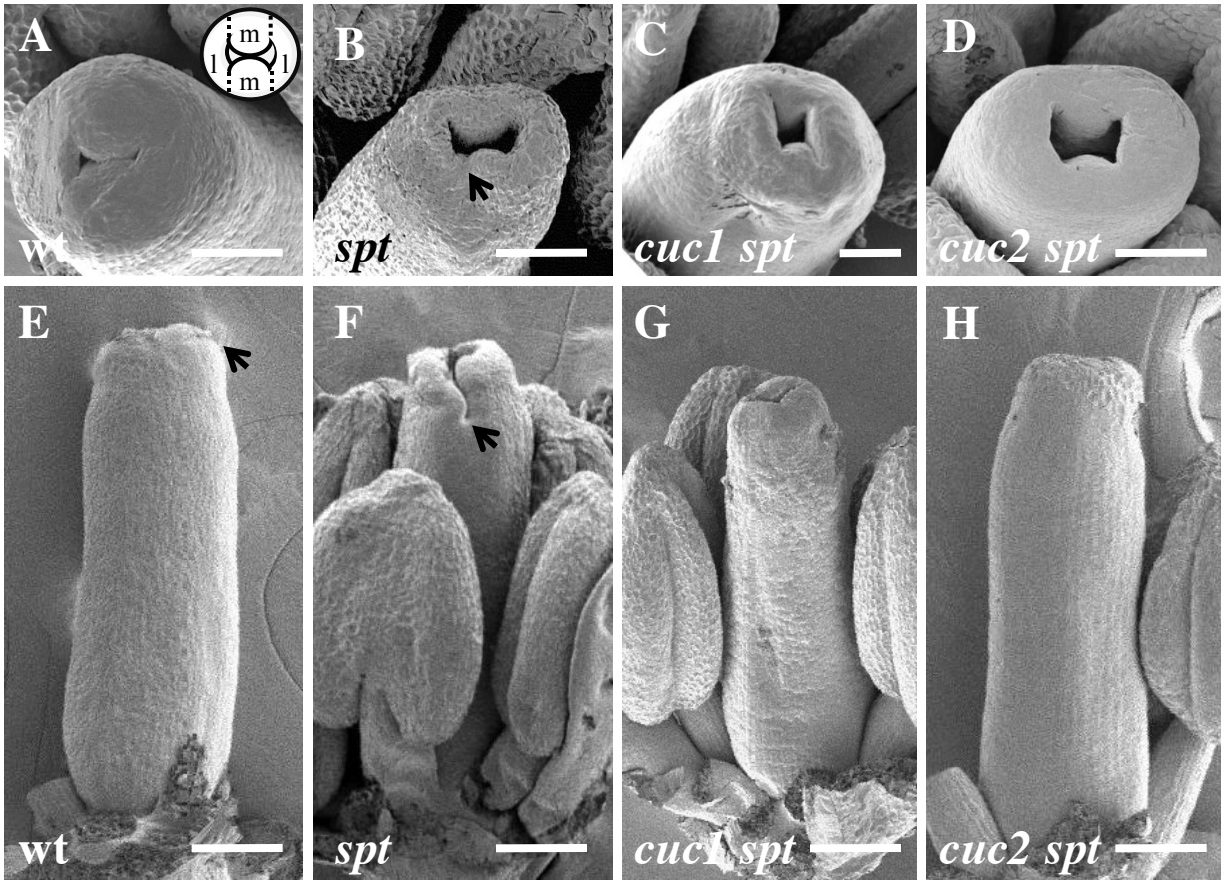

Fig. 7 Early gynoecium development in wt, *spt*, *cuc1 spt* and *cuc2 spt*

(A-D) Stage 8 gynoecia viewed from above. (E-H) Lateral view of stage 11 gynoecia. (A) In 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 contact due to reduced growth of the medial ridges. (E) In 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.

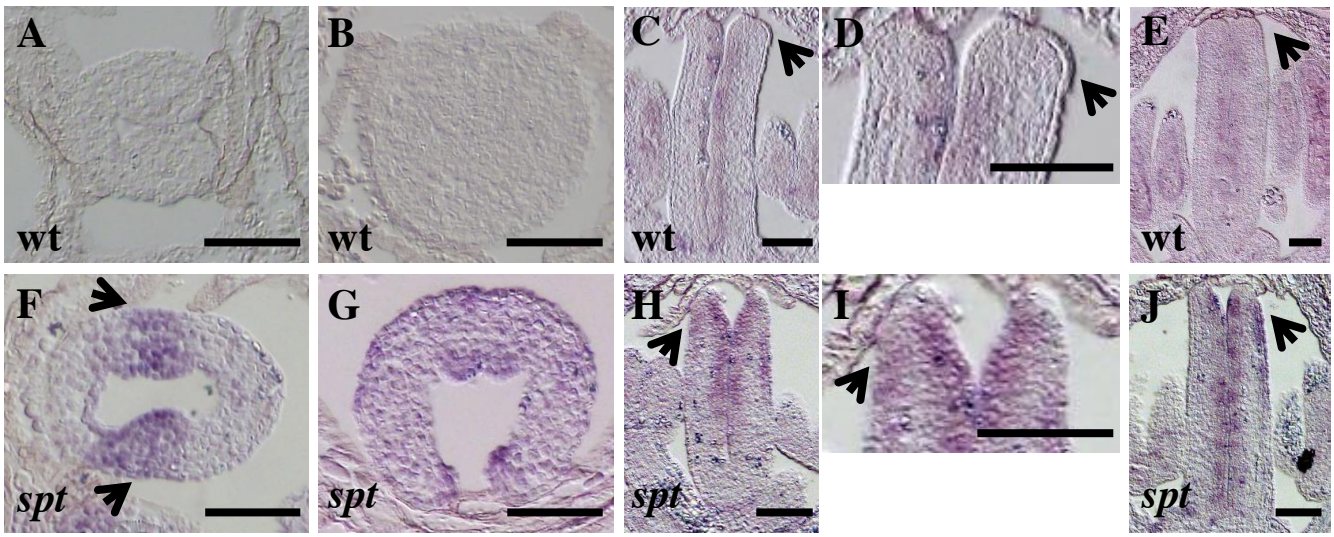

Fig. 8 *CUC1* expression in the apical region of wild type and *spt* gynoecia

All transverse sections represent the apical part of the gynoecium. Probes detect (A–J) *CUC1* transcripts, (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 (H) at stage 8 (I) close up views of the apical gynoecium of (H) and (J) stage 10; arrows indicate ectopic expression.

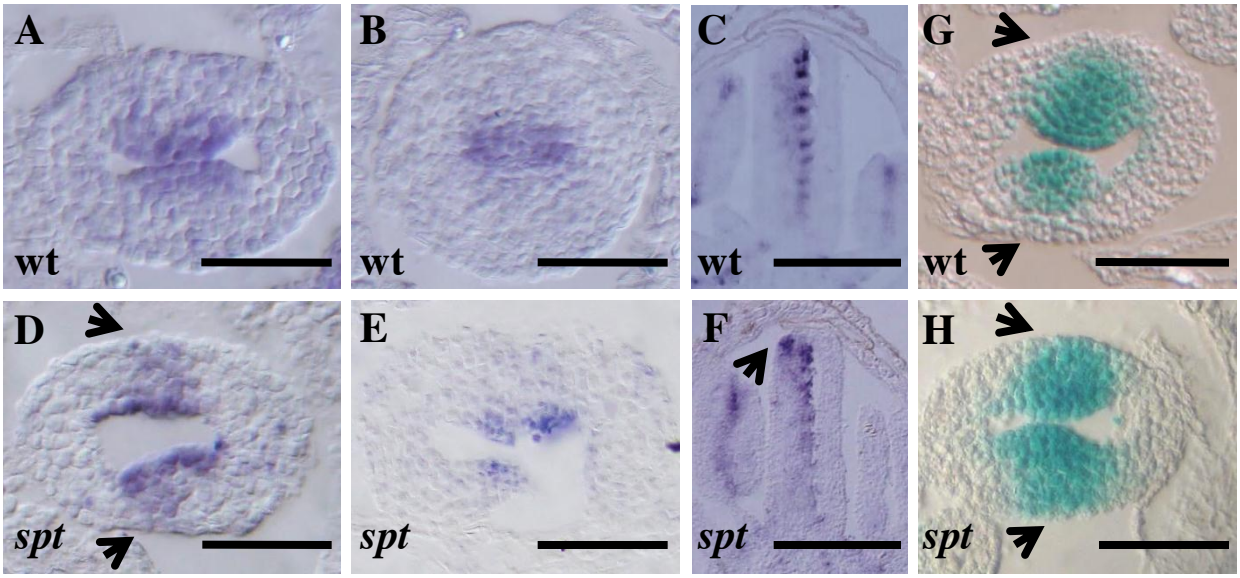

Fig. 9 *CUC2* expression in the apical part of the wild type and *spt* mutant

(A-C) *CUC2* expression in wild type at (A) stage 8, (B) stage 10 (transverse sections) and (C) stage 10 (longitudinal section). (D-F) *CUC2* expression in *spt* at (D) stage 8, (E) stage 10 (transverse sections) and (F) stage 10 (longitudinal section); arrows indicate the ectopic expression. (G-H) *ProCUC2::GUS* expression in wild type at (G) stage 8; note that GUS activity is not detected on the abaxial side (arrows). (H) *ProCUC2::GUS* activity is detected ectopically on the abaxial side at stage 8 (arrows). Bars, A, B, D, E, G and H= 50 μm; C and F = 130 μm; wt, wild-type.

3.2 Part 2. Interaction between *CUC1*, *CUC2* and *SPT* in carpel margin derived organ development

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

Shortly after the initiation of the gynoecial tube development, 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* 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 these three genes, I compared the septum phenotypes of *cuc1 spt* and *cuc2 spt* double mutants and compared it with those of the wild type and *spt* mutants. In the wild type and each single mutant of *cuc1* and *cuc2*, most gynoecia had an intact septum (Fig. 10A, E, F, M), whereas more than 85% of *spt* gynoecia showed a mild septum defect in which the unfused region covered less than half of the entire ovary length (Fig. 10B, G, H, M).

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. 10C, I, J, M) whereas the remaining 60% showed a milder defect. In *cuc2 spt*, 90% of the gynoecia displayed a strong septum defect (Fig. 10D, K, L, M) 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 portion of these ovules develop into seeds, whereas neither *cuc1* nor *cuc2* single mutation affects ovule number (Ishida et al. 2000). I 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 as compared to 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.

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

Genotype	No. of ovules	No. of seeds	Percent 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 8 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 $P < 0.01$ (**).

3.2.2 *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, I examined the effect of the *spt* mutation on *CUC1* and *CUC2* expression 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. 11A, 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. 11B, 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. 11C, I). In the *spt* mutant, expression of *CUC1* and *CUC2* was very similar to wild type throughout these stages (Fig. 11D, E, F, J, K, L), except that *CUC* transcripts showed relatively weak expression in presumptive transmitting tract cells in the septum (Fig. 11F). These results indicate that *CUC1* and *CUC2* do not require *SPT* in the ovary of the gynoecium.

3.2.3 *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, I carried out in situ hybridization in 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 wild-type, where it extended from the adaxial to the abaxial side (Fig. 12A). 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. 12B, C;

Heisler et al. 2001). By contrast, in the *cuc1 cuc2* double mutant, *SPT* transcript accumulation was observed in the apical medial domain, as for wild type (Fig. 12D), *SPT* expression was missing lower down within the ovary throughout these stages (Fig. 12E, F). Taken together, these results show that *CUC1* and *CUC2* are redundantly required for *SPT* expression in the medial ridge of the ovary.

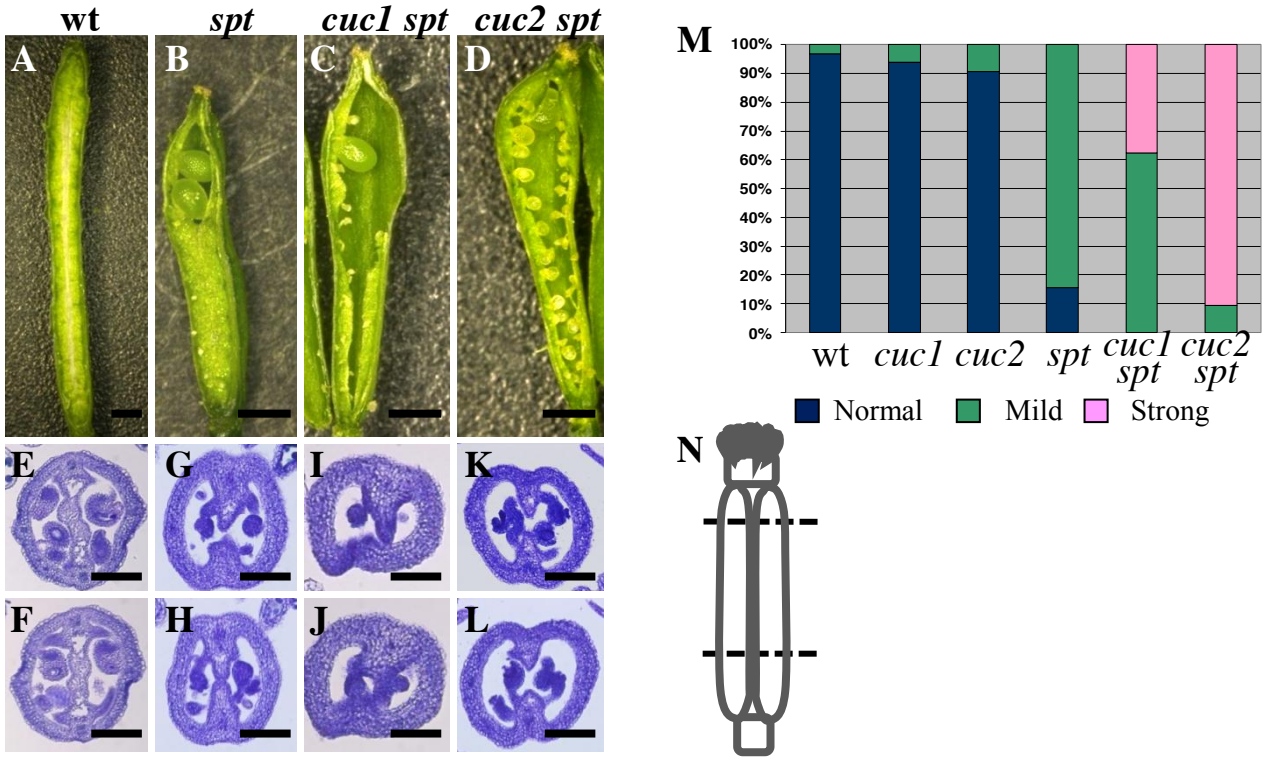

Fig. 10 Septum development in wild type, *spt*, *cuc1 spt*, *cuc2 spt*

(A-D) Lateral views of mature siliques at stage 17 with one valve removed. Developing seeds in the front side are also removed. (E-L) Transverse sections (see N) showing the septum phenotype in apical (E, G, I, K) and basal (F, H, J, L) 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 unfused. No fusion occurred between the septa across the apical-basal axis of the ovary in (C) *cuc1 spt*, (D) *cuc2 spt* . Transverse sections show no septum defect in apical (E) and basal (F) parts of the wild type ovary. In *spt*, septum development is defective in the apical part (G), but normal in the basal part (H), of the ovary. Severe septum defects were observed throughout the ovary in *cuc1 spt* (I, J), *cuc2 spt* (K, L). (M) Percentage of septum defects. The first 8 flowers on the main inflorescence of 4 plants were scored. (N) Schematic diagram of the mature gynoecium. Dotted lines (upper, apical; lower, basal) indicate approximate positions of the section plane in E to L. Bar in A-D = 250 μm and E-L = 50 μm. wt, wild-type.

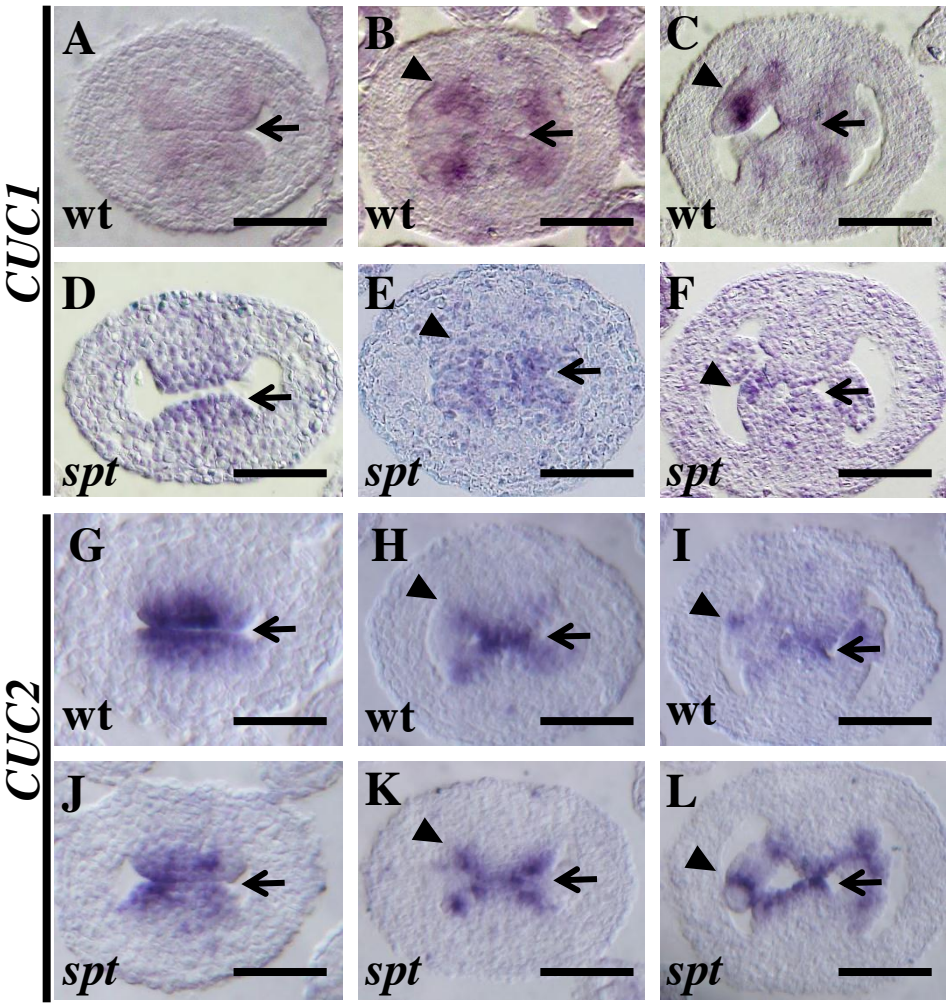

Fig. 11 *CUC1* and *CUC2* expression in the basal region of wild type and *spt* gynoecium

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 e*xpression 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.

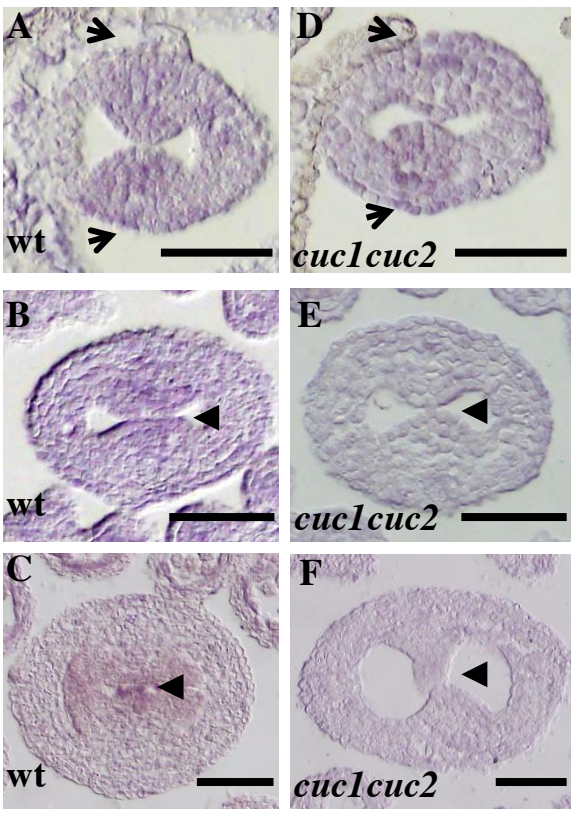

Fig. 12 *SPT* expression in wild type and *cuc1 cuc2* gynoecium

All images are of transverse sections. Sections from the apical (A, D) and basal (B-C, E-F) regions of the gynoecium. (A) In the apical region at stage 8, *SPT* expression extends from the adaxial to abaxial sides of the medial domain of wild-type (A, arrows). (B, C) In the basal gynoecium of wild type, *SPT* expression is restricted to the medial ridges on the adaxial side at stage 8 (B, arrowhead) and in the developing septum at stage 10 (C, arrowhead). (D) At stage 8 in *cuc1 cuc2* double mutant *SPT* expression covers abaxial to adaxial region of apical medial domain. (E, F) 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.

CHAPTER 4

DISCUSSION

4.1 *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). However, how *SPT* executes this function has remained largely unknown. I 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. 7B, 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 postgenital fusion (Fig. 7B, 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. 7B, 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. 12A). In the same domain, *CUC1* is not expressed and *CUC2* expression is restricted to the central 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. 13, orange arrows). On the other hand, no detectable contribution of *CUC1* and *CUC2* in the medial ridge growth and subsequent postgenital fusion in the centrally apical gynoecium of *spt*, indicating that only *SPT* is involved in postgenital fusion to form a solid style (Fig. 13, green arrows).

It has been demonstrated that *CUC1* and *CUC2* are under the 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.

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). Second, 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). Third, 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.

4.2 *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 (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. 10). The *spt* mutant defect in these organs is associated with the reduction of the medial ridge size, suggesting that *SPT* 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 suggests that *CUC1* and *CUC2* act upstream of *SPT* to initiate or maintain its expression. By contrast, both *CUC1* and *CUC2* transcripts are detected in the medial ridges as well as in the margin-derived organs of *spt*, 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).

My 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 *SPT* 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.

4.3 Relation between *CUC*/ *SPT* functions and gynoecium patterning

My 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 appear to be involved in partitioning the gynoecium primordium into distinct regions. Complex genetic networks ensure the maintenance of the meristem-primordia, central-peripheral, 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. 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.

Moreover, carpel is the progenitor organ of the fruit and a defining feature of angiosperm. Diversity of fruit structure is truly remarkable in angiosperm evolution. Although many key questions remain to be answered in this processes, this analysis will facilitate our understanding of how the position specific tissue differentiation contributes to the evolutionary success of angiosperms.

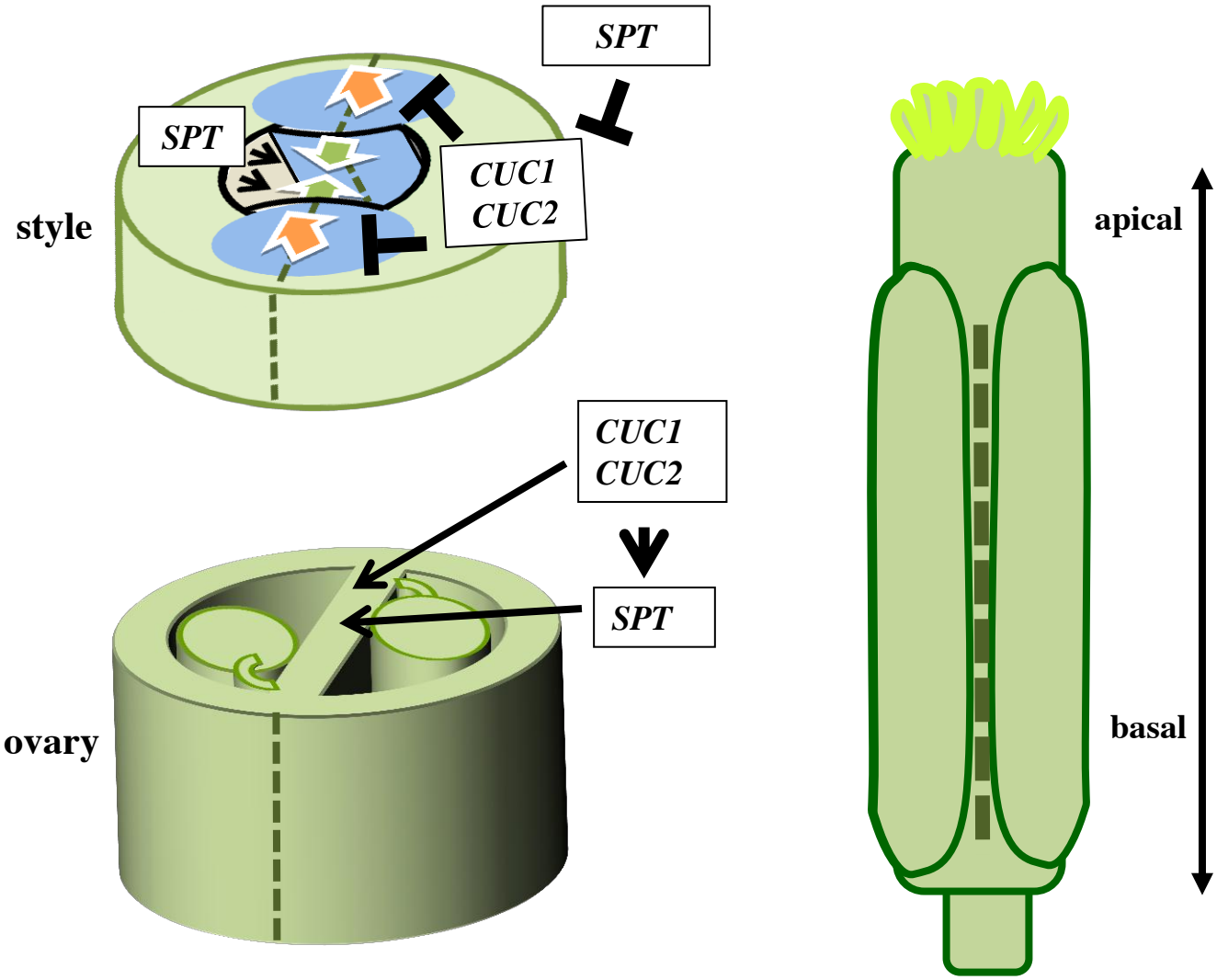

Fig. 13 A model for the interaction between *SPT*, *CUC1* and *CUC2* in the *Arabidopsis* gynoecium.
The closure of the apical gynoecium in the region of the style and stigma results from the combination of two types of growth activities: upward growth of the gynoecial tube ensures congenital fusion of carpels along their lateral margins (dashed darker olive green 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* and *CUC1* and *CUC2* activities are not involved in this development. On the other hand in the ovary *CUC1*, *CUC2* and *SPT* function coordinately for septum development. Reduced *SPT* expression in *cuc1 cuc2* ovary indicate *SPT* could be the downstream factor of *CUC1* and *CUC2* in the ovary of the gynoecium.

CHAPTER 5

CONCLUSIONS AND PERSPECTIVE

Carpel fusion is essential for closed gynoecium, and is associated with the development of septum, ovules and styles derived from the meristematic carpel margin. The present knowledge of carpel fusion as well as margin-derived organ development is still very fragmented.

In this study, I have shown *CUC1* and *CUC2* expression are restricted by *SPT* in the apical gynoecium. On the other hand I found *CUC1*, *CUC2* and *SPT* function collectively promoting carpel margin-derived organ development in the ovary. My analysis also demonstrated that *SPT* differentially interacts with *CUC1*, *CUC2* in the apical-basal part of the gynoecium. Based on the present results, the following experiments will be suggested for future.

I. Analysis of interaction among *CUC1*, *CUC2* and auxin related genes:

The present study suggests that *cuc1*and *cuc2* partially rescued the apical split phenotype of *spt*. Previously it was observed that *SPT* function has linked to auxin (Groszmann et al. 2008, Eklund et al. 2010). *SPT* gene has been proposed to mediate auxin signalling, since chemical inhibition of polar auxin transport restores normal gynoecium development in *spt* mutants (Nemhauser et al. 2000). Accordingly, auxin might be a key regulator in the *CUC* pathway in carpel fusion. To further understand the mechanisms of action among *CUC1*, *CUC2* and *SPT* genes in apical gynoecium it would be important to determine whether *CUC1* and *CUC2* interact with auxin related genes in gynoecium development.

II. Search for the new factors those are regulated differentially in the apical and basal axis of the gynoecium:

Present analysis demonstrated that the effect of *SPT* on *CUC1* and *CUC2* expression differs between the apical and basal regions of the gynoecium primordia. *SPT* suppresses the expression of *CUC1* and *CUC2* in apical region, whereas in the basal region *SPT* does not affect expression of either gene. Thus the actions of *SPT* on *CUC1* and *CUC2* expression is likely influenced by additional factors. It would be interesting to find out other new factors that act differentially in apical and basal axis of the gynoecium by mutant screening or microarray analyses.

III. Analysis of interactions among *CUC1*, *CUC2* and other carpel genes:

Another possible extension of this work would be to know the interaction of *CUC1* and *CUC2* genes with other carpel genes have similar functions like *SPT*. By making double and triple mutant with the combination of *cuc* will uncover the genetic network involved in carpel fusion of the gynoecium.

Further analysis between *CUC1*, *CUC2* and *SPT* will reveal the molecular mechanism of regulatory network responsible for the patterning of gynoecium. As we know, fruits are plant parts of major economic and agricultural interest. The basis for fruit development is established during the formation of the carpel (Roeder and Yanofsky 2005, Sorefan et al. 2009). The present study will extent the understanding that how genetic networks have been modified through evolution to generate diversity of fruit forms found in nature.

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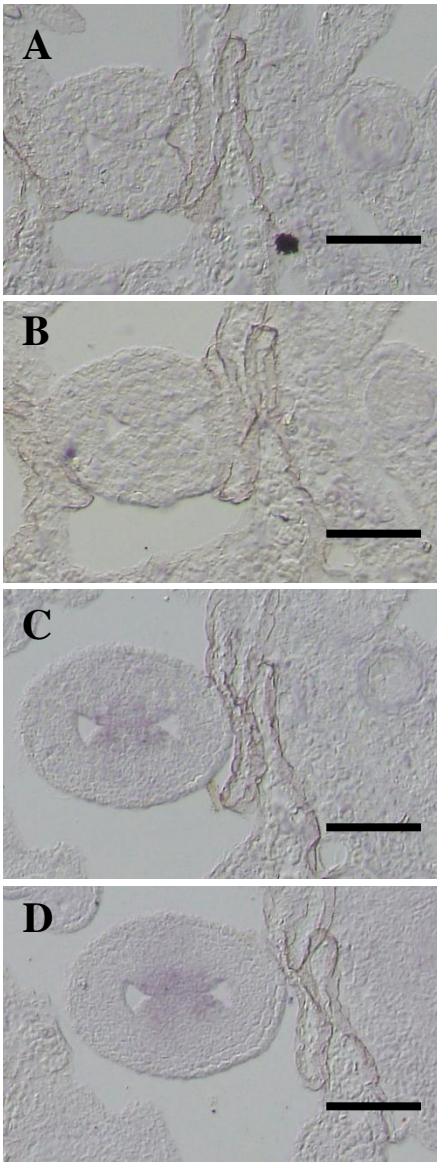

Supplementary Fig. S1 Expression of *CUC1* at the top of the wild type gynoecium

Serial sections were prepared by sectioning from top to the base of the stage 8 gynoecium; the first and second sections, as shown in (A, B) contain no expression. Subsequent sections (C, D) show expression in adaxial medial ridges. The original photograph of Fig S1A is the same as that of Fig 8A. Bars = 50 μm.