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"A genetic and Hormonal Framework for Early Gynoecium Development of *Arabidopsis*"

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1. RESUMEN

La flor es la estructura reproductiva en las angiospermas, cuyo origen y evolución ha contribuido al gran éxito evolutivo de estas. La parte reproductiva femenina de la flor se llama gineceo y comprende al carpelo que es responsable de la generación y protección de los óvulos. El gineceo de *Arabidopsis thaliana* es una compleja estructura compuesta de dos carpelos fusionados congénitamente, que tiene su origen en el centro de la flor. Durante el desarrollo del gineceo de *A. thaliana* existen dos principales eventos: el establecimiento de los ejes de desarrollo y la formación de la región Media. En la región media se encuentra una zona meristemática llamada Meristemo del Margen del Carpelo (CMM en inglés), que es la responsable del desarrollo de los tejidos internos tales como óvulos, septum y el tracto de transmisión (TT). A pesar de la importancia del CMM en el desarrollo del carpelo, poco se sabe sobre los mecanismos moleculares que causan su desarrollo y mantenimiento. Varios genes están involucrados en el establecimiento de los ejes de desarrollo y la formación de la región Media. Las fitohormonas auxinas y citocininas desempeñan un papel central en los diferentes procesos de desarrollo de las plantas. Sin embargo a pesar de la importancia de la interacción de las auxinas y citocininas en el desarrollo y la gran cantidad de información genética de genes involucrados en la fusión Postgenital (PG), aún se desconoce cómo interactúan las fitohormonas y los genes en la formación de la región Media. Aquí presento un modelo para el desarrollo temprano del gineceo que une la distribución auxinas y citocininas con la regulación genética durante el establecimiento de los ejes de desarrollo y la formación de la región Media. En nuestro modelo, la presencia de la *SPATULA* (*SPT*) y los factores de trascripción ARR tipo B son esenciales para el establecimiento de un máximo de señalización de citocinina en el CMM, proporcionando de ese modo la actividad meristemática necesaria para el crecimiento y desarrollo del gineceo. Por otra parte, las citocininas promueven, a través de ARR tipo B y SPT, la biosíntesis de auxina mediante la activación de la expresión de *TAA1*. A continuación, la auxina sintetizada se transporta lejos del centro del gineceo por la expresión del transportador de eflujo de auxinas PIN-FORMED 3 (PIN3) para mantener un mínimo de auxina en el CMM. Mientras tanto, la señalización de citocinina se limita a la CMM por la expresión de AHP6, ARR16 y ETT en el domino lateral.

1 ABSTRACT

The flower is the reproductive structure in angiosperms and its origin has contributed to the evolution and the great success of the angiosperms. The female reproductive part of the flower is called gynoecium, which comprises the carpel that is responsible for ovule generation and protection. In *Arabidopsis*, the gynoecium is a complex structure composed of two congenitally fused carpels that arise from a single primordium at the center of the flower. Two key events during gynoecium development, and crucial for reproductive competence, are the establishment of axes and the medial region. In the medial region, there is a meristematic zone called the Carpel Margin Meristem (CMM), which produces the internal tissues, such as the ovules, septum and transmitting tract. Several genes are involved in the establishment of the axes and the medial region in *Arabidopsis* gynoecium development, however, the roles of auxin and cytokinin in this process are largely unknown. Here, I present a molecular framework model for early gynoecium development, linking auxin-cytokinin distribution patterns and the gene regulation network during the establishment of axes and the medial region of the gynoecium. In the model, the presence of *SPATULA* (*SPT*) and the cytokinin type-B ARR transcription factors are essential to establish a cytokinin signaling output maximum at the CMM, thereby providing it with the meristematic activity necessary for growth. Moreover, cytokinin promote, through type-B ARRs and SPT, auxin biosynthesis by activating the expression of *TAA1.* Next the synthesized auxin is transported away from the gynoecium center by the expression of the auxin efflux transporter PIN-FORMED 3 (PIN3) at the CMM to maintain an auxin minimum in this region. Meanwhile, intense cytokinin signaling is restricted to the CMM by AHP6, ARR16, and ETT, absent from the medial domain (CMM), but present at the lateral (valve) domain of the developing gynoecium.

2 BACKGROUND

2.1 FLOWER DEVELOPMENT

The flower is the reproductive structure in angiosperms and its origin has contributed to the evolution and the great success of the angiosperms (Zahn *et al.*, 2005).

The origin and evolution of the flower has been considered as a major mystery in botany (Darwin, 1879). The major reasons for this are a rapid rise and diversification of the flower, the limited number of fossil records and no obvious series of morphological intermediates (Theissen and Melzer, 2007; Pennisi, 2009).

Flowers generally have four distinct organs which arise in concentric rings, called whorls, attached to the tip of a short stalk called gynophore (Fig. 1). From the outside to the center of the flower: The first whorl consists of sepals which enclose and protect the rest of the flower. The second whorl is composed of petals which attract insects and animals that help the process of pollination. The third whorl is the androecium, the male structure of the flower and consists of stamens. Finally, the fourth whorl is the gynoecium which is the female structure of the flower and consists of one or more carpels (Krizek and Fletcher, 2005).

Flowers arise from a meristem called the floral meristem. The floral meristem is a population of actively dividing cells, which differentiate to give to all floral organs. In response to floral inductive cues the floral meristem passes through a series of transformations into an individual flower. This process occurs through a series of steps: The Inflorescence Meristem induction (IM), Flower Meristem induction (FM), whorls primordial induction and whorls differentiation (Zik and Irish, 2003; Krizek and Fletcher, 2005).

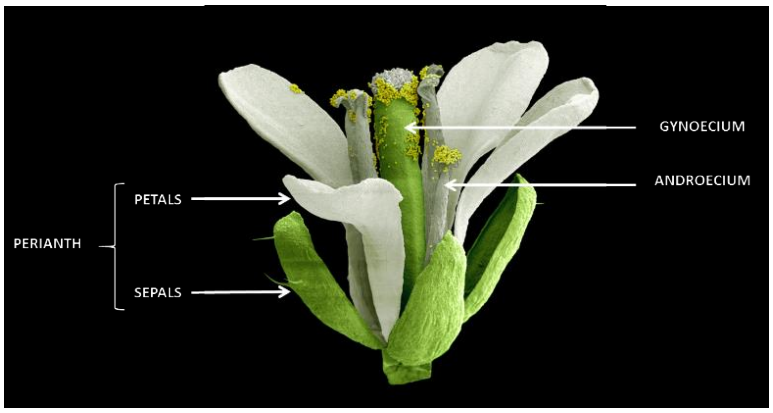


Figure 1. *Arabidopsis thaliana* flower. The flower of *Arabidopsis thaliana* consists of four whorls of organs: Sepals, Petals, Androecium (male reproductive organs; anthers) and Gynoecium (female reproductive organs; carpels). The carpels are fused and form the ovary, which harbours the ovules.

2.1.1 Inflorescence Meristem induction

The appropriate timing of flowering is crucial for reproductive success of the plants and for this reason the plants have precise mechanisms to respond to environmental cues such as day length (photoperiod), temperatures (vernalization) and humidity (Andres and Coupland, 2012).

The floral meristem arises from the Shoot Apical Meristem (SAM). The SAM is the meristem that has pluripotent cells that generate all aerial vegetative organs such as leaves and branches (Krizek and Fletcher, 2005). In *Arabidopsis* the SAM is made up of three distinct cell layers with the stem cells residing in the center of the meristem in an area called central zone (Barton, 2010). The stem cells in the SAM are maintained by a signaling pathway involving the homeodomain protein WUSCHEL (WUS) and the CLAVATA (CLV) ligand-receptor system. The expansion of WUS expression is prevented by the CLV signaling pathway, in which the CLV3 peptide is transcriptionally induced by WUS in the stem cells (Ito and Bo, 2015). After a period of vegetative growth and in response to environmental cues the SAM becomes an Inflorescence Meristem (IM). Therefore the control of the switch from vegetative to reproductive development is crucial for plant reproductive success. In *Arabidopsis* different genes are involved in floral transition such as *GIGANTEA* (*GI*), *FLAVIN KELCH F BOX 1* (*FKF1*), *CONSTANS* (*CO*), *FLOWERING LOCUS T* (*FT*), *FLOWERING LOCUS M* (*FLM*) and *PHYTOCROME INTERACTING FACTOR 4* (*PIF4*), among others (Andres and Coupland, 2012; Pose *et al.*, 2013).

2.1.2 Flower Meristem induction

Once IM is initiated, it is necessary to ensure that IM adopts a flower fate. The transition of IM to FM is strongly regulated by the floral meristem identity genes, for example, in *Arabidopsis LEAFY* (*LFY*) and *APETALA1* (*AP1*) carry out this process. Mutations in these genes result in a partial conversion of flowers into shoot-like structures whereas ectopic expression of *LFY* and/or *AP1* causes a rapid transformation of the IM to FM. Interestingly, the ectopic expression of *LFY* can cause a conversion to a floral meristematic fate in a variety of species, suggesting a conserved function in this process (Zik and Irish, 2003; Krizek and Fletcher, 2005; Alvarez-Buylla *et al.*, 2010).

Another important process in the FM induction is the loss of pluripotent capacity by production a finite set of organs. In *Arabidopsis* the floral stem cell activity is regulated for two major pathways: The CLV-WUS pathway and the AGAMOUS (AG)-WUS pathway. The CLV-WUS pathway regulates floral stem cells mainly in early floral stages (stage 1–6) through spatial restriction of WUS. The AG-WUS pathway is involved in the determination of meristems at floral stage 6 through temporal repression of *WUS* (Zik and Irish, 2003; Alvarez-Buylla *et al.*, 2010; Ito and Bo, 2015).

2.1.3 Whorl primordia induction

A flower starts its development as a group of undifferentiated cells (the floral meristem) which arises on the flank of the inflorescence meristem (Gomez-Mena *et al.*, 2005; Alvarez-Buylla *et al.*, 2010). The correct specification of the floral organs depends on the combinatorial action of the well conserved genes called ABC. The ABC model proposes that three regulatory gene classes (A, B, C) work in a combinatorial fashion to confer floral identity (Honma and Goto, 2001; Zik and Irish, 2003; Krizek and Fletcher, 2005). Thereby, A-function genes specify the development of the sepals, A and B the petals, B and C the stamens, and C alone the carpels (Bailey and Meyerowitz, 1991). In *Arabidopsis*, *APETALA1* (*AP1*) and *APETALA2* (*AP2*) are class A genes, *PISTILLATA* (*PI*) and *APETALA3* (*AP3*) are class B genes, and *AGAMOUS* (*AG*) is class C gene. Moreover, the model also predicts a negative regulation between the A and C domains, such that the expression of C suppresses the A effects. Finally, the model extends to new class E

genes. In *Arabidopsis* the E genes encode four redundant proteins SEP1, 2, 3, 4 that are required to specify petals, stamens and carpels (Fig. 2) (Bailey and Meyerowitz, 1991; Honma and Goto, 2001; Krizek and Fletcher, 2005; Zahn *et al.*, 2005).

2.1.4 Whorl differentiation

Once that the expression of the A, B, C, E genes occurs, the FM begins to undergo a series of changes that conclude in the formation of the floral organs. In the FM, the floral organs are initiated in centripetal sequence; that is, the first to develop are the outermost organs such as sepals, and last to develop are the innermost organs such as carpels. During this period the A, B, C genes activate genes necessary for each floral organ identity. Among the targets of the ABC genes are diverse phytohormone-related genes and transcription factors involved in differentiation (Alvarez-Buylla *et al.*, 2010). In *Arabidopsis* the process of formation from flower to fruit is divided into 20 stages using a series of landmark events (Smyth *et al.*, 1990; Alvarez-Buylla *et al.*, 2010).

2.2 GYNOECIUM EVOLUTION AND DEVELOPMENT

The female reproductive part of the flower is called gynoecium, which is a highly complex organ with a great diversity of forms (Igersheim and Endress, 1997; Staedler *et al.*, 2009). The gynoecium is composed mainly of carpel(s) which are specialized structures responsible for seed production, protection and dispersal (Endress and Igersheim, 2000). The carpel confers a number of selective advantages to the angiosperm evolution such as physical protection of the ovules and an efficient collective and selective pollen system (Lorts *et al.*, 2008; Williams, 2009). The vast diversity of size, shape of gynoecium has been mainly associated with pollination and seed dispersion (Endress and Igersheim, 2000; Lorts *et al.*, 2008).

2.2.1 Gynoecium evolution

The angiospermy is the key innovation in the angiosperms, which means that the ovules are completely enclosed in a gynoecium. However, the way in which the gynoecium was originated is unknown today. Various hypotheses about the origin and evolution of gynoecium have been proposed but the leaf-like structure origin is the most accepted (Igersheim and Endress, 1997; Endress and Igersheim, 2000).

It has been long discussed whether the primitive carpels in angiosperms are plicate or ascidiate (Fig. 2) (Endress and Igersheim, 2000). The Plicate carpel: Folding of the carpels with ovules inside resembles a folded leaf. The Ascidiate carpel: invagination of carpel to form a tubular or pitcher-like hollow structure with the ovules inside (Endress and Igersheim, 2000; Endress and Doyle, 2009). Based on morphological studies in the living basal-most angiosperms, the ANITA clade (Amborellaceae, Nymphaeales, and Austrobaileyales), is generally assumed that ascidiate character was the first carpel and plicate carpel is a derivate character that probably evolved from progressive elongation of the asymmetric mouth of the sac-like primitive carpel (Fig. 2a) (Endress and Igersheim, 2000; Endress and Doyle, 2009; Hawkins and Liu, 2014). However, it is important to take into account that ANITA clade flowers form a true gynoecium, so is probable that ascidiate carpel also evolved from plicate carpel through fusions along of the margin (Fig. 2b).

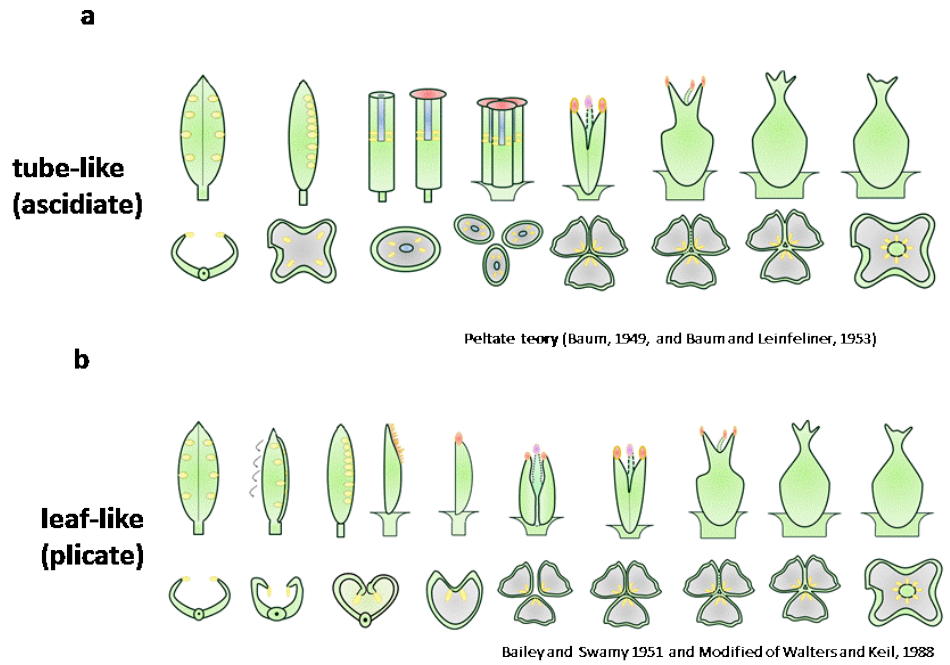


Figure 2. Gynoecium evolution. Two theories as to about how a gynoecium has evolved have been proposed: **a**, Tube-like (ascidiate) carpel theory and **b**, leaf-like (plicate), resembling leaves that have folded with the opposite edges sealed together.

A key event in carpel evolution is the carpel closure (CC), whose origin and early evolution is closely tied to its role in inner surface of carpel, stigma, transmitting tract (TT) and style (Staldal *et al.*, 2008; Soltis *et al.*, 2009; Williams, 2009). The CC comes when epidermal cells of surface begin to fuse and re-differentiate into parenchyma (Endress and Igersheim, 2000; Ferrándiz *et al.*, 2010). In some angiosperms the gynoecium undergoes two fusion events, the congenital (CG) and postgenital (PG) fusion. The first occurs early in development at primordia emergence, whereas the second occurs late in development by a process of fusion of inner margins (Bowman *et al.*, 1999; Bowman and Smyth, 1999; Ferrándiz *et al.*, 2010). In most dicotyledons both CG and PG carpel fusion can co-occur during development; however, in the basal groups of angiosperms frequently there is only one (Endress and Igersheim, 2000).

2.2.2 Medial tissue evolution

Medial tissue development is the major innovation in the gynoecium evolution, and was essential for angiosperm success. The origin of medial tissues led to the novelty of inner surface

tissues of carpel such as placenta, septum and transmitting tract (TT) (Endress and Igersheim, 2000; Williams, 2009).

The carpel of basal-most angiosperms the ANITA clade (Amborellaceae, Nymphaeales and Austrobaileyales) tends to be ascidiate carpels (pitcher-like hollow) in which the medial tissues are only in a small region encompassing the placenta and ovules. In the ANITA group there are diverse placentation patterns: (1) linear placentae, where the ovules are arranged in a line on each side of the carpel; (2) diffuse placentae, laminar-diffuse, with the ovules dispersed over a broad region of the carpel flanks; or they may be protruding-diffuse, with the ovules dispersed over a protruding part of parietal or axile placentation.

In the magnoliids group, the carpels are completely plicate (leaf-like former), but in some groups are occasionally partially ascidiate, notably in the basal species. In many species of magnoliids the placenta is inserted close to their plicate carpel zone. Where postgenital fusion occurs, in the inner surface a canal filled with secretion is formed, which serves as a pollen tube transmitting tract (Igersheim and Endress, 1997; Staedler *et al.*, 2009). In Lauraceae the gynoecium consists of a single carpel; therefore, there is no septum (Endress and Igersheim, 1997).

An important specialization of medial tissues is the septum. The origin and evolution of the septum is complex since it has evolved many times in numerous plant groups, for example at least 4 times in Monocots and core Eudicots. A fundamental process for septum evolution is the synorganization (the fusion of two or more organs to form a functional unit). In syncarpous gynoecium the carpels are fused laterally and separated from each other by the septum, in many species the placenta is inserted at the septum (Endress, 2011).

2.3 *Arabidopsis* GYNOECIUM DEVELOPMENT

The *Arabidopsis* gynoecium is a complex syncarpic structure composed of two CG fused carpels which form a solid cylinder. The gynoecium continues to grow until it starts to close by PG fusion of the two medial ridges to form the septum (Roeder and Yanofsky, 2006; Colombo *et al.*, 2010). When mature, it consists of an apical stigma, a short style, an ovary and basal gynophore (Fig. 3). During gynoecium development, the organ identity is established by the three axes: the apical–basal, the adaxial–abaxial and the medial–lateral axes (Fig. 3). Along its medial/lateral axe the mature gynoecium can be divided into three main regions: the replum and septum in the middle and the valves at the edges (Bowman *et al.*, 1999; Colombo *et al.*, 2010).

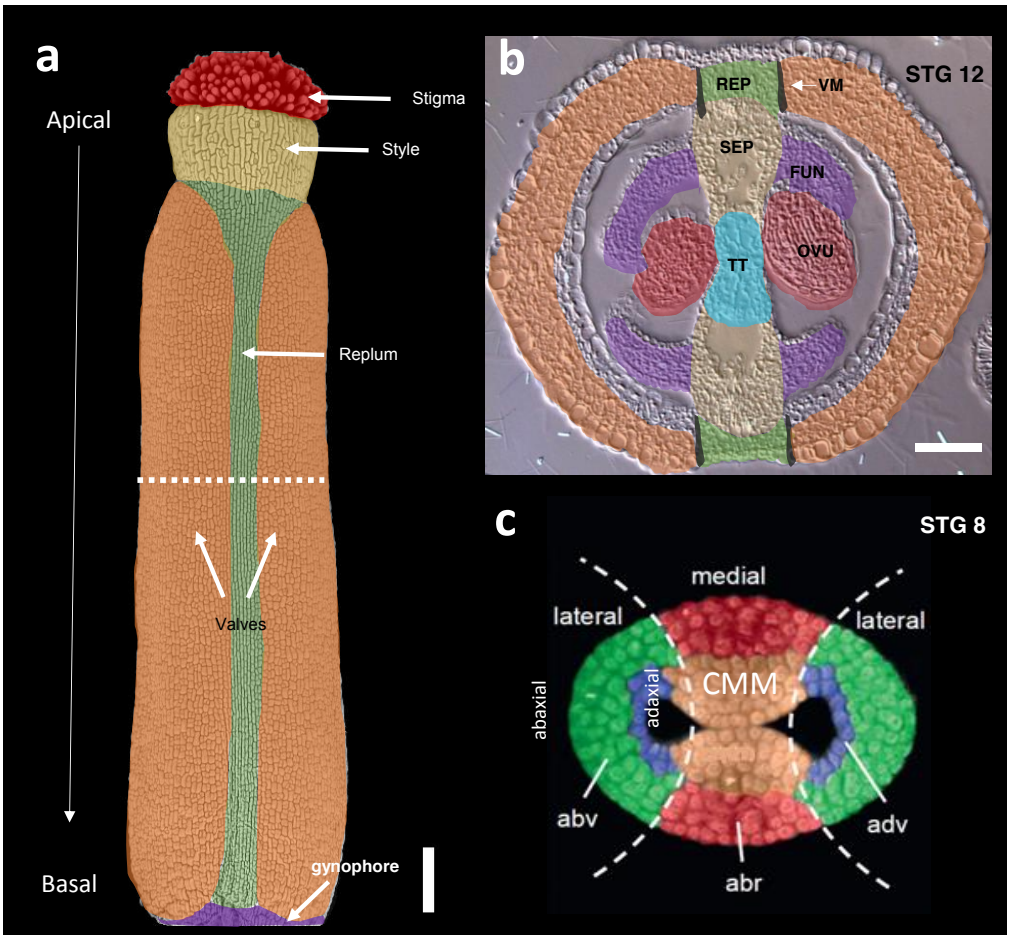


Figure 3. Overview of *Arabidopsis thaliana* gynoecium development. a, False-coloured longitudinal scanning electron microscope image of a stage 12 gynoecium. False-coloured transverse ovary section of a stage 12 (**b**) and a stage 8 (**c**) gynoecium. CMM, carpel margin meristem; SEP, Septum; OVU, Ovule; F, Funiculus; TT, Transmitting tract; Va, Valve; VM, Valve

margin; REP, Replum; abv, Abaxial valve; adv, Adaxial valve, abr Abaxial replum. Scale bars = 100 µM (a, c), 10 µM (b). Modified from (Nole-Wilson et al., 2010; Reyes-Olalde et al., 2015).

Flower and fruit development is divided into 20 stages using a series of landmark events (Smyth *et al.*, 1990). However, in this project I will describe only the developmental stages 5 to 12, because in these stages the gynoecium completes its development. In stage 5, stamen and petal precursors appear, later gynoecium primordia will arise (Fig. 4a). In stage 6, the sepals completely cover the bud, and at this time the gynoecium arises as ridge cells at the center of the floorwalker meristem (Fig. 4b). During stage 7 the gynoecium is a hollow tube that begins to grow vertically, while the Carpel Margin Meristem (CMM) is clearly visible (Fig. 4c). The beginning of stage 8 is defined as the moment in which anther locules are visible, while the gynoecium continues to grow in width and length and the ovules primordia develop on both sides of the septum primordia (Fig. 4d). In stage 9 all organs have a rapid lengthening, especially the tongue-shaped petals. Then the gynoecium continues to elongate and the apex starts to close, the first rounded stigmatic papillae cells appear at the top of the gynoecium. The valve, placenta and septum primordia cells begin to differentiate and PG fusion occurs in septum cells margin (Fig. 4e). Stage 10 begins when the fast-growing petals reach the top of the short stamens, furthermore the gynoecium septa continues to grow out from the middle of the medial ridge and the cells start to differentiate as TT cells (Fig. 4f). Finally, at stages 11 and 12 the apical part of the gynoecium is completely closed, the stigmatic papillae cells and the septum are completely developed and the valves and valves margins begin to differentiate and expand laterally to become clearly distinct from the narrow apical style (Fig. 4g, h) (Roeder and Yanofsky, 2006; Ferrándiz *et al.*, 2010; Reyes-Olalde *et al.*, 2013).

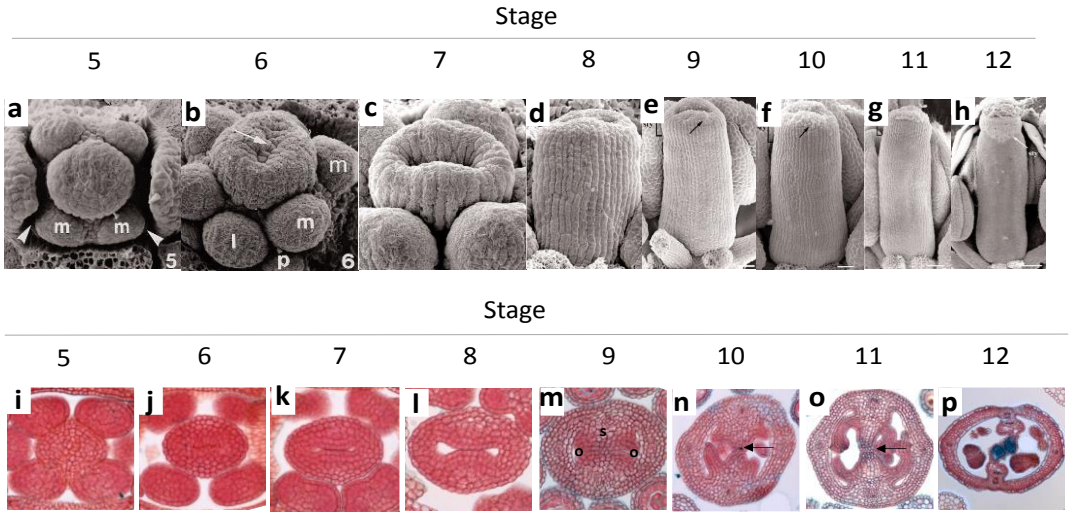


Figure 4: Gynoecium development of wild type *Arabidopsis*. Stages 5-12 (**a-p**). (a) SEM of a late stage 5 floral meristem. Arrowheads point to the petal primordia and two of the medial stamens are labelled m. (i) Transverse section. (b, j) stage 6, (b) SEM showing the beginning of formation of the gynoecium (arrow), (j) Transverse section. (c, k) Stage 7, (c) SEM shows the 6 vertical growth of the gynoecium, (k) Transverse section of a late-stage 7. (d, l) Stage 8, d) SEM of a stage 8 gynoecium, (l) Transverse section of a late-stage 8 gynoecium. (e, m) stage 9, (e) SEM show the style region (bracketed) and a few of the stigmatic papillae become visible, (m) Cross section show ovule primordia (o) arise from the placentas and in the center the septum (s). (f, n) stage 10, F) SEM show more stigmatic papillae (arrow), which has closed over, (n) Cross section show smaller darkly staining cells TT precursors (arrow). (g, o) stage 11, (g) SEM, the stigma show a carpel covered with many papillae, (o) Cross-section show the septum (arrow) with small darkly staining cells that will form the TT. (h, p) stage 12, (h) SEM of a mid-stage 12 gynoecium where the stylar epidermal cells are clearly distinct, (p) Cross-section show the TT in the middle of the septum stains darkly. Modified from (Roeder and Yanofsky, 2006; Reyes-Olalde *et al.*, 2013).

2.4 GENES INVOLVED IN *Arabidopsis thaliana* GYNOECIUM DEVELOPMENT

In *Arabidopsis thaliana* several genes are involved in gynoecium development and organ identity (reviewed in Reyes-Olalde *et al.*, 2013), which according to gene expression patterns and their gynoecium development defects, can be classified into three different categories: the apical–basal, the adaxial–abaxial and the medial–lateral genes. However, the understanding of how these genes determine the formation of the three axes is incomplete today, since many genes that are expressed in one specific axis affect the establishment of other axes.

Gynoecium organogenesis begins at stage 8-10, once the three axes are established. During gynoecium organogenesis the cells interact with each other to produce tissues and organs. These interactions create privileged sites called stem cell niches such as the CMM region. The CMM provides paracrine factors (auxin and cytokinin) that allow cells residing within them to remain relatively undifferentiated. Each stem cell that is adjacent to CMM divides to produce a daughter cell that divides a number of times, which acquire a specific differentiation status according to positional signals. At the medial region the CMM gives rise to the carpel marginal tissues, which include the placenta, ovules, septum, transmitting tract, style, and stigma (Reyes-Olalde *et al.*, 2013).

2.4.1 The apical–basal axis

The apical-basal axis can be divided into four domains: The apical part is the stigma consisting of a single layer of elongated cells called papillae, below is a solid cylinder called the style, then there is the ovary which is the more complex part of the gynoecium containing the ovules, and in the basal part the gynophore is located, which is a short stalk-like structure connecting the gynoecium with the rest of the plant (Balanza *et al.*, 2006; Roeder and Yanofsky, 2006; Zuñiga-Mayo *et al.*, 2014). Different *Arabidopsis* mutants have been reported showing apical–basal defects such as valves defects (Sohlberg *et al.*, 2006; Zuñiga-Mayo *et al.*, 2014) and Style-Stigma defects (Staldal *et al.*, 2008).

The analyses of apical–basal mutants indicate that the phytohormone auxin plays an important role in the determination of the apical-basal axis (Nemhauser *et al.*, 2000; Staldal *et al.*, 2008; Hawkins and Liu, 2014), since many auxin synthesis or response mutants show alterations in the length of the ovary, style and gynophore regions. For example the auxin biosynthesis double *yuc1yuc4* mutant, likewise, double mutant of *TAA1/TAR* family genes exhibit a complete loss of valves, named “valveless” phenotype (Cheng *et al.*, 2006; Stepanova *et al.*, 2008; Stepanova *et al.*, 2011; Zuñiga-Mayo *et al.*, 2014). The auxin efflux transport PIN-FORMED (PIN) family, the PINOID (PID), and the AGC3-type protein kinase are all implicated in auxin transport, and corresponding mutants exhibit a complete loss of valves (Okada *et al.*, 1991; Benkova *et al.*, 2003; Friml *et al.*, 2004; Huang *et al.*, 2010).

Other important molecules implicated in apical–basal axis determination are transcription factors (TF) such as *STYLISH1* and *2* (*STY1/2*) and members of the *NGATHA* (*NGA*) family. The *STY1/2* and *NGA* TFs are implicated in the regulation of auxin biosynthesis through the direct activation of *YUCCA* (*YUC*) genes (Cheng *et al.*, 2006; Sohlberg *et al.*, 2006). The *sty* and *nga* mutations cause severe defects in the establishment of the apical-basal axis such as style-stigma and valve reduction defects. Another important TF is *ETTIN* (*ETT*) that encodes the *Auxin Response Factor 3* (*ARF3*), which has an important role in auxin signaling. The ARFs are a family of transcription factors that bind with specificity to auxin response elements (AuxREs) in promoters of primary or early auxin-responsive genes (Roeder and Yanofsky, 2006). In the *ett* mutant, the gynoecium morphology shows defects in the apical-basal axis; the ovary is reduced in size whereas the gynophore, stigma and style are increased (Sessions *et al.*, 1997; Nemhauser *et al.*, 2000).

Finally, we recently reported that exogenous application of cytokinin causes severe defects in apical-basal patterning and this effect was enhanced in auxin signaling and transport mutants (Zuñiga-Mayo *et al.*, 2014). These data suggest that cytokinin and auxin act together to specify the apical-basal patterning.

2.4.2 The adaxial–abaxial axis

Lateral organs of seed plants, such as leaves and flowers are polarized along their adaxial-abaxial axis. Adaxial and abaxial tissues have characteristic anatomical and histological distinctions, including vascular polarity, cell differentiations such as trichomes or mesophyll cells and distinct patterns of expression of genes (Kerstetter *et al.*, 2001; McConnell *et al.*, 2001; Fukuda, 2004). In leaves the adaxial side is close to the shoot apical meristem (SAM), and the abaxial side is far from the SAM (Siegfried *et al.*, 1999; Kerstetter *et al.*, 2001).

The *Arabidopsis* gynoecium is a solid cylinder with reproductive organs (ovules) at the adaxial side (inside). According to anatomical and histological features the adaxial–abaxial gynoecium axis can be divided into: abaxial valve (abv.), adaxial valve (adv.), abaxial replum (abr.), and adaxial replum (adr) (Fig. 5). The abv encompassed outer epidermal layer of the valves (exocarp) and mesocarp, adaxial valve encompassed inner epidermis the endocarp (enc.) and second

endocarp layer, enb. The abr of the medial domain of the gynoecium differentiates into the replum. Finally, the adr encompasses the CMM that gives rise to the reproductive organs such as ovules (Fig. 5) (Roeder and Yanofsky, 2006; Alvarez-Buylla *et al.*, 2010; Reyes-Olalde *et al.*, 2013) .

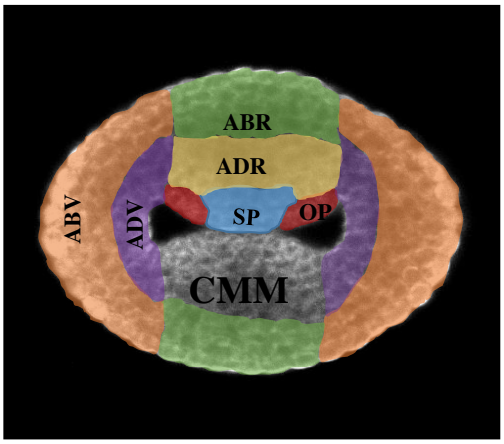


Figure 5. Schematic representation and false-coloured of a gynoecium at stage 8. Transverse sections of gynoecia at stage 8, the different domains are indicated in false-coloured. Abbreviations: CMM: Carpel Margin Meristem; ABR: abaxial replum; ADR: adaxial replum; SP: septum primordia; OP: ovule primordia; ADV: adaxial valve; ABV: abaxial valve. Modified from (Reyes-Olalde *et al.*, 2013).

Several transcription factors and microRNAs are involved in adaxial–abaxial gynoecium axis determination. Interestingly, many of these genes are also involved in adaxial–abaxial determination in leaves, denoting a common origin. Here, I describe the function of some adaxial and abaxial identity genes.

Adaxial-identity genes

The mutation of three HD-ZIP III transcription factors, *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*) and *REVOLUTA* (*REV*) causes the transformation of abaxial to adaxial fates in leaves (McConnell *et al.*, 2001). However, in the gynoecium the effects of single or triple mutants has been studied very little, because the single mutants do not show a noticeable gynoecium defect phenotype and *phb phv rev* triple mutant plants do not produce flowers, because of seedling lethality caused by the fusion of cotyledons that block the formation of the shoot apical meristem (SAM) (McConnell *et al.*, 2001; Prigge *et al.*, 2005; Roeder and Yanofsky, 2006).

In the gynoecium *REV* is expressed in adaxial portions at adv and ovule primordia regions at stage 7-9 (Otsuga *et al.*, 2001; Azhakanandam *et al.*, 2008). The *rev-9* single mutant occasionally displays short apical-basal defects characterized by reduced valve length, a phenotype that is enhancement in the *ant rev* double mutant (Nole-Wilson *et al.*, 2010).

Another important gene in Adaxial-identity is *PHB,* which is expressed in the internal regions (adaxial) where the adaxial valve (adv) and ovule primordia arise at stages 7–9 (Azhakanandam *et al.*, 2008). In the gynoecium, the PHB gain-of-function mutant causes that ovules are produced on the outside of the base of the gynoecium (McConnell *et al.*, 2001).

Abaxial-identity genes

The YABBY gene family consists of transcription factors that are expressed in the abaxial side of lateral organs and have been linked to Abaxial-identity. The YABBY gene family is composed of six members, however, only *FILAMENTOUS FLOWER (FIL), CRABS CLAW (CRC)* and *FIL's* closest homologue *YABBY3 (YAB3)* are expressed strongly in the gynoecium during floral stages 6-11 (Bowman and Smyth, 1999; Eshed *et al.*, 1999; Siegfried *et al.*, 1999; Dinneny *et al.*, 2005; Sarojam *et al.*, 2010). *FIL* and *YAB3* expression is confined to abaxial portions of the valve (Siegfried *et al.*, 1999; Nole-Wilson *et al.*, 2010). *CRC* is expressed at stage 7 and 8 in the abaxial epidermis of the gynoecium in medial and lateral domains (Bowman and Smyth, 1999). The mutations of these genes cause severe loss of abaxial-identity (Eshed *et al.*, 1999; Siegfried *et al.*, 1999).

The *KANADI* genes are transcriptions factors that belong to the GARP family and are expressed on the abaxial side of all lateral organs. The *KAN* genes originally were identified in a screening that enhanced the floral phenotype of *crabs claw* (*crc*). The *Arabidopsis* genome encodes two *KAN* related sequences. In the gynoecium, *KAN1* is expressed strongly during floral stages 6-7 in abaxial epidermis and in septum primordia (Eshed *et al.*, 2001; Kerstetter *et al.*, 2001). The *kan* mutant has gynoecium defects characterized by proliferation of replum tissue, production of ectopic ovules and ectopic formation of style, or stigmatic tissue (Eshed *et al.*, 1999; Kerstetter *et al.*, 2001).

Another important gene involved in Abaxial-identity is *ETT*. *ETT* shows an abaxial expression pattern in the gynoecium similar to *CRC* (Sessions *et al.*, 1997). In the *ett* mutant, the gynoecium morphology shows defects in the apical- basal axis characterized by a reduction in ovary size and increase of the gynophore, stigma and style (Sessions *et al.*, 1997; Nemhauser *et al.*, 2000). Interestingly, the *ett arf4* double mutant phenotypes are strikingly similar to those of *kan1 kan2,* suggesting similar functions in gynoecium abaxial-identity (Pekker *et al.*, 2005).

Finally, microRNAs are important post-transcriptional gene regulators in animals and plants. In the gynoecium, microRNAs are implicated in the regulation of different transcription factors (Sieber *et al.*, 2007; José Ripoll *et al.*, 2015). In adaxial–abaxial polarity the *miR166/165* are important, which are involved in the regulation of the HD-ZIP III transcription factors, PHABULOSA (PHB), REVOLUTA (REV), and PHAVOLUTA (PHV). The *miR166/165* genes are expressed in the abaxial side of the leaf (McConnell *et al.*, 2001; Rhoades *et al.*, 2002; Kidner and Martienssen, 2004). In the gynoecium, *miR166/165* genes are expressed in different organs such as ovules and stigma (Jung and Park, 2007).

2.4.3 The medial–lateral axis

The medial-lateral axis can be divided into the medial region and the lateral region. The medial region encompasses the CMM and all its derivatives. Studies of different *Arabidopsis* mutants affected in derived tissues from CMM have contributed to the identification of several genes involved in the CMM determination (Reyes-Olalde *et al.*, 2013). Here, I describe the function and expression of several genes such as *CRABS CLAW* (*CRC*)*, SPATULA* (*SPT*)*, ALCATRAZ* (*ALC*), *INDEHISCENT* (*IND*)*, HECATE* (*HEC*), *CUP-SHAPED COTYLEDON* (*CUC*)*, SHOOT MERISTEMLESS, SHOOTLESS* (*STM*)*, ETTIN* (*ETT*)*, MONOPTEROS* (*MP*)*, Arabidopsis Response Regulator* (*ARR*) *type-B* and *type-A* and *PIN-FORMED* (*PIN*). Interestingly, many of these genes mediate auxin-cytokinin related processes (Staldal *et al.*, 2008; Reyes-Olalde *et al.*, 2013; Moubayidin and Ostergaard, 2014; Marsch-Martinez and de Folter, 2016).

Medial-identity genes

CRC encodes a transcription factor belonging to the YABBY protein family and plays an important role in carpel and nectary development (Alvarez and Smyth, 1999; Bowman and

Smyth, 1999). The members of YABBY family are involved in specifying abaxial cell fate in plant lateral organs such as leaves. *CRC* expression is mostly limited to nectary and abaxial epidermis of carpels and controls the development of these structures. The *crc* mutant has several alterations on carpel development; characterized by wider and shorter gynoecium, reduced amount of style tissue and loss of PG fusion at the apex (Bowman *et al.*, 1999; Bowman and Smyth, 1999; Alvarez and Smyth, 2002). In addition, *CRC* shows a highly conserved carpel expression pattern in *Arabidopsis* and basal angiosperms suggesting that *CRC* plays an ancestral role in carpel development (Fourquin *et al.*, 2007). Another important data is that overexpression of *STY1*, an auxin biosynthesis regulator gene, can restore *crc-1* style developmental defects suggesting that *CRC* may act downstream or parallel with the auxin pathway (Staldal *et al.*, 2008).

Another important gene involved in the CMM development is *SPT,* encoding a transcription factor belonging to the bHLH (basic Helix-Loop-Helix) protein family (Heisler *et al.*, 2001). Members of the bHLH protein family are involved in the regulation of a wide variety of developmental processes, for example *ALCATRAZ (ALC)* and *INDEHISCENT (IND)* are implicated in valve margin development (Sorefan *et al.*, 2009; Girin *et al.*, 2011). In the gynoecium, *SPT* is expressed in the medial domain in a region that corresponds to the CMM at stages 6–7. This expression is limited to the internal regions (adaxial) where the septum arises at stages 8–9 and finally in stages 10-12 the expression is confined to the transmitting tract and ovule primordia, but decreases in these regions at stage 13. The *spt* mutant shows severe alteration in gynoecium development characterizes by a reduction in stigmatic tissues, severe disruption of the septum and TT and the lack of fusion of the two carpels at the top (Heisler *et al.*, 2001; Groszmann *et al.*, 2010). Moreover, *SPT* has been associated with other processes such as seed dormancy, light response and germination response to temperature (Penfield *et al.*, 2005; Josse *et al.*, 2011; Reymond *et al.*, 2012; Vaistij *et al.*, 2013). Finally, it has been demonstrated that SPT physical interaction with several transcriptions factors that are involved in gynoecium development such as HECATE (HEC), IND, ALC and with the intracellular repressors of GA responses DELLA proteins (Girin *et al.*, 2011; Groszmann *et al.*, 2011; Josse *et al.*, 2011).

The *ALCATRAZ (ALC)* gene is the closest homolog to *SPT* and is involved in early gynoecium and fruit development. The *ALC* gene is required for both the lignified and separation layers of the dehiscence zone and septum development (Rajani and Sundaresan, 2001; Groszmann *et al.*, 2011). In the gynoecium, *ALC* is expressed in the CMM at stage 8, at the inner (adaxial) sides that correspond to septum primordia and in the outer (abaxial) medial domain where the later replum differentiates. At stages 10-12, *ALC* expression is seen in the developing septum and becomes confined to the epidermis and valve margins (Liljegren *et al.*, 2004; Groszmann *et al.*, 2011). Finally, IND and SPT interact both genetically and through physical protein–protein contact (Groszmann *et al.*, 2011).

The *INDEHISCENT* (*IND*) gene encodes a bHLH transcription factor involved in the valve margin differentiation. In the gynoecium, *IND* is expressed in the medial region at stage 9 and in valve margins at stage 12 (Liljegren *et al.*, 2004; Girin *et al.*, 2011). It is assumed that *IND* is responsible for the establishment of an auxin minimum necessary for specification of the valve margin (Sorefan *et al.*, 2009). IND directly and positively regulates the expression of *SPT*, and IND and SPT also interact by physical protein–protein contact. Moreover, *SPT* and *IND* control auxin distribution through the repression of the protein kinase PID (Sorefan *et al.*, 2009; Girin *et al.*, 2011).

Other important bHLH members are the *HECATE (HEC)* genes. They are transcription factors with a highly redundant function. The *Arabidopsis* genome has three paralogous genes *HEC1, HEC2* and *HEC3.* In the gynoecium all three *HEC* genes are expressed in the stigma and septum during stages 8 to 12. The single *hec3* mutant displays a moderate phenotype characterized by smaller fruit and a modest reduction in fertility, whereas the combination of *hec1 hec3* double and *HEC2-RNAi hec1 hec3* leads to severe defects in septum and TT development, phenotypes similar as observed in the *spt* mutant (Gremski *et al.*, 2007). Moreover, HEC has physical protein–protein interaction with SPT, suggesting a teamwork to regulate septum and TT development (Gremski *et al.*, 2007).

The *CUC* genes are a group of transcription factors with a redundant function, which belong to the NAC protein family (Ishida *et al.*, 2000; Hibara *et al.*, 2006). They are expressed in organ boundaries, where they repress growth and differentiation to allow organ separation (Hibara *et*

al., 2006; Ferrándiz *et al.*, 2010). In the gynoecium, the *CUC* genes are expressed in the inner part of the presumptive septal and ovules regions before swelling of septal primordia, which occurs at stage 8 (Goncalves *et al.*, 2015). This expression continues until stage 11 where it also spreads to ovules (Ishida *et al.*, 2000; Takada *et al.*, 2001; Nahar *et al.*, 2012; Kamiuchi *et al.*, 2014). Single *cuc* mutants do not show any phenotype, whereas the combination of two *CUC* genes leads to defects in boundary formation such as fusion of the cotyledon margins (Takada *et al.*, 2001). The *cuc1 cuc2* double mutant shows severe defects in development and is unable to flower. However, *cuc1 cuc2* plants produced from calli are able to flower, and flowers derived from calli are defective in carpel development. The gynoecium of *cuc1 cuc2* plants exhibit defects in marginal tissue development and lacked fused septa (Ishida *et al.*, 2000). Little is known about the regulatory mechanism of *CUC* gene expression, but it has been suggested that a candidate for regulating of *CUC* expression is *STM*, a member of the KNOTTED 1 protein family (Ishida *et al.*, 2000; Spinelli *et al.*, 2011). On the other hand, *CUC1* and *CUC2* expression are required for correct *STM* expression in the CMM (Kamiuchi *et al.*, 2014), suggesting a positive feedback between *CUC* and *STM.* Recently, it has been reported that there is a regulatory relationship between CUC2 and PIN1 during leaf margin serrations, which stabilizes the position of auxin maxima (Bilsborough *et al.*, 2011). Finally, a genetic association of *SPT* with the *CUCs* has been reported (Nahar *et al.*, 2012), as it is necessary to have *CUC* expression to have *SPT* expression in the CMM.

Another key regulator of CMM development is *STM*, which regulates many developmental processes and is required for the establishment and maintenance of meristematic cells in the Shoot Apical Meristem (SAM). STM is a transcription factor belonging to KNOTTED homeobox class 1 (Long *et al.*, 1996). *STM* expression is mostly limited to vegetative, axillary, inflorescence and floral meristems. In carpels, *STM* is expressed in medial tissues at the early stages of gynoecium development (Long *et al.*, 1996; Scofield *et al.*, 2007). The *stm* mutant fails to develop a SAM during embryogenesis, which is why it is difficult to analyze the effects in flower and carpel development. To test the role of *STM* in flower development, Scofield *et al.*, (2007) used an inducible RNAi line. In some cases the RNAi plants showed reduced formation of placental tissues, loss of carpel fusion and in several cases complete loss of carpel development.

ETT is an ARF transcription factor and plays an important role in abaxial identity, however, this gene interacts with genes involved in the CMM development and has several defects in organs derived from the CMM (Sessions and Zambryski, 1995; Sessions *et al.*, 1997), making *ETT* an important candidate gene in the study of medial region and development of the CMM. In weak alleles of *ett* mutants, the gynoecium shows outgrowth in the medial plane with stigmatic charters (Sessions and Zambryski, 1995). Moreover, the patterning defects of *ett* gynoecia are almost completely restored when *SPT* is mutated; because *SPT* is ectopically expressed in *ett* gynoecia (Heisler *et al.*, 2001). Taken together, this data suggests that *SPT* is epistatic to *ETT* (Sessions *et al.*, 1997). Recently, it has been demonstrated that AG modulates *ETT* expression indirectly and promotes the repression of *WUS* expression (Liu *et al.*, 2014), suggesting a possible role of *ETT* in flower determination.

MONOPTEROS (MP) is another important ARF that has been implicated in apical-basal gynoecium specification and ovule development. In the gynoecium *MP* is expressed in the CMM region, mainly in the placenta primordia. In gynoecium, MP is an important regulator of ovule development through the direct activation of *CUC* genes, which are required for both correct *PIN1* expression and PIN1 localization (Galbiati *et al.*, 2013).

The Arabidopsis Response Regulator (ARR) type-B proteins are a family of 12 transcription factors that contain a Myb-like DNA binding domain called ARRM (type B). The ARR type-B transcription factors are functioning in a highly redundant manner, regulating the responses to cytokinins and participate in diverse developmental programs such as root elongation, lateral root formation, callus induction and the maintenance of meristem cells in the SAM (Hwang and Sheen, 2001; Mason *et al.*, 2005; Argyros *et al.*, 2008; Dello Ioio *et al.*, 2008). Due to the high level of functional redundancy between type-B ARR transcription factors the single loss-of-function mutants do not show strong phenotypic alterations (Mason *et al.*, 2005). However, the *arr1,10,12* triple mutant shows severe defects in the cytokinin signalling and displays poor growth (Argyros *et al.*, 2008; Choi *et al.*, 2014). On the other hand, the dominant repressor version *35S::ARR1-SRDX* displays reduction in flower and fruit size (Heyl *et al.*, 2008). Taken together these data suggest that type-B *ARR* genes could play an important role in CMM development.

Type-A Arabidopsis response regulators (ARRs) are a family of 10 genes that are rapidly induced by cytokinin and they act as negative regulators of cytokinin signalling due to lacking a DNA-binding motif. The type-A ARR proteins act as repressors of cytokinin signalling. However, the effects of single or higher-order mutants in gynoecia have been little studied, due to high level of functional redundancy (To *et al.*, 2004; Ren *et al.*, 2009).

The PIN-FORMED (PIN) proteins are important efflux transporters involved in polar auxin transport (PAT) (Krecek *et al.*, 2009). PIN proteins have asymmetric subcellular localization to determine the directionality of the auxin flux (Blilou *et al.*, 2005; Petrasek *et al.*, 2006; Wisniewska *et al.*, 2006). The *Arabidopsis* genome encodes eight PIN-related sequences, five of which are mainly located in the plasma membrane (PIN1-4 and PIN7) and mediate auxin flux between cells. The other three PIN proteins (PIN5, PIN6 and PIN8) are localized in the endoplasmic reticulum (ER) and it is believed that they regulate the auxin flux in the inside of the cell (Petrasek *et al.*, 2006; Mravec *et al.*, 2009). *PIN1, PIN3* and *PIN7* are expressed during the earliest stages of gynoecium development (Sorefan *et al.*, 2009; Larsson *et al.*, 2014; Moubayidin and Ostergaard, 2014). The single *pin1* mutant has abnormalities in the inflorescence axis, flowers, and leaf development. The *pin1* mutant also displays several abnormalities in gynoecium development such as the lack of ovule and septum development (Okada *et al.*, 1991; Reinhardt *et al.*, 2003). Furthermore, the double *pin3 pin7* mutant has severe floral defects and abnormal gynoecium development (Benkova *et al.*, 2003). This data supports that polar auxin transport might play an important role in CMM development and differentiation during early gynoecium development.

Finally, the *MICRORNA164* family is a group of redundant miRNA genes involved in the regulation of the expression of members of the NAC family (Sieber *et al.*, 2007). In the gynoecium their mutation or overexpression causes severe alteration similar to the phenotype of mutation or overexpression of *CUC* genes (Sieber *et al.*, 2007). Interestingly, CUC2g-m4 (miR164-resistant *CUC2*) plants show ectopic proliferation in the replum tissue that resembles those observed in the *kan1 kan2* double mutant and *ett* mutant, suggesting that regulation of the *CUC* expression by *miR164* is important for correct formation of the CMM. In the

gynoecium, *miR164c* is expressed in the CMM region at stage 6-7 (Baker *et al.*, 2005; Nikovics *et al.*, 2006; Sieber *et al.*, 2007).

Lateral identity genes

Currently, little is known about the molecular mechanisms that control valve development. However, the discovery of different *Arabidopsis* mutants affected in valves has contributed to the identification of several genes involved in valves determination (Roeder and Yanofsky, 2006).

FRUITFULL (*FUL*) is a transcription factor belonging to the extended family of MADS-box genes (Gu *et al.*, 1998). In the gynoecium, *FUL* is strongly expressed in the valves from stage 8. *FUL* controls valve development and dehiscence in *Arabidopsis* by promoting cell elongation and differentiation (Roeder and Yanofsky, 2006). Mutation in the *FUL* gene results in small, compact fruits that fail to elongate after fertilization because valve cell development is dramatically altered and the valve cells fail to expand. In contrast, the overexpression of *FUL* results in cells of the outer replum and valve margin to adopt a valve cell fate, thus, the cells on the entire surface have the appearance of valve cells (Gu *et al.*, 1998; Roeder and Yanofsky, 2006).

Another gene involved in valve determination is the *FILAMENTOUS FLOWER (FIL)* gene, which is strongly expressed during floral stages 6-11 in the gynoecium, in the valves and in cells that will probably contribute to the formation of the valve margin (Siegfried *et al.*, 1999; Dinneny *et al.*, 2005). The *fil* single mutant does not show any major defects in valve or dehiscence development; however, the valves and valve margins are markedly affected in the *fil yab3* double mutant (Chen *et al.*, 2001; Dinneny *et al.*, 2005).

The *ASYMMETRIC LEAVES1* (*AS1*) gene is a MYB transcription factor that acts as a negative regulator of class 1 *KNOTTED1-like* homeobox (*KNOX*) genes in leaf primordia. The *as1* mutant shows defects in gynoecium development such as large repla and a reduction in valve width. The *AS1* gene is strongly expressed in the fruit valves (Alonso-Cantabrana *et al.*, 2007). Recently, AS1 has been associated with repression of *ETT* in the leaves (Takahashi *et al.*, 2013), this suggests a similar role of AS1 in the gynoecium.

2.5 AUXIN-CYTOKININ PHYTOHORMONES

The auxin and cytokinin phytohormones are important inducers of development in plants and act together to regulate different developmental processes such as stem cell maintenance and root development (Frigerio *et al.*, 2006; Muller and Sheen, 2008; Zhao *et al.*, 2010; Su *et al.*, 2011; Schaller *et al.*, 2015). Many functions of auxin and cytokinin depend on their differential distribution. This distribution can produce a local maximum or gradient between the cells. The gradient acts as a morphogen that activates different developmental programs (Friml *et al.*, 2003; Muller and Sheen, 2008; Sorefan *et al.*, 2009; Wolters and Jurgens, 2009; Bishopp *et al.*, 2011).

2.5.1 Auxins

Auxin coordinates important developmental processes such as the formation of the embryo apical-basal axis, vascular development, tropisms, opening of fruits and many other aspects of plant growth and developmental (Benkova *et al.*, 2003; Friml *et al.*, 2003; Blilou *et al.*, 2005; Teale *et al.*, 2006; Sorefan *et al.*, 2009). Therefore, any alteration in the homeostasis of auxin leads to drastic changes in plant development. To prevent alterations in auxin homeostasis in plants, there are multiple regulatory steps that contribute to the differential auxin distribution within tissues at different developmental stages such as biosynthesis, transport, perception, and signaling (Su *et al.*, 2011; Schaller *et al.*, 2015).

Indole-3-Acetic Acid (IAA) synthesis occurs through several metabolic pathways, but most of the routes for the synthesis of auxin use tryptophan as the main intermediary (Zhao, 2014). In most plants the main auxin is in the form of indole-3-acetic acid (IAA). The existence of multiple metabolic pathways for the synthesis of IAA and genetic redundancy has hampered the search for genes involved in the synthesis and gene regulation. However, now several important genes in the IAA synthesis are characterized such as the *YUCCA* (*YUC*) genes and the Tryptophan Aminotransferase (*TAA*) genes, which are two independent auxin biosynthesis pathways (Woodward and Bartel, 2005; Zhao, 2014). Interestingly, *YUC* and *TAA* genes show tissue specific expression patterns in the gynoecium, suggesting that the regulation of auxin biosynthesis is very important in gynoecium development (Larsson *et al.*, 2014).

Auxin transport is mostly directional and is highly regulated, which contributes to the generation of a differential distribution of auxin. Different proteins are involved in auxin transport, among these they are: the family of amino acid permease protein-like AUXIN-RESISTANT1 / LIKE AUX1 (AUX1 / LAX) that mediate the input transport of the auxins (influx), the PIN family proteins that are involved in output transport of the auxins (efflux), and another important set of proteins that mediate efflux transport are the ATP-binding cassette subfamily B (ABCB)-type transporters of the multidrug resistance / phosphoglycoprotein (ABCB / MDR / PGP). In the gynoecium PIN and PGP proteins are expressed during the earliest stages of development, suggesting an important role during the establishment of the gynoecium (Petrasek and Friml, 2009; Friml, 2010; Larsson *et al.*, 2014).

The final level of auxin regulation is through the auxin signaling response. The auxin signaling network is mediated by three groups of well-studied genes: the auxin response factor (ARF) family of transcription factors, the Aux/IAA family of transcriptional repressors, and the TIR1/AFB1-AFB5 F-box components of the SCF complex (Teale *et al.*, 2006; Sauer and Friml, 2011; Schaller *et al.*, 2015). The ARFs are a family of 23 transcription factors in *Arabidopsis* that bind with specificity to auxin response elements (AuxREs) in promoters, either activate or repress their activity (Roeder and Yanofsky, 2006; Teale *et al.*, 2006; Sauer and Friml, 2011). The Aux/IAA proteins are negative regulators of auxin signaling, which consist of 29 members in *Arabidopsis.* The Aux/IAA proteins interact specifically with ARFs and render them transcriptionally inactive when auxin concentrations are low (Teale *et al.*, 2006; Sauer and Friml, 2011; Vernoux *et al.*, 2011). In high auxin concentration, auxin joins to the TIR1 receptor promoting the degradation of the Aux/IAA via the 26S proteasome (Dharmasiri *et al.*, 2005; Sauer and Friml, 2011; Calderon Villalobos *et al.*, 2012).

2.5.2 Cytokinins

Another important phytohormone in plant development is cytokinin, which acts either synergistically or antagonistically with auxin in several significant developmental processes such as maintenance of stem-cells, vascular, gametophyte, photomorphogenic and root development (Muller and Sheen, 2008; Zhao *et al.*, 2010; Bishopp *et al.*, 2011). Little is known about the role of cytokinin and its interaction with auxin in gynoecium development. However,

recently it has been proposed that it functions synergistically with auxin during the CMM and septum development (Marsch-Martinez *et al.*, 2012b; Marsch-Martinez *et al.*, 2012a).

Like auxin, the correct regulation of cytokinin homeostasis is also vital to plant growth and development. In plants, cytokinin homeostasis is mainly regulated by a fine balance between synthesis and catabolism (Su *et al.*, 2011). Cytokinin participate in local and long-distance signalling, it is generally assumed that cytokinins are transported in the xylem (Kieber and Schaller, 2014). However, in contrast to the well-defined polar transport of auxins, the molecular mechanisms that control cytokinin transport is poorly understood.

Cytokinins are adenine derivatives and depending on substitutions in the N^6 position. They are classified as isoprenoid or aromatic cytokinins (Kakimoto, 2003; Kieber and Schaller, 2014). The first step in the biosynthesis of cytokinin is catalyzed by the enzyme *isopentenyl transferase* (*IPT*), which catalyzes the transfer of an isopentenyl group from dimethylallyl diphosphate to an adenine nucleotide (ATP, ADP, or AMP). In *Arabidopsis* there are nine *IPT* genes, of which at least *IPT1* and *IPT7* are expressed in the gynoecium (Kakimoto, 2003; Miyawaki *et al.*, 2004; Su *et al.*, 2011). The second step in the biosynthesis of cytokinin is mediated by LONELY GUY (LOG) family proteins which catalyze the last step in the conversion of cytokinin riboside 5´-monophosphates to free-base form that is the active forms of cytokinins. In *Arabidopsis* eight *LOG* genes have been identified. Enzymes involved in the catalysis of cytokinins are the CYTOKININ OXIDASE/DEHYDROGENASE (CKX) proteins. The *ckx3 ckx5* double mutant results in larger floral organ size and increased seed yield due to an increase in meristem size and ovule-forming placenta activity (Kurakawa *et al.*, 2007; Kuroha *et al.*, 2009; Bartrina *et al.*, 2011; Kieber and Schaller, 2014; Schaller *et al.*, 2015).

The cytokinin signal transduction pathway is a two-component response system that involves three components. When cytokinins binds to the receptor(s), it causes the autophosphorylation of the membrane bound receptors ARABIDOPSIS HISTIDINE KINASES (AHK2, AHK3, AHK4/CRE1), followed by a phosphorelay cascade. The phosphate gets relayed from the receptors to the ARABIDOPSIS HISTIDINE PHOSPHOTRANSFERASE proteins (AHP1-AHP5), with AHP6 competing for the phosphotransfer, i.e., interfering with cytokinin signalling. The AHP1-AHP5 proteins shuttle between the cytosol and the nucleus, and in the nucleus they phosphorylate the

ARABIDOPSIS RESPONSE REGULATOR (ARR) proteins. Phosphorylated type-B ARR proteins work as transcription factors activating cytokinin-responsive genes, including the type-A ARR genes, which form a negative feedback loop for cytokinin signaling (Hwang and Sheen, 2001; Kieber and Schaller, 2014; Schaller *et al.*, 2015).

2.6 AUXIN-CYTOKININ CROSSTALK GENES

Cytokinin and auxin act together to regulate different developmental processes, thus, many genes are regulated by both hormones (Su *et al.*, 2011). In this sense, many of these genes contain regulatory elements that are commonly regulated by the two hormones. On the other hand, auxin and cytokinin are mutually controlled through multiple regulatory steps such as biosynthesis and signal transduction pathways (Bishopp *et al.*, 2006; Wolters and Jurgens, 2009). In addition, it has been suggested that GA signalling is an important mediator of phytohormone auxin-cytokinin crosstalk (Wolters and Jurgens, 2009). Here, I present an overview of some genes that have interaction between auxin and cytokinin.

Auxin is an important inducer of root development through specific gene expression activation, one of these genes is *MONOPTEROS (MP)/ARF5* (Su *et al.*, 2011). *MP* is an ARF transcription factor that has been involved in the establishment of the embryonic axial trough the activation of TARGET OF MP 5 (TMO5), TMO7 and PIN1 (Schlereth *et al.*, 2010). Recently it has been demonstrated that TMO5 induces the expression of *LONELY GUY 4* (*LOG4*) during embryogenesis and post-embryonic root development (De Rybel *et al.*, 2014). This observation suggests the possibility that *MP* promotes cytokinin synthesis at least indirectly. Moreover, type-A ARRs ARR7 and ARR15 are expressed during root embryogenesis and it has been demonstrated that auxin signaling activates the transcription of them through direct activation mediated MP, suggesting that cytokinin signaling is strongly regulated in roots by MP (Muller and Sheen, 2008; Zhao *et al.*, 2010; De Rybel *et al.*, 2014).

The PIN proteins control auxin distribution during plant organogenesis; thus, any changes in the expression of PINs will alter the spatial pattern of intercellular auxin distribution (Reinhardt *et al.*, 2003). The transcriptional regulation of several *PIN* genes by cytokinins has been demonstrated in the *Arabidopsis* roots (Dello Ioio *et al.*, 2008; Pernisova *et al.*, 2009; Bishopp *et al.*, 2011). In roots cytokinins perform a negative regulation in PIN expression through direct transcriptional activation of the Aux/IAA protein SHY2 by ARR1 (Dello Ioio *et al.*, 2008). In roots cytokinins regulate auxin distribution also through other points of crosstalk such as inhibiting the endocytic recycling of PIN (Marhavy *et al.*, 2014) or through the type-A ARRs activation (Zhang *et al.*, 2011). Furthermore, the reduction of PIN1 expression in *cre1-12 ahk2-2 ahk3-3*

ovules and their increase expression after treatment with the exogenous cytokinins in ovules and carpels (Bencivenga *et al.*, 2012; Zuñiga-Mayo *et al.*, 2014), suggests a complex role of cytokinins in roots and aerial organs.

A key regulator of cytokinin signalling is *AHP6*, which functions as a cytokinin signalling inhibitor, since it lacks the conserved histidine residue that is required for the phosphotransfer cascade (Mahonen *et al.*, 2006; Schaller *et al.*, 2015). The *ahp6* mutant displays defects in root development and phyllotaxis pattern. In addition, it has been demonstrated that auxin rapidly activates the expression of *AHP6* in roots and the SAM. Moreover, the finding that MP binds to three out of six locations of putative ARF binding sites in the *AHP6* promoter, suggests that this regulation is directly mediated by MP (Bishopp *et al.*, 2011; Besnard *et al.*, 2014).

The *type-A ARR* genes form a negative feedback loop for cytokinin signaling. They are active or repressed by auxin according to the tissue (Su *et al.*, 2011; Schaller *et al.*, 2015). In roots the type-A *ARR 7* and *15* are expressed in early embryonic root development activated by auxin in the hypophysis region the pre-root embryonic structure (Muller and Sheen, 2008). On the other hand, in the SAM the repression of the type-A *ARR* is necessary for SAM maintenance. This is obtained through two modes: the direct repression mediated by *WUS (Leibfried et al., 2005)* and the auxin repression mediated by MP (Zhao *et al.*, 2010). The complex role of type-A ARR proteins in roots and the SAM supports the notion that auxin and cytokinin have apparently opposite roles in the SAM and the root (Schaller *et al.*, 2015).

An important phytohormone involved in auxin-cytokinin crosstalk is GA. Auxin and cytokinin often have antagonistic roles in regulating the GA pathway in specific developmental process. For example, in the SAM high auxin response induces GA biosynthesis genes, whereas high cytokinin signalling maintains low GA biosynthesis through inhibition of the production of GA via downregulation of *GA20ox* biosynthesis gene (Sun, 2008; Wolters and Jurgens, 2009). On the other hand, in roots it has recently been demonstrated that DELLA proteins are recruited by type-B ARR proteins to the promoters of cytokinin-regulated genes (such as DELLA self), where they act as transcriptional coactivators (Marín-de la Rosa *et al.*, 2015). On the other hand, auxin promotes DELLA protein degradation in roots (Wolters and Jurgens, 2009). All this data suggests an important role of GA signalling in auxin-cytokinin crosstalk.

Cytokinin and *STM* play essential roles in the maintenance and generation of the novo stem-cell system in the SAM. *STM* induces cytokinin synthesis through indirect activation of the *ISOPENTENYL TRANSFERASE 7 (IPT7)* gene (Yanai *et al.*, 2005). Conversely, overproduction or application of cytokinin increases the expression level of *STM* and can rescue weak *stm* mutant alleles (Rupp *et al.*, 1999; Jasinski *et al.*, 2005; Yanai *et al.*, 2005). In addition, *STM* has a role in the generation of de novo stem-cells through the induction of *CYCD3* (Scofield *et al.*, 2013). Moreover, STM represses the biosynthesis of GAs to maintain normal meristem function (Jasinski *et al.*, 2005). On the other hand, auxin suppresses the expression of *STM* and the STM-related factors, such as BREVIPEDICELLUS (BP) in the lateral organ primordia (Heisler *et al.*, 2005; Hay *et al.*, 2006; Heidstra and Sabatini, 2014).

The HECs, SPT, IND, ALC, PIFs, TMO5 and TMO7 are important developmental genes. They regulate different developmental processes through phytohormone regulation. For example, *HEC* has been associated with meristem activity maintenance through CLV3 repression and the type-A ARR activation and the activation of PINs (Schuster *et al.*, 2014; Schuster *et al.*, 2015). Moreover, *SPT* relations with auxin signaling has been documented. For example, the patterning defects of *ett* gynoecia are almost completely restored by mutations in *SPT*; otherwise SPT is ectopically expressed in the *ett* gynoecia. In addition, application of an inhibitor of PAT to the *spt* mutant apex restores closure of the apex as in the wild type (Nemhauser *et al.*, 2000; Heisler *et al.*, 2001; Staldal *et al.*, 2008). On the other hand, *SPT* has also been associated with the regulation of gibberellin homeostasis through different mechanisms. For example, the negative regulation of *GA3OX* expression during germination (Penfield *et al.*, 2005) or DELLA protein interactions have been reported (Josse *et al.*, 2011).

Other important genes in gynoecium development are *KANADI* and class III *HD-ZIP* genes, which have been associated with auxin signaling. Their expression patterns are similar to auxin distribution patterns and they are induced by auxins. Furthermore, they have been implicated in PAT by regulating the PIN1 protein (Izhaki and Bowman, 2007; Ilegems *et al.*, 2010; Brandt *et al.*, 2012). Moreover, the HD-ZIPIII PHB and PHV activate cytokinin-dependent cell differentiation pathways in the roots through the activation the cytokinin biosynthesis gene *IPT7*.

3 QUESTIONS TO ANSWER IN GYNOECIUM DEVELOPMENT

Despite recent significant advances in our understanding of genes affecting early gynoecium development and auxin-cytokinin signaling, there are still many questions to be resolved. These include: How are the phytohormones auxin and cytokinin mutually regulated?, How do the phytohormones auxin and cytokinin affect CMM development? What is the role of *SPT, ETT, CRC* in the process of CMM development? How do auxin-cytokinin and SPT-ETT interact with genes guiding CMM development? Does the expression of medial region genes change in CMM mutants? How do STM, CRC, SPT, CUC1/2 and type-B ARR proteins interact to guide correct CMM development? And how do abaxial-adaxial genes interact during CMM development?

4. HYPOTHESIS

The interactions between the phytohormones auxin and cytokinin, and the *SPT-CRC* genes, play a key role in CMM development in coordination with transcription factors expressed in the medial region of the gynoecium.

5 OBJECTIVES

5.1 MAIN OBJECTIVE

The goal of this study is to investigate the link between the patterns of auxin-cytokinin distribution and the gene regulatory network during CMM development in the gynoecium.

5.2 SPECIFIC OBJECTIVES

1. Identify and characterize auxin/cytokinin distribution in wild type, *crc-1* and *spt-2* mutant plants during CMM development.

2. Analyze the effect of auxin and cytokinin application on the marker lines *TCS* and *DR5* in wild type, *crc-1* and *spt-2* mutant plants.

3. Determine and analyze the expression patterns of selected genes involved in medial region development at stages 7-9 during CMM development in wild type and *spt-2* mutant plants.

4. Generate a protein-protein interaction network of CRC and CUC1/2 proteins.

6 MATERIALS AND METHODS

PLANT MATERIALS AND GROWTH CONDITIONS

The *spt-2* (CS275), *crc-1* (CS3814), *arr1-3 arr10-5 arr12-1* (CS39992), *pMIR164c::GUS* (CS65827) and *DR5rev::GFP* (CS9361) lines were obtained from the *Arabidopsis* Biological Resource Center (Ohio State University, Columbus). The *AHK3::GUS* and *AHK4::GU*S were obtained from Tatsuo Kakimoto. The *AHK2::GUS* line was obtained from Chia-Yi Cheng. The *pAHK4::AHK4-GFP* was obtained from Jianru Zuo lab. The *ahk2, ahk3, cre1-12* and double mutants were supplied by José López- Busio. The *ARR16::GUS* line was obtained from Takeshi Mizuno. The *spt-12* (CS923132), *35S::SPT*, and *35S::SPT-HA* lines were obtained from Karen Halliday. The *pin3-4* and *pin3 pin7* mutants were obtained from Eva Benková. The *PIN3::PIN3-GFP* was obtained from Lars Østergaard. The *PIN1::PIN1-GFP* and *PIN7::PIN7-GFP* lines were obtained from Luis Herrera-Estrella. The *PIN4::PIN4-GFP* line was obtained from Elena Alvarez-Buylla. The *TCS::GFP* marker line was obtained from Bruno Muller. The *AHP6::GFP* line was obtained from Ykä Helariutta. The *TCS::GFP* in the *ahp6-1* background was obtained from Teva Vernoux. The translational fusion *pSPT-6253:GUS* was obtained from David Smyth. The translational protein fusion construct *35S::ARR1ΔDDK:GR* was supplied by Raffaele Dello Ioio and Sabrina Sabatini. The *TAA1::GFP-TAA1* line was obtained from Anna Stepanova. The Enhancer trap lines *E2023* of the *KAN2* gene was supplied from Stewart Gillmor. The *pCRC::CRC:GFP* marker line was supplied by Charles Scutt. The lines *pMP::SV40-3xeGFP* and *pETT::SV40-3xeGFP* were supplied by Dolf Weijers. *35S::STM-GR* line was supplied by Simon Scofield and James Murray. The lines *YUC4::GUS, IPT1::GUS, PID::PID::GFP* and *stm-2* mutant were supplied by Cristina Ferrándiz. The transcriptional lines *pmiR165A::GFP* and *pmiR166B::GFP* were obtained from Philip N. Benfey. The transcriptional line *REV::REV:VENUS* was supplied by Elliot M. Meyerowitz. The lines *gFUL-GFP*, *STM::GUS*, *WUS::GUS* were available in the lab. *Arabidopsis thaliana* plants were grown in soil under normal greenhouse conditions or in a growth chamber (~22°C, long day light regime).

CYTOKININ TREATMENTS

Plants were grown and when flowering started (approximately 3.5 weeks) treated with the cytokinin 6-Benzylaminopurine (BAP) using the previously described protocol in (Zuñiga-Mayo *et*

al., 2014). In summary, one week after bolting drops were placed on the inflorescences once a day for 1, 2, 5 or 10 consecutive days with BAP solution. The BAP solution contains 100 µM 6-Benzylaminopurine (BAP; Duchefa Biochemie) and 0.01% Silwet L-77 (Lehle Seeds). Mock treatments contained only distilled water and 0.01% Silwet L-77. All treated plants with their respective controls were grown simultaneously under the same conditions.

GENE EXPRESSION ANALYSIS

For qRT-PCR analysis, stage 8-10 gynoecia or inflorescence with only floral buds were collected and total RNA was extracted using TRIzol (Invitrogen). After DNAse I (Invitrogen) treatment, cDNA was prepared using SuperScript III Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. First-strand cDNA was prepared using reverse specific primers (Table 1). The cDNA was analyzed in an ABI PRISM 7500 sequence detection system (Applied Biosystems) with SYBR Green Master Mix (Applied Biosystems) according to the manufacturer's instructions. The individual reactions were done in triplicate (biological replicates) with each four technical replicates. Data was analyzed using the (Pfaffl, 2001). Expression levels were normalized with ACTIN2. Primer sequences are listed in Table 1.

IN SITU HYBRIDIZATION

In situ hybridization was carried out as previously described (Gonzalez-Reig *et al.*, 2012) with the following modifications. The template for the DIG-labeled antisense and sense probe synthesis for *ARR1* mRNA was generated by PCR using specific primers (Table 1) and inflorescence wild type cDNA. The resulting PCR amplicon was purified, sequenced and used as template to transcribe the antisense probe with the T7 RNA polymerase (Promega) and the sense probe with the SP6 polymerase (Promega), respectively.

ChIP ANALYSIS

Chromatin Immunoprecipitation (ChIP) assays experiments were performed as previously described (Matias-Hernandez *et al.*, 2010) and confirmed by using the Plant ChIP-seq kit (Diagenode Inc., USA) on two different biological replicates. Between 0.2 and 1 g of floral buds of a 35S::SPT-HA line and Col-0 were collected for each experiment. In the first ChIP experiment,

monoclonal mouse anti-HA (Sigma; H3663) was used according to manufacturer instructions (2 µg per sample), and in the second ChIP experiment (using the Plant ChIP-seq kit) rabbit anti-HA (Invitrogen; 71-5500) was used according to manufacturer instructions (5 µg per sample) and was handled in parallel to a sample lacking antibody (negative control). ChIP efficiency was determined using ABI5 region as a positive control5. Primers used for ChIP analyses are listed in Table 1.

LUCIFERASE ACTIVITY ASSAY

Promoter regions of *PIN3* (2.3 kb, -2310 nt to ATG), *SPT* (2kb, -2077 nt to ATG), *ARR1* (2.1 kb, -2116 nt to ATG) and *TAA1* (2 kb, -2087nt to ATG) were amplified from Arabidopsis Col-0 genomic DNA with specific primer pairs (Table 1), cloned into pGEM-T vector (Promega), digested with SmaI and NcoI restriction enzymes, and ligated into pGREEN-LUC (Hellens *et al.*, 2005) to generate *pPIN3::LUC*, *pSPT::LUC, pTAA1::LUC*, and *pARR1::LUC* reporters for transient expression assays in *Nicotiana benthamiana* leaves. The *TCS::LUC* reporter has been previously described (Marín-de la Rosa *et al.*, 2015).

The *35S::SPT* effector construct that was coinfiltrated with the reporters was generated by transferring *SPT* into the pEARLY100 vector (Earley *et al.*, 2006) through Gateway reactions. The *SPT* ORF was previously cloned in the pDONR221 vector (Invitrogen). The *35S::HA-ARR1* has been previously described (Marín-de la Rosa *et al.*, 2015).

Luciferase assay: The transient expression assays were performed by transient transformation of *N. benthamiana* leaves by Agrobacterium infiltration, which was carried out following the protocol previously described (Espley *et al.*, 2009) with minor modifications (Ballester *et al.*, 2015). At least three plants at the same developmental stage were used for each treatment, and the experiments were repeated at least three times.

TISSUE PREPARATION AND CONFOCAL ANALYSIS

To observe fluorescence signal, carpels were dissected and observed as previously described in (Reyes-Olalde *et al.*, 2015). In summary, gynoecia were observed longitudinally or cut transversely using a scalpel and mounted in 50% glycerol. Propidium iodide (Fluka), 50 µM PI for

30-60 seconds, was used as counterstain. All imaging was done using a Zeiss LSM 510 META inverted confocal microscope (Carl Zeiss, Germany) with either a 20X or 40X air objective. GFP was excited with a 488 nm line of an Argon laser and propidium iodide (PI) with a 514 laser line. GFP emission was filtered with a BP 500-520 nm filter and PI emission was filtered with a LP 575 nm filter.

SCANNING ELECTRON MICROSCOPE ANALYSIS

Fresh tissue samples were visualized in a Zeiss scanning electron microscope EVO40 (Carl Zeiss) using the VPSE G3 or the BSD detector with a 15–20 kV beam.

GUS ANALYSIS

Gynoecia of different developmental stages were dissected and pre-fixed with cold acetone for 20 min, then rinsed and transferred into GUS substrate solution: 50 mM sodium phosphate pH 7, 5 mM K3/K4 FeCN, 0.1% (w/v) Triton X-100, and 2 mM 5-bromo-4-chloro-3-indolyl-beta-GlcUA (Gold BioTechnology Inc). After application of vacuum for 5 min, samples were incubated at 37°C, for 4 hrs to *SPT::GUS,* 12 hrs to *ARR16::GUS, STM::GUS,* cytokinin receptor GUS fusions, *WUS::GUS* and *YUCCA4::GUS* and 24 hrs. to *IPT1::GUS*.

HISTOLOGY

Tissues were fixed in FAE (3.7% formaldehyde, 5% glacial acetic acid and 50% ethanol) with vacuum (15 min, 4°C) and incubated for 60 min at room temperature. The material was rinsed with 70% ethanol and incubated overnight at 4°C, followed by dehydration in a series of alcohol solutions (70, 85, 95, and 100% ethanol) for 60 min each and embedded in Technovit as previously described (Marsch-Martinez *et al.*, 2014). Pictures were taken using a Leica DM6000B microscope (Leica).

TRANSMITTING TRACT (TT) ANALYSIS

Transmitting tract staining was performed as previously described (Zuniga-Mayo *et al.*, 2012). In summary, tissue sections were stained with a solution of 0.5% alcian blue (pH 3.1; Sigma-

Aldrich) for 25 min and counterstained with a solution of 0.5% neutral red (Sigma-Aldrich) for 5 min. Slides were rinsed in water, air dried, mounted, and observed under an optic microscope.

YEAST TWO-HYBRID

Protein-protein interactions were tested with the yeast two-hybrid (Y2H) system. The *SPT* cDNA was cloned in the pENTR/D TOPO vector (Invitrogen) and sequenced verified. Using Gateway LR recombination reactions SPT was introduced into the LexA DNA-binding domain encoding bait vector (pBTM116c-D9). The type-B ARRs fused to the Gal4 activation domain in pACT2 (Clontech, Mountain View, CA, USA) are previously described (Dortay *et al.*, 2006). Successive yeast transformation were performed following the protocol described (de Folter and Immink, 2011) using the L40ccaU strain (MATa his3D200 trp1-901 leu2-3 112 LYS:: (lexAop)4-HIS3 URA3::(lexAop)8-lacZ, ADE2::(lexAop)8- URA3 GAL4 gal80 can1 cyh2) (Goehler *et al.*, 2004). The assay was done on SD-Gluc medium lacking Leucine, Tryptophan, and Histidine complemented with 3 mM 3-Amino-1,2,4-triazole. Protein-protein interactions were scored after growing yeast at 25°C for 5 days.

BIMOLECULAR FLUORESCENCE COMPLEMENTATION ASSAY

SPT and ARR1 coding sequences in entry vectors were recombined with pYFC4317 to generate a N-terminal fusion with the C-terminal part of the YFP and pYFN4317 (N-terminal fusion with the N-terminal part of YFP) respectively. The experiment was performed using young *Nicothiana tabacum* leaves as previously described in (Marsch-Martinez *et al.*, 2014). YFP signal was assayed 3 days after infiltration using a confocal microscope.

PCR PROGRAMS

*35S::ARR1ΔDDK:*GR genotype

<table>
<tr><th>Process</th><th>Temperature</th><th>Number of cycles</th></tr>
<tr><td>Initialization step</td><td>94°C 2 min</td><td>1</td></tr>
<tr><td rowspan="2">Annealing step</td><td>94°C 2 min</td><td rowspan="2">30</td></tr>
<tr><td>58.8°C 45 sec.</td></tr>
</table>

	72°C 1 min	
Extension/elongation step	72°C 5 min	1

pin3-4 genotype

Process	Temperature	Number of cycles
Initialization step	94°C 2 min	1
Annealing step	94°C 2 min	30
	53°C 45 sec.	
	72°C 1 min	
Extension/elongation step	72°C 5 min	1

GFP genotype

Process	Temperature	Number of cycles
Initialization step	94°C 2 min	1
Annealing step	94°C 30 min	27
	60°C 45 sec.	
	72°C 45 min	
Extension/elongation step	72°C 5 min	1

TABLE 1: PRIMERS USED IN THIS STUDY.

Gene	Accession	Forward primer	Reverse primer	Purpose	Reference
ARR12	AT2G25180	CACGATGAAGCAGGAACAGA	TTCTGAGTGAACTAAACCCTCCA	qRT-PCR	(Muller and Sheen, 2008)
ARR1	AT3G16857	GCAAGTCACCTCCAGAAATACC	ATCCTGACCCGTCATAAACG	qRT-PCR	(Muller and Sheen, 2008)
ARR10	AT4G31920	GACACAGGAACAGAGCCAATC	TATGCATGTTCCGAGTGAGC	qRT-PCR	(Muller and Sheen, 2008)
ARR16	AT2G40670	CCTGTAACGTTATGAAGGTGAGTC	GACTCCTTCACTTTCTTGAGTAGC	qRT-PCR	(Muller and Sheen, 2008)
ACT2/7	AT1G49240	CGTACAACCGGTATTGTGCTGGAT	GCTTGGTGCAAGTGCTGTGATTTC	qRT-PCR	This study
SPT	AT4G36930	CAGCTCCAAGTTCAGATGTT	GAGCTTGTTCCCCGGTTATG	qRT-PCR	(Ichihashi *et al.*, 2010)
PIN3	AT1G70940	GACCAGGTGATGCCGAATA	CTGATGCTGGTCTTGGAATG	qRT-PCR	(Bennett *et al.*, 2006)
STM	AT1G62360	CAACCCTTGCTCCTCTTCC	CCTGTTGGTCCCATAGATGC	qRT-PCR	This study
ETT	AT2G33860	GGTCCCAAGAGAAGCAGGATTGGCT	GCAAGACCCTCTGGAATCTCAATG	qRT-PCR	This study
AHP6	AT1G80100	TAACGTCTGCGTTGCCTTT	CCTCCAGTCCTCTCAAGCAC	qRT-PCR	This study
PIN3 a	AT1G70940	AGTCCAAAGATCAGAGTAAACAGAGG	CACGCGTCTTGTCCATAATGAAAG	ChiP	This study
PIN3 b	AT1G70940	CACGTGTTTAAGCCACCAGTAGCC	CACGTGAACAAAAGTATACGGTTTAGCC	ChiP	This study
TAA1	AT1G70560	CCATTTAAAAAGCAGATACCAGTC	AGAGAATAGTAGGTCGAAGTGT	ChiP	This study
ARR1	AT3G16857	CGATAGATGGAGAGGTCGATGC	CTATTGCGACACGTGTCCACC	ChiP	This study
ABI5	AT2G36270	TTAGGTCGCTGGTTCGATTC	CATGATTCCGAACTTCCATTG	ChiP	(Vaistij *et al.*, 2013)
ACTIN2/7	AT1G49240	CCAATCGTGAGAAAATGACTCAG	CCAAACGCAGAATAGCATGTGG	ChiP	(Matias-Hernandez *et al.*, 2010)
ETT a	AT2G33860	CCAAATTCTCACTTCAGAGTCC	GTATTTCCTTTGTTCTTGCCC	ChiP	This study
ETT b	AT2G33860	CTTCAGCTTCTGTGTCTGTG	CATACGTGAAGCTTAACATCG	ChiP	This study
ARR1	AT3g16857	ATTTAGGTGACACTATAGATTACTTCACGGTGTCCCCACG	TAATACGACTCACTATAGGGGTGTCTTGACATGGACGAAGAAGAG	*In situ* hybridization	This study
pPIN3	AT1G70940	CCCGGGAGAGATTATTAAAACATCAATTAACGTCA	CCATGGCCACGTAGAGAGGA ATCACGGCGGT	LUC assay	This study
pSPT	AT4G36930	CCCGGGAAACTACCAACGAACAACTTAAAAAACA	CCATGGCTCTCTGCTTCTTCTCTTCTCTTTCTT	LUC assay	This study
pARR1	AT3g16857	CCCGGGAAAGCTTCTCAGCAACGTGATT	CCATGGCTCTCTCTATGTAGCTCGAA	LUC assay	This study

pTAA	AT1G70560	GGATAGAGCGACTCTCACGTC	CTTCTTCTTCTTGGTTTGGTCGTTTG	LUC assay	This study
SPT	AT4G36930	ATGATATCACAGAGAGAAGAAA	TCAAGTAATTCGATCTTTTAGG	35S::SPTLUC assay	This study
SPT	AT4G36930	CACCTTTTTTGTTGTTGGTGTAATGATAT	GGACACTGTTCAAGTAATTCG	Y2H and BiFC	This study
pin3-4	AT1G70940	TGCCACCTTCAATTCAAAAAC	TGAGAAAATCCAACGCTTCAC	Genotyping	(Benkova *et al.*, 2003)
spt-12	AT4G36930	TTCGCTCATGTGTTGAGC	CGTGTCGGAGATTTCTCTGAG	Genotyping	(Schuster *et al.*, 2015)
35S::ARR 1:GR	AT3g16857	CAATCCCACTATCCTTCGCAAGACCC	ATCCTGACCCGTCATAAACG	Genotyping	This study
ARR16:: GUS	AT2G40670	TTTGATGCAATCTCTTCCCC	GGCACAGCACATCAAAGA	Genotyping	This study
GFP		ATGCCTGAGGGATACGTGC	GTGGTCTCTCTTTTCGTTGGG	Genotyping	This study

7 RESULTS

7.1 Functional analysis of SPATULA, a bHLH transcription factor involved in carpel and fruit development in Arabidopsis

Cytokinin signalling is involved in ectopic outgrowths from the medial domain

Recent studies indicate that cytokinins play an important role in CMM development (Marsch-Martinez *et al.*, 2012b). However, it is unknown how cytokinins are involved in this process. To address this lack of information, we first analyzed the phenotypes of the cytokinin receptor mutants on CMM development (Higuchi *et al.*, 2004).

As reported previously, the single cytokinin receptor mutants do not show a noticeable gynoecium defect phenotype and the *cre1-12 ahk2-2 ahk3-3* triple mutant is difficult to grow or produces few flowers (Higuchi *et al.*, 2004; Nishimura *et al.*, 2004; Kinoshita-Tsujimura and Kakimoto, 2011). Thus, we used the cytokinin receptor *cre1-12 ahk2-2 ahk3-3* triple mutant and the *cre1 ahk2, cre1 ahk3*, and *ahk2 ahk3* double mutants. In the gynoecium, at stage 12, the three double mutants apparently did not show significant differences from wild type (Sup. Fig. 1a). However, when we analyzed cross-sections from double mutants and the triple mutant they showed some defects in the transmitting tract, characterized by a size reduction and alcian blue dye defects (less staining) (Fig. 6a-e).

Next, we studied the expression pattern of the three cytokinin receptors in gynoecia and for this we analyzed the *pAHK4::GUS*, *pAHK2::GUS*, and *pAHK3::GUS* marker lines (Nishimura *et al.*, 2004). All three GUS lines showed expression in the gynoecium. The promoters of *AHK2* and *AHK3* are broadly expressed from early developmental stages on (Fig. 6f-g), whereas, *pAHK4::GUS* showed a more restricted expression in the vascular bundles of the medial region and the ovules (Fig. 6h). However, the *pAHK4::AHK4-GFP* line showed GFP signal also in other tissues such as the valves and ovule primordia (Sup. Fig. 3b). This suggests the possibility that *AHK4* transcription takes place in the medial region and the resulting protein moves to the valve cells.

Then, the effects of exogenous cytokinin application were evaluated on the cytokinin receptor mutants (see Methods). Inflorescence apices of the cytokinin receptor double mutants were treated with 100 µM BAP for 5 days. Newly opened flowers were examined after 3–4 weeks of treatment. In wild type, most gynoecia developed outgrowths from the repla (Fig. 6i, as previously described (Marsch-Martinez *et al.*, 2012b). In contrast, the cytokinin receptor mutants showed a decrease in BAP response, and an absence of response in the *ahk2 ahk4* double mutant (Fig. 6j-m). This data is consistent with the idea that cytokinin receptors are important for the cytokinin-induced overproliferation effect.

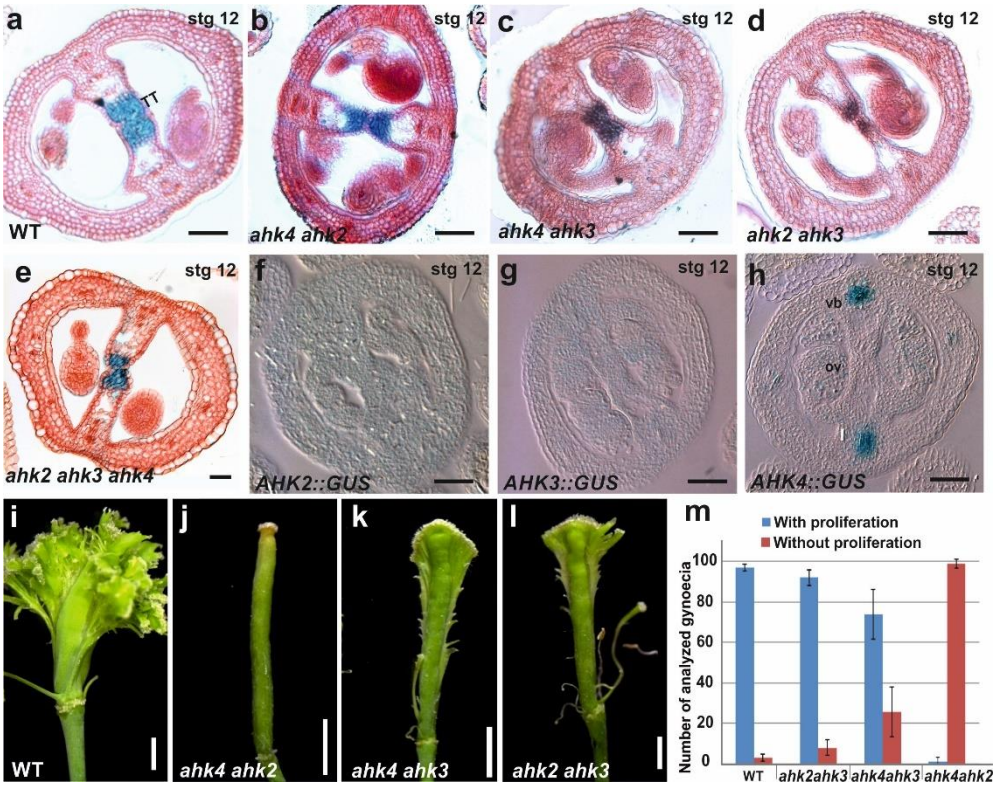


Figure 6. Cytokinin signalling is necessary for proper gynoecium development. (**a-e),** Septum and transmitting tract (TT) phenotypes in transverse sections of stage 12 gynoecia of wild type (a) and the mutants *ahk4 ahk2* (b), *ahk4 ahk3* (c), *ahk2 ahk3* (d), *ahk2 ahk3 ahk4* triple mutant (e). (**f-h)**, Expression of the transcriptional GUS reporter lines *AHK2::GUS* (f), *AHK3::GUS* (g), and *AHK4::GUS* (h). Vascular bundles (vb) and; ovules (ov) are indicated. (**i–m)**, wild type (i), *ahk4 ahk2* (j), *ahk4 ahk3* (k), *ahk2*

ahk3 (l) gynoecia three to four weeks after receiving a BAP treatment for five days and apical-basal gynoecium patterning phenotype frequency of BAP treatments in wild type and mutant backgrounds (m). Scale bars: 5 mm (i-l), 100 µm (a-g), 10 µm (h).

SPT gene expression is necessary for the cytokinin-response in the CMM

We previously showed that cytokinin signalling is involved in gynoecium and fruit development. However, it remains unknown how the cytokinin regulates this process (Marsch-Martinez *et al.*, 2012b). Striking resemblances can be observed between the cytokinin signalling output pattern (*TCS::GFP*) (Marsch-Martinez *et al.*, 2012b) and the *SPT* expression pattern (Fig. 7b, c), suggesting a connection between them. Therefore, we investigated whether *SPT* participates in cytokinin signalling during gynoecium development. In order to analyze this possible interaction, the fluorescence marker *TCS::GFP* was first analyzed, which has a synthetic promoter containing *type-B ARR* binding sites (Muller and Sheen, 2008), in a *spt-2* and *35S::SPT* background.

At stage 9, the CMM gives rise to the placenta and septum primordia (Fig.7a). The *TCS* signal is detected in two regions in the CMM: the septum primordia and the provasculature cell region (Fig. 7c). In the provasculature cell region TCS signal forms a ring engulfing the presumptive provasculature cells (Fig. 7c). In the septum primordia TCS expression appears to be restricted to the edges in putative progenitor cells of the septum, consistent with previous analysis (Fig. 7c) (Marsch-Martinez *et al.*, 2012b). In a transverse section at stage 12, *TCS* signal is detected in the valve margin and the transmitting tract tissue (Sup. Fig. 3a). Furthermore, we observed that the *TCS* fluorescence increased and is localized to the transmitting tract develop tissue in the septum (Sup. Fig. 3a).

In contrast to wild type, *TCS::GFP* in the *spt-2* mutant background at stage 9 showed strong reduction of fluorescence signal, although it was still observed at the presumptive provasculature cells region. In comparison, at the septum primordia the *TCS* fluorescence was lost (Fig. 7d and Sup. Fig. 6A). Notably, during stages 10 to 12, the *TCS* signal in *spt*

increased and could be observed at the edges of the defective septa, which indicates that this later signal is independent of *SPT* expression (Fig. 7f and Sup. Fig. 6A).

Since the *TCS* signal in *spt-2* is reduced in the CMM, we wanted to test whether the opposite effect occurs in a *35S::SPT* overexpression line. Indeed, an increase in the *TCS::GFP* signal was observed in stage 9 gynoecia of a *35S::SPT* overexpression line. *TCS* activity was increased in the CMM and septum primordia region, this strong expression pattern continued during stage 12 (Fig. 7e and Sup. Fig. 6A). Interestingly, despite the ectopic expression of *SPT*, we did not detect ectopic expression of *TCS* signal in other tissues outside the CMM region (Sup. Fig. 6A), which suggests a negative regulation either of *SPT* or cytokinins signalling outside of the medial region.

To test whether *SPT* was involved in cytokinin signalling, inflorescence apices of *spt-2 TCS::GFP* and wild type *TCS::GFP* were treated with 100 µM of BAP twice for 48 hrs. 12 hrs. after the last treatment, the flowers were examined by confocal microscopy (Methods). The gynoecia of wild type *TCS* plants treated with BAP at stage 9 showed an expansion and increase of the *TCS* signal in the CMM, mainly in provasculature cells and the septum primordia regions (Fig. 7g and Sup. Fig. 4). In contrast, in the *spt* mutant the *TCS* signal only showed a weak response at the provasculature cells region, while in the septum primordia region the *TCS* response was absent (Fig. 7h and Sup. Fig. 7). However, the response of *TCS spt* at stage 12, showed an increase in GFP fluorescence (Sup. Fig. 7), suggesting that this later signal response was independent of *SPT*.

It has been demonstrated that *spt* apical defects are restored when auxin homoeostasis changes (Heisler *et al.*, 2001; Staldal *et al.*, 2008). To test whether auxin application can change the cytokinin response in *spt*, inflorescence apices of *spt-2 TCS* were treated with 100 µM of Indole 3-Acetic Acid (IAA). In control plants, the *TCS* signal was increased in presumptive provasculature cells and septum primordia (Fig. 7i and Sup. Fig. 5). In contrast, the *spt-2 TCS::GFP* treated plants showed an absence of *TCS* signal in CMM and septum primordia at stage 9 and 12 (Fig. 7j and Sup. Fig. 7). In summary, the results indicate that *SPT* is necessary for cytokinin signalling activation in the CMM and septa

primordia and the correct expression of *SPT* is a central hub to balance the local auxin-cytokinin signalling.

Application of exogenous cytokinins recovers the apical *spt* defects

We next investigated the functional relevance and nature of the relationship between *SPT* and cytokinin signalling in the gynoecium. Repeated cytokinin applications result in overproliferation causing ectopic outgrowths from the repla (Marsch-Martinez *et al.*, 2012b). Using this assay as a tool to evaluate the cytokinin response competence of gynoecia, we treated inflorescence apices of the *spt-2* mutant and its respective wild type *Landsberg erecta* (L*er*) with 100 µM of BAP for 48 hrs. and for 5 days and newly opened flowers were examined after 3–4 weeks of treatment (see Methods) (Fig. 7k-p).

In wild type plants, the treatment for 48 hrs. resulted in shorter fruits (Fig. 7k). In transverse cross-sections of wild type fruits treated for 48 hrs., the septum and transmitting tract region showed a significant widening (Fig. 7m,o). After 5 days of BAP treatment, the gynoecia often developed outgrowths from the replum (Fig. 7k); close observation of these outgrowths showed that they consist of cells displaying characteristics of ectopic-stylar tissues, as previously described (Marsch-Martinez *et al.*, 2012b). Sometimes BAP applications induced trichome development on carpels in the *Ler* background (Sup. Fig. 2).

Interestingly, the treated *spt-2* gynoecia showed complete fusion of the apical region, characterized by the suppression of the stylar split phenotype and enhanced stigma development (Fig. 7 n, p). However, when we analyzed the cross-sections, the style region showed only partially restored phenotype, but the central region remained hollow (Sup. Fig. 2). Furthermore, in the ovary-region, the septum fusion defects persisted and never produce transmitting tract tissue (Fig. 7 n, p). On the other hand, when *spt* gynoecia was treated for 5 days with BAP, striking phenotype appeared in which the gynoecium was completely unfused at the apical end (Fig. 7l). The apical region resulted in reduced style and stigma development, displaying extensive tissue proliferation at the top. From this point on we call this phenotype “bazooka” (Fig. 7l). Overall, the results indicate that the

defects observed in *spt* are probably not caused by reduced levels of cytokinin, and that the main role of SPT may not be the induction of cytokinin biosynthesis in these tissues. Worthwhile noting is that the response to cytokinin is different in the style and stigma region than the ovary region.

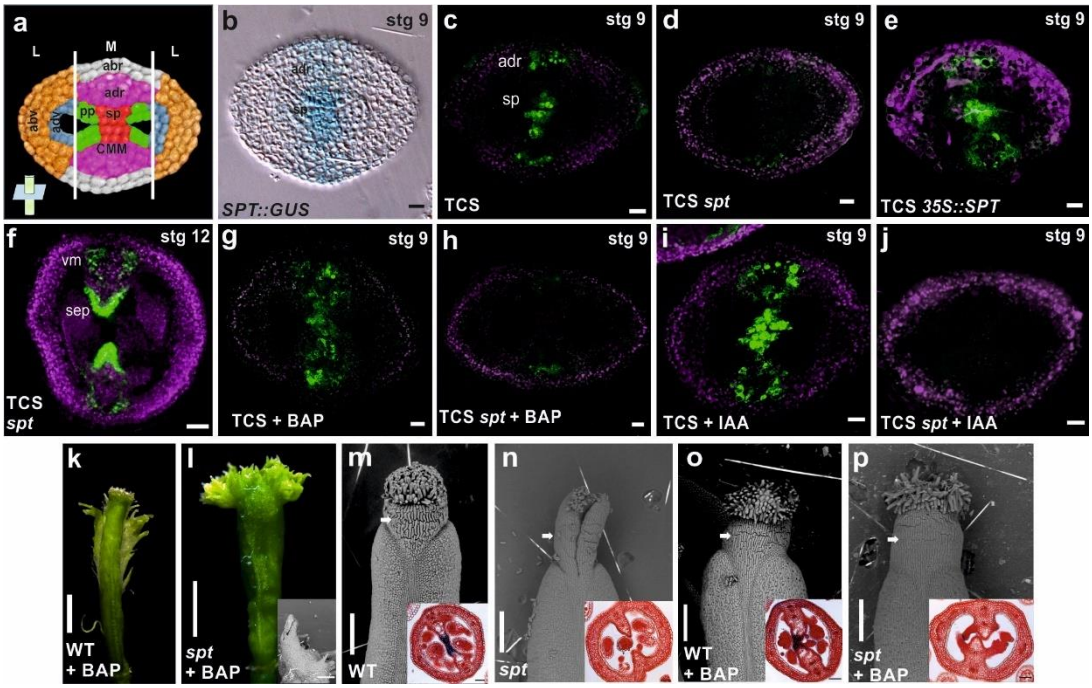


Figure 7. Cytokinin signalling and *SPT* are important for early gynoecium development. a, Schematic overview and false-coloured transverse section of a stage 8. Orange, abaxial valve (abv); blue, adaxial valve (adv); white, abaxial replum (abr); pink, adaxial replum (adr); green, placenta primordia (pp); red, septum primordia (sp); CMM, carpel margin meristem; L, lateral domain; M, medial domain. **b**, Expression of the transcriptional reporter *SPT::GUS* line in a transverse section of a stage 9 gynoecium. **c-j**, Expression of the transcriptional reporter *TCS::GFP* in transverse sections of stage 9 gynoecia of wild type (c), *spt-2* (d), *35S::SPT* (e) and stage 12 gynoecia of *TCS::GFP spt-2* (f). Wild type gynoecia treated for 48 hours with the synthetic cytokinin 6-benzylaminopurine (BAP) (g), *spt-2* treated for 48 hours with BAP (h). Wild type gynoecia treated for 48 hours with Indole 3-Acetic Acid (IAA) (i), *spt-2* treated for 48 hours with IAA (i). **k–l**, Wild type (k) and *spt-2* (l) gynoecia three to four weeks after receiving a BAP treatment for five days. **m–p**, Scanning electron microscopy image of wild type and *spt-2* stage 12 gynoecia one day after either receiving mock treatment (m, n) or 48 hours of BAP treatment (o, p). Insets show a transverse section of the ovary. Scale bars: 5 mm (k-l), 100 µm (m-p), 50 µm (insets in m-p), 10 µm (b-j).

SPT mediates *type-B ARR* expression

Because *TCS* is a synthetic promoter that contains the *type-B ARR* binding sites (Muller and Sheen, 2008) and *TCS* is expressed in the CMM and septum primordia, we hypothesized that *type-B ARRs* are involved in CMM and septum development. Therefore, in order to obtain deeper insights about the relevance of the *type-B ARR* transcription factors during early gynoecium development, we analyzed plants with impaired *type-B ARR* genes, in which the cytokinin signalling output is affected (Schaller *et al.*, 2015).

The loss-of-function mutants for single *type-B ARR* genes have weak phenotypic alterations (Mason *et al.*, 2005). However, the *arr1 arr10 arr12* triple mutant displays phenotypes similar to those observed in the triple *ahk2 ahk3 ahk4* triple mutant, such as poor growth (Yokoyama *et al.*, 2007). For this reasons we study the *arr1 arr10 arr12* triple mutant gynoecium phenotype. The *arr1 arr10 arr12* triple mutant shows a drastic reduction in gynoecium and replum length (Fig. 8a-c and Sup. Fig. 1), as well as reduced ovule, seed and replum cell number (Sup. Fig. 1). Moreover, transverse sections of gynoecia of the *arr1, 10, 12* triple mutant showed a drastic reduction or absence of transmitting tract tissue (Fig. 8d-e) and some have subtle defects in septum fusion (Sup. Fig. 1).

Since the type-B ARRs are transcription factors involved in cytokinin signalling, we analyzed the response of the *arr1, 10, 12* triple mutant to exogenous cytokinin application. The *arr1, 10, 12* triple mutant was treated with 100 µM of BAP for 5 days. After 5 days of treatment, as expected, the *arr1, 10, 12* mutant did not show any response in contrast to wild type gynoecia (Fig. 8f-g).

Taken together, the close resemblances between *spt* and *arr1, 10, 12* mutant phenotypes in the ovary, the coexpression of *SPT* and *TCS*, and the BAP-response of *spt* and *arr1, 10, 12* mutants, we hypothesized that *SPT* participates in the transcriptional regulation of *type-B ARR* genes. To explore this possibility, we analyzed the transcript levels of *ARR1, ARR10,* and *ARR12* in *spt-12*, *35S::SPT* and in Col-0 dissected gynoecia. We found that the expression level of the *type-B ARR1* was decreased (Fig. 8h), and to a lesser extent the

expression of *ARR12* in the *spt* mutant compared to wild type, while the expression level of *ARR10* was not altered. Interestingly, in *35S::SPT* gynoecia, we observed a decrease of all three *type-B ARR* genes, which might be due to negative feedback (Sup. Fig. 1e). After observing this reduction and because *ARR1* transcript abundance showed the most conspicuous reduction in *spt*, we performed an *in situ* hybridization for *ARR1* in wild type gynoecia and *spt* mutant (in collaboration with Dr. Juan José Ripoll of the laboratory of Dr. Martin Yanofsky, UCSD, USA). In wild type plants *ARR1* is expressed in septum primordia and placenta primordia during stage 8 to 9 (Fig. 8i and Sup. Fig. 3A). At stage 10 to early stage 12, the hybridization signal was localized to the transmitting tract (Fig. 8i and Sup. Fig. 3A) and to the developing stigma-style region (Fig. 8i). In contrast, in the *spt* mutant no or reduced expression levels of *ARR1* was detected (Fig. 8j and Sup. Fig. 6A).

The decrease of *ARR1* expression in the *spt* mutant suggests that *SPT* regulates directly or indirectly the expression of *ARR1*. To test whether this regulation could be direct, we first performed a luciferase reporter assay in tobacco leaves (in collaboration with Patricia Ballester of the laboratory of Dr. Cristina Ferrándiz, CSIC, Valencia, Spain). We found that transiently expressed SPT is able to activate an *ARR1::LUC* reporter (Fig. 8k), suggesting that SPT interacts with the *ARR1* promoter and can activate *ARR1* expression. Subsequently, we confirmed direct binding of SPT to the *ARR1* promoter sequences by chromatin immunoprecipitation (ChIP) with an anti-Hemagglutinin (HA) antibody using a *35S::SPT-HA* line (Josse *et al.*, 2011) (In collaboration with Dr. Dario Paolo and Dr. Ignacio Ezquer of the laboratory of Dr. Lucia Colombo, University of Milan, Italy). The ChIP assays revealed that SPT is able to bind to one identified G-box region present in the *ARR1* promoter (Fig. 8l).

In order to analyze the effects of overexpression of *ARR1* in the *spt* mutant and in an overexpression line of *SPT*, we crossed a glucocorticoid inducible construct of *ARR1* (*35S::ARR1ΔDDK:GR*) (Sakai *et al.*, 2001) with *spt-12* and *35S::SPT*. These crosses and analyses are in progress.

In summary, these data support the idea that *SPT* expression is required for direct regulation of *ARR1* expression in the ovary, explaining the observed lack of cytokinin-induced *TCS* signal at the CMM and septa primordia.

Cytokinin positively regulates *SPT* expression

Interestingly, when exploring the relationship between cytokinin and *SPT*, we found that cytokinin signalling is also required for *SPT* expression, because, first, *SPT* is significantly decreased based on qRT-PCR results in inflorescences of the type-B triple *arr1, 10, 12* mutant (Fig. 8m), and secondly, *SPT* was moderately induced in inflorescence apices of Col-0 wild type treated with 100 µM BAP for 30 min (Fig. 8n). In another experiment *pSPT::GUS* plants (Groszmann *et al.*, 2010) were treated with 100 µM BAP for 48 hrs. The results of these experiments showed that *SPT* expression was increased and that the pattern became slightly more abaxialized in the gynoecium upon application of exogenous cytokinin (Fig. 8o-p), confirming the qRT-PCR results. Altogether, this demonstrates that cytokinin affects the expression and localization of *SPT*. This data is consistent with the recent observation that after 15 min of exogenous cytokinin treatment *SPT* expression is increased and this increase is depended on *ARR1* (Ramireddy *et al.*, 2013).

Having established a regulatory interaction between ARR1 and *SPT*, we next asked whether this interaction is direct. To test this we used a luciferase reporter assay in tobacco leaves (In collaboration with Patricia Ballester of the laboratory of Dr. Cristina Ferrándiz, CSIC, Valencia, Spain). Transiently expressed ARR1 is able to activate a *SPT::LUC* reporter (Fig. 8q), suggesting that ARR1 interacts with the *SPT* promoter and can activate *SPT* expression. To provide further support for ARR1 binding to the *SPT* promoter in *vivo*, an assay using chromatin immunoprecipitation (ChIP) assays with an anti-rat glucocorticoid receptor (GR) using *35S::ARR1ΔDDK:GR* plants (Sakai *et al.*, 2001), is under development.

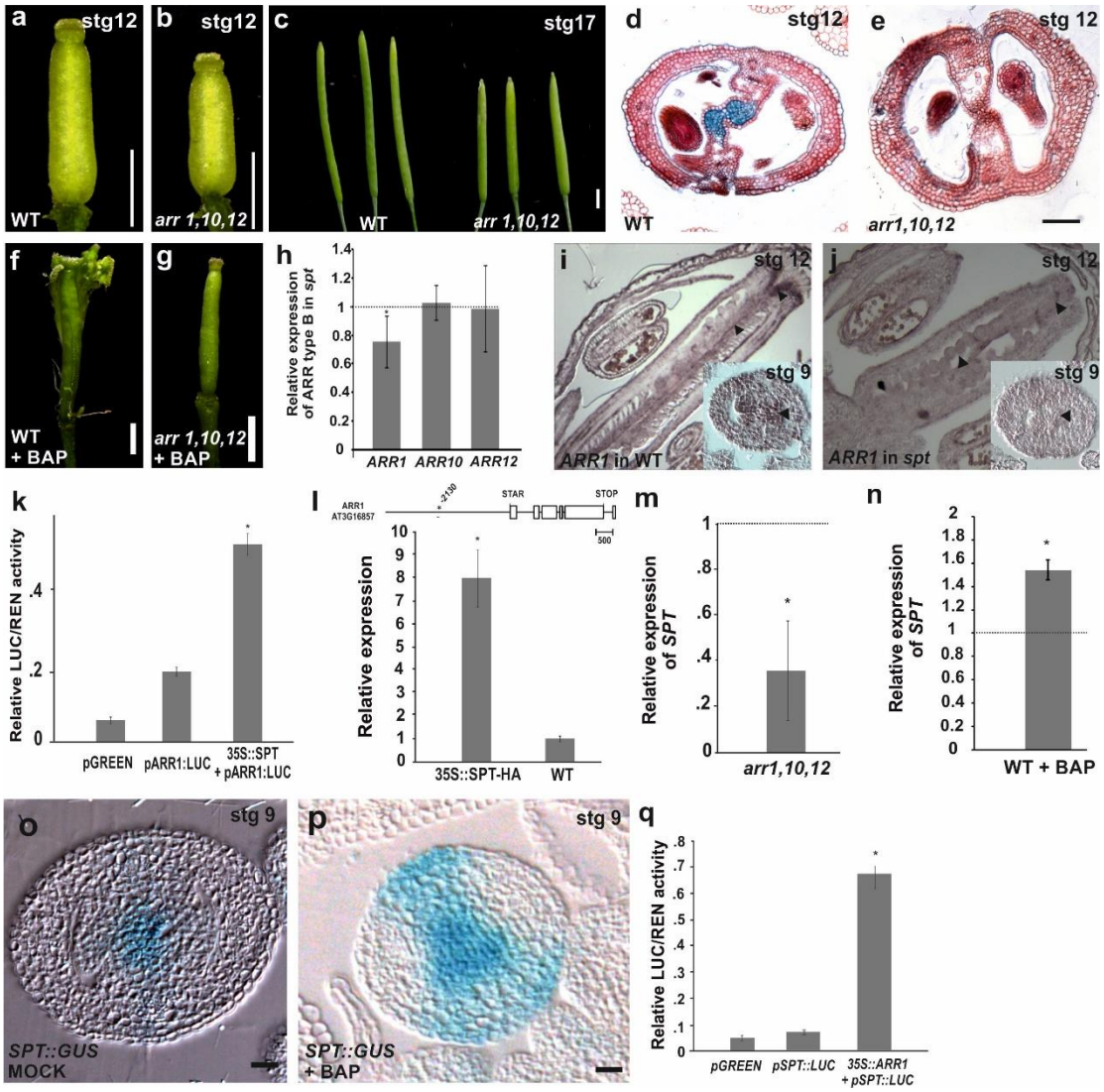


Figure 8. SPT enables a cytokinin response during early gynoecium development and also responds to cytokinin a-e, Phenotypes of the type-B *arr1 arr10 arr12* triple mutant compared to wild type (WT): mature gynoecium size (a-b), fruit size (c), transverse sections of stage 12 gynoecia of wild type (d) and *arr1, 10, 12* (e) mutant. **f, g**; wild type (f) and *spt-2* (g) gynoecia three to four weeks after receiving a BAP treatment for five days. **h**, qRT-PCR of *ARR1*, *ARR10* and *ARR12* of wild type and *spt-12* inflorescences. **i–j**, In situ hybridization of the type-B *ARR1* in wild type (i) and in *spt-2* stage 9 and stage 12 flowers (j). **k**, Ratio of firefly luciferase (LUC) to Renilla luciferase (REN) activity in *N. benthamiana* leaves co-transformed with *35S::SPT* and *pARR1::LUC*. **l**, ChIP experiment against the *ARR1* promoter region using a *35S::SPT-HA* line. ACT2/7 served as a negative control. **m-n**, qRT-PCR of *SPT* in *arr1 arr10 arr12* triple mutant inflorescences (m) and in wild type inflorescences treated with BAP for 30 min (n). **o-p**, Expression of the transcriptional reporter *SPT::GUS* in transverse sections of ovaries of mock-treated (o) and BAP-treated (p) stage 9 gynoecia. **q**, Ratio of LUC/REN

activity in *N. benthamiana* leaves co-transformed with *35S::ARR1* and *pSPT::LUC*. Error bars represent the s.d. for LUC assays and for qRT-PCR analyses based on three biological replicates. A ChIP result of one representative experiment is shown; error bars represent the s.d. of the technical replicates. *P < 0.05 (qRT-PCR and qPCR: ANOVA; LUC: Student-t test). Scale bars: 5 mm (a-c, f, g), 100 µm (d–e), 50 µm (insets in d–e), 10 µm (o, p).

The *spt* mutant exhibits defects in response to auxin

Many functions of cytokinin in plant development are controlled by auxin interaction (Dello Ioio *et al.*, 2008; Muller and Sheen, 2008; Zhao, 2014; Schuster *et al.*, 2015). In order to dissect the interaction between auxin response and *SPT*, we crossed the auxin reporter line *revDR5::GFP* with the *spt-2* mutant and the *35S::SPT* line. At stage 9, *DR5* is seen as a ring in the abaxial gynoecium (Fig. 9a, c and Sup. Fig. 3A), this pattern persists in the apex of the gynoecium until the apical part of the gynoecium is completely closed (Fig. 9a, c). In a transverse view, the *DR5* signal is refined at stage 9 to presumptive provasculature cells of the CMM and presumptive ovule primordia (Fig. 9c and Sup. Fig. 3A). Interestingly, no GFP signals were detected in the septum primordia region or transmitting tract at stage 12 (Fig. 9a and Sup. Fig. 3A). The absence of *DR5* signal in septum primordia strongly coincides with a high *TCS* signal (Fig. 7c and Sup. Fig. 3A), suggesting an antagonistic relation.

In contrast, in the *spt-2* mutant, the *DR5* signal in the gynoecial apex was limited to two spots in the lateral domains, consistent with previous analysis (Fig. 9b) (Girin *et al.*, 2011; Moubayidin and Ostergaard, 2014). In a transverse view, the *DR5* signal in presumptive provasculature cells was not clearly defined and in some occasions the *DR5* signal was extremely weak compared to wild type, (Fig. 9d and Sup. Fig. 6A). Conversely, in plants that overexpress *SPT* from the constitutive 35S promoter have lower *DR5* signal levels in the provasculature cells (Fig. 9e and Sup. Fig. 6A). Although, *DR5* signal activity is low, apparently is sufficient for a correct functioning of the CMM. Furthermore, at stage 12, *DR5* expression is limited to the apical tip of the gynoecium in the stigma, however, in *35S::SPT* the *DR5* signal is expanded to the style and stigma (Fig. 9n, o). In summary, this

data strongly suggests that *SPT* is important in establishing the auxin-signaling response in the gynoecium.

Next, we tested the effect of cytokinin on the auxin-response reporter *DR5::GFP*. We treated *DR5* in wild type and the *spt DR5::GFP* mutant background with 100 µM BAP. In wild type treated plants, *DR5* signal conserved the same pattern, however, the auxin response showed a considerable increase in the presumptive provasculature cells and in the valves (Fig. 9f and Sup. Fig. 4). While in the *spt* mutant, an increase in the *DR5::GFP* signal was observed in presumptive provasculature cells, and septum primordia (Fig. 9g and Sup. Fig. 7).

Next, the effect of IAA on the auxin response in the gynoecium was evaluated. Whereas the IAA treated wild type plants, the *DR5* signal pattern remained constant (Fig. 9h and Sup. Fig. 5), the auxin response in the presumptive provasculature cells frequently showed a small increase in *DR5* signal (Fig. 9h and Sup. Fig. 5). In contrast, in the *spt* mutant gynoecia treated with IAA, severe abnormalities in the *DR5* distribution were observed, for instance the enhanced and expanded *DR5* signal in septum primordia (Fig. 9i and Sup. Fig. 7). All the data suggest a synergy between the two hormones in the CMM region and support the notion that *SPT* is important during this process.

SPT regulates the synthesis of auxin

It has been shown that *SPT* interacts with elements of the auxin pathway (Moubayidin and Ostergaard, 2014), however, it is unknown whether these interactions also occur in the medial domain of the gynoecium. The *TRYPTOPHANE AMINOTRANSFERASE OF ARABIDOPSIS 1* (*TAA1*) gene encodes an auxin biosynthesis enzyme and is expressed in the same region as *SPT* during early stages of gynoecium development (Fig. 9j) (Stepanova *et al.*, 2008). Therefore, we tested if *SPT* regulates local auxin production. In order to understand the interaction between auxin synthesis and *SPT,* we analyzed the *TAA1::TAA1:GFP* in the *spt-12* mutant background (Moubayidin and Ostergaard, 2014). These results show that at stage 9 wild type gynoecia, *TAA1* expression was detected in the adaxial medial domains, most strongly in septum primordia and ovule primordia (Fig.

9j and Sup. Fig. 3C). At stage 12 wild type gynoecia, *TAA1* signal was detected in ovules, vascular tissues and transmitting tract tissue (Sup. Fig. C). In *spt-12* gynoecia, *TAA1::TAA1:GFP* is still expressed, however, the *TAA1* expression showed a reduction in the septum primordia and transmitting tract tissue at late stages (Fig. 9k and Sup. Fig. 6A), which suggests the possibility that SPT regulates *TAA1* expression in septum primordia and the transmitting tract.

To test whether *TAA1* expression is dependent on SPT, we used a luciferase reporter assay in tobacco leaves (In collaboration with Patricia Ballester of the laboratory of Dr. Cristina Ferrándiz, CSIC, Valencia, Spain). Indeed, we found that transiently expressed *SPT* is able to activate the *TAA1::LUC* reporter (Fig. 9p), suggesting that SPT interacts with the *TAA1* promoter and can activate *TAA1* expression in septum primordia. To provide further support for SPT binding to the *TAA1* promoter in *vivo*, we will develop chromatin immunoprecipitation (ChIP) assays with an anti-Hemagglutinin (HA) antibody using *35S::SPT:HA* plants.

Cytokinin regulates the *TAA1* expression

The overlap in expression patterns between *TCS::GFP* and the *TAA1* gene and the recent demonstration that the auxin synthesis *TAA1* gene is a putative cytokinin-responsive gene (Bhargava *et al.*, 2013; Reyes-Olalde *et al.*, 2013), suggests an interaction between cytokinin signalling and auxin biosynthesis. To test whether *TAA1* is induced by cytokinin, inflorescence apices of *TAA1::TAA1:GFP* were treated with 100 µM BAP twice within a period of 48 hrs. In wild type treated plants, *TAA1-GFP* showed an expanded and increased signal (Fig. 9l and Sup. Fig. 4), suggesting the possibility that cytokinin treatment activates *TAA1* expression. To test whether cytokinin induction of *TAA1* is dependent on ARR1, we used a luciferase reporter assay in tobacco leaves (In collaboration with Patricia Ballester of the laboratory of Dr. Cristina Ferrándiz, CSIC, Valencia, Spain). Transiently expressed ARR1 was able to activate the *TAA1::LUC* reporter. This suggests that ARR1 interacts with the *TAA1* promoter and can activate *TAA1* expression (Fig. 9 p). To provide additional evidence for ARR1 binding to the *TAA1* promoter in *vivo*, we will develop

chromatin immunoprecipitation (ChIP) assays with an anti-rat glucocorticoid receptor (GR) antibody using *35S::ARR1ΔDDK:GR* plants (Sakai *et al.*, 2001).

Since we have found that *SPT* enables cytokinin signalling at the medial region of the ovary (Fig. 7 d, l),we also evaluated *TAA1* response to cytokinin in the absence of *SPT*. In contrast to wild type gynoecia, where *TAA1* is induced by cytokinin, in the *spt* background *TAA1* is not induced (Fig. 9m and Sup. Fig. 7). This suggests that SPT is able to activate *TAA1*.

In summary, these results indicate that cytokinin, through ARR1, can activate *TAA1* at the medial region of the ovary and that this activation requires *SPT* expression. Therefore, *ARR1* and *SPT* can integrate the cytokinin signalling pathway and auxin biosynthesis at the CMM and septa primordia.

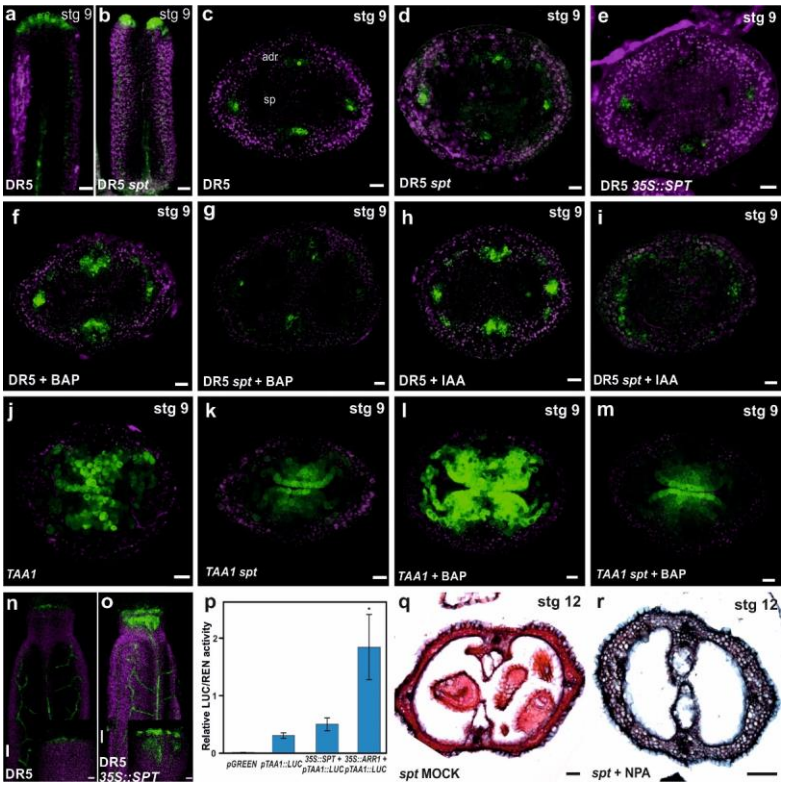


Figure 9. Cytokinin signalling and *SPT* regulate Auxin Distribution. a-i, Expression of the transcriptional reporter *DR5::GFP* line in wild type (a, c), in *spt-2* (b, d), and in *35S::SPT* (e). Wild type gynoecia treated for 48 hours with the synthetic cytokinin 6-benzylaminopurine (BAP) (f), *spt-2* treated for 48 hours with BAP (g). Wild type gynoecia treated for 48 hours with Indole 3-Acetic Acid (IAA) (h),

spt-2 treated for 48 hours with IAA (i). **j-m**, Expression of the translational fusion *TAA1::GFP–TAA1* in a transverse section of a stage 9 wild type gynoecium (j) and *spt 12* (k). Wild type gynoecia treated for 48 hours with the synthetic cytokinin 6-benzylaminopurine (BAP) (l), *spt-2* treated for 48 hours with BAP (m).**n-o**, *DR5::GFP* expression in stage-12 gynoecia of Wild type gynoecia (n) and *35S::SPT* (o). **p**, Ratio of LUC/REN activity in *N. benthamiana* leaves co-transformed with *35S::SPT* and *pTAA1::LUC*. **q, r**; *spt-2* gynoecium that received mock treatment (q) or 48 hours of NPA treatment (r). Error bars represent the s.d. for the LUC assays based on three biological replicates. *P < 0.05 (qRT-PCR and qPCR: ANOVA; LUC: Student-t test). Scale bars: 100 µm (q, r), 50 µm (n, o), 20 µm (a, b and insets in n, o), 10 µm (c-m).

NPA treatment partially rescued the ovary defects of *spt* mutants

It has been demonstrated that apical gynoecium defects in *spt* are restored when auxin homoeostasis changes with the addition of the auxin transport inhibitor 1-N-Naphthylphthalamic acid (NPA) (Nemhauser *et al.*, 2000). However, the effects of NPA in the ovary have not been evaluated, therefore, we investigated whether NPA affects ovary development in the *spt* mutant. In untreated *spt-2* gynoecia, the ovary showed severe disruption of septum and transmitting tract development. In contrast, in a preliminary experiment, in NPA-treated *spt* mutant gynoecia, the septum fusion is restored, as in the wild type (Fig. 9q, r). In summary, NPA treatment rescues the split style defects and possibly also the internal septum fusion.

***PIN3* is expressed in the CMM and septum primordia**

Intriguingly, the expression of TAA1 at the medial tissues of the ovary did not coincide with the expression of the auxin reporter DR5rev::GFP, which was not detected in these tissues (CMM, septa primordia, septum and transmitting tract) (Fig. 9 c and Sup. Fig. 3A). One possible explanation for this discrepancy is that the auxin synthesized by TAA1 is transported outside these tissues by PIN auxin efflux transporters. In order to test this, the spatial and temporal expression patterns of several PIN reporter lines (PIN1, PIN3, PIN4 and PIN7) were analyzed in stage 7-12 gynoecia (Sup. Fig. 8). We found that *PIN1* and *PIN3* are expressed in the medial region (Fig. 10a, b and Sup. Fig. 3A), however, *PIN3* is the only one that is expressed continuously in presumptive provasculature cells, septum primordia,

septum and transmitting tract (Fig. 10b and Sup. Fig. 3A). Therefore, *PIN3* is a strong candidate to be involved in CMM and septum development.

At stage 9, *PIN3* is expressed in the distal end of presumptive style-stigma zone and in the center of the gynoecium (Sup. Fig. 8). In transverse sections, *PIN3* is mainly localized in the CMM region such as provasculature cells, septum primordia and the ovule region (Fig. 10b and Sup. Fig. 3A). In valves fluorescence signal is detected in putative vasculature. At stage 12, *PIN3* signal was stronger in the replum and transmitting tract region and in valves the *PIN3* signal was retained in vasculature (Sup. Fig. 3A).

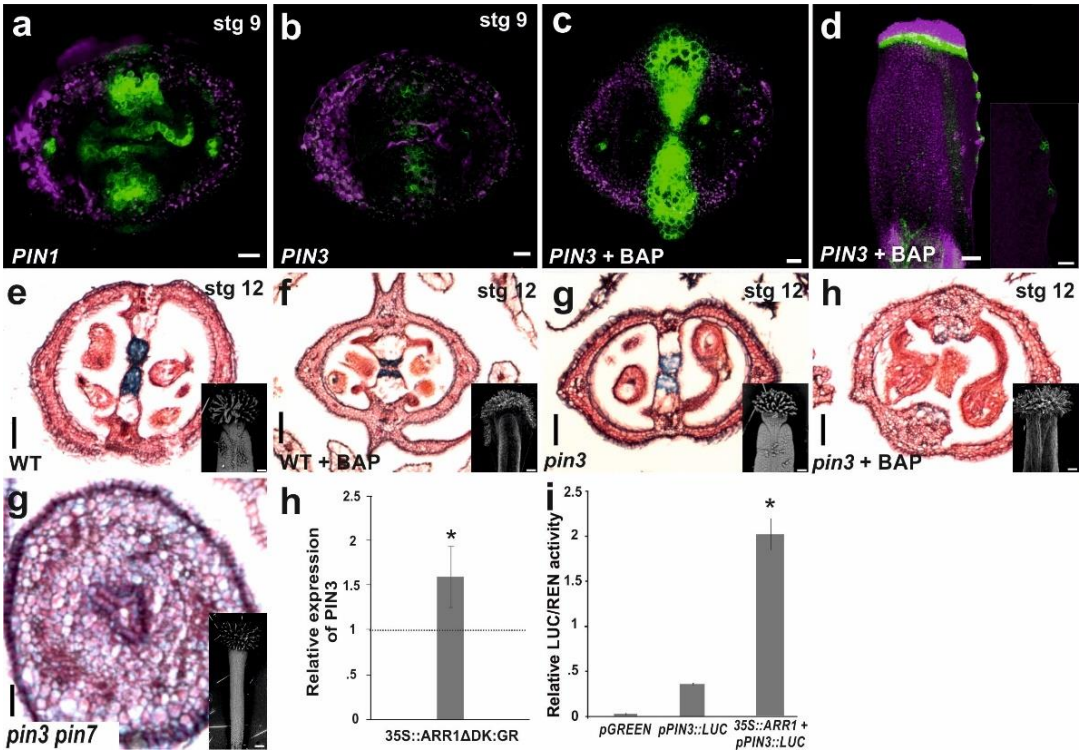


Figure 10. Cytokinin signalling is necessary for *PIN3* activation in the CMM. a, Expression of the translational fusion PIN1::PIN1-GFP line in a wild type stage 9 gynoecium. **b–d**, *PIN3* expression in transverse sections of stage 9 *PIN3-GFP* gynoecia that either received mock treatment (b) or 48 hours of BAP treatment (c), and in a longitudinal view after 48 hours of BAP treatment; the inset shows a magnified view of the proliferating tissue, arrows indicate the possible auxin flow (d). **e–h**, Transverse sections of stage 13 gynoecia of wild type (e) and *pin3-4* (g), phenotypes three to four weeks after wild type (f) and *pin3-4* (h) received applications of BAP for five days. Insets show a scanning electron microscopy image of the gynoecium. Observed gynoecia phenotypes in

the *pin3 pin7* double mutant (g). **h**, qRT-PCR of *PIN3* on *35S::ARR1ΔDDK:GR* with induction with dexamethasone and in the presence of cycloheximide. **i**, Ratio of LUC/REN activity in *N. benthamiana* leaves co-transformed with *35S::ARR1* and *pPIN3::LUC*. Error bars represent the s.d. for the LUC assays and for the qRT-PCR analyses based on three biological replicates. *P < 0.05 (qRT-PCR and qPCR: ANOVA; LUC: Student-t test). Scale bars: 100 µm (e–g, insets e–g) 20 µm (d) 10 µm (a-d).

PIN3 expression is involved in cytokinin response

The coexpression pattern of *TCS::GFP* and *PIN3* (Fig. 7c and Sup. Fig. 3A) suggests a possible cytokinin regulation. To shed light on this, we treated inflorescence apices of *PIN3::PIN3:GFP* with 100 µM BAP for 2 times during 48 hrs. One day after the BAP treatment, interestingly, increased *PIN3-GFP* fluorescence was observed in the epidermis cells of the CMM and septum primordia (Fig. 10c and Sup. Fig. 4).

Mutations in *PIN3* alter phototropism and gravitropism responses (Friml *et al.*, 2002; Ding *et al.*, 2011). However, little is known about its role in early gynoecium development. In order to determine the role of *PIN3*, we examined the effect on the development of the gynoecium in the *pin3-4* loss-of-function mutant. Stage 12 gynoecia of the *pin3-4* mutant apparently did not show significant differences from wild type (Fig. 10e, g). However, when we analyzed cross-sections of gynoecia, we observed that transmitting tract tissues were reduced in size as compared to the wild type and it had more intercellular spaces in the region of the septum (Fig. 10e, g). According to these observations, we reasoned that if PIN3 was required for the formation of the ectopic outgrowths from the repla, it would not be produced in a *PIN3* defective background. Indeed, only apical-basal defects were observed in 78.2% of the cases (n=330) when cytokinin (100 uM BAP) was applied to the *pin3* mutant (Fig. 10f, h and Sup. Fig. 9). In the other 21.8% of the gynoecia only a very minor effect was observed. The cross-sections showed an absence or diminished response to BAP (Fig. 10f, h and Sup. Fig. 9). Together, the data indicates an important role for PIN auxin transporters and in particular for *PIN3* in early gynoecium development. The expression pattern of *PIN3*, the increased *PIN3* expression induced by BAP, and the lack of BAP-induction of proliferating tissue in the *pin3* mutant, supports the notion that *PIN3* is

important for the cytokinin response in the CMM and septa primordia and probably acts downstream in the cytokinin perception.

Despite that the transmitting tract is defective in *pin3* gynoecia, the single *pin3* mutant does not show severe defects in CMM development, septum fusion or ovule development, which suggest functional redundancy with other PIN proteins. To test this, we analyzed the *pin3 pin7* double mutant. Flower and gynoecium defects have been reported for this double mutant (Benkova *et al.*, 2003). The *pin3 pin7* gynoecia showed increased apical-basal defects characterized by absence or diminished size of the valves, and sometimes multiple carpels were fused (Fig. 10g and Sup. Fig. 9). Gynoecia cross-sections showed severe defects in CMM development such as septum and placentae reduction or absence (Fig. 10g and Sup. Fig. 9).

To test whether cytokinin induction of *PIN3* is dependent on ARR1, the inducible dexamethasone (DEX) line *35S::ARR1ΔDDK:GR* (Sakai *et al.*, 2001) was used to analyze *PIN3* induction in inflorescence tissue. After 30 min of treatment with 10 uM dexamethasone in the presence of 10 uM cycloheximide (Fig. 10h), an increase in *PIN3* expression in the inflorescence of *ARR1ΔDDK:GR* plants was observed, which is compatible with BAP induction (Fig. 10c). To determine if ARR1 activates *PIN3* expression by directly binding to its promoter, we used a luciferase reporter assay in tobacco leaves (In collaboration with Patricia Ballester of the laboratory of Dr. Cristina Ferrándiz, CSIC, Valencia, Spain). Transiently expressed ARR1 was able to activate the *PIN3::LUC* reporter (Fig. 10i), suggesting that ARR1 interacts with the *PIN3* promoter and in turn activates *PIN3* expression. To provide further support for ARR1 binding to the *PIN3* promoter in vivo, we are developing chromatin immunoprecipitation (ChIP) assays with an anti-rat glucocorticoid receptor (GR) using *35S::ARR1ΔDDK:GR* plants (Sakai *et al.*, 2001).

SPT regulates *PIN3* expression in the CMM and septum primordia

Recently, *HEC,* a close relative of *SPT*, has been linked to auxin transport in style-stigma development (Schuster *et al.*, 2015). To investigate whether SPT could also activate the auxin transporter *PIN3* in the medial region, we analyzed *PIN3* expression in *spt-12* and

35S::SPT gynoecia by qRT-PCR (Fig. 11a). The results showed a decrease in PIN3 expression in the *spt* background and an increase in the *35S::SPT* background (Fig. 11a), suggesting a positive regulation. In order to analyze the effects of *SPT* on *PIN3*, we crossed the *PIN3::PIN3:GFP* with the *spt-2* mutant and the *35S::SPT* line. In contrast to wild type, the *spt-2* mutant showed a significant reduction in PIN3-GFP signal in the center of the gynoecium and in the style-stigma, where it is limited to two spots in the lateral domains (Fig. 11c and Sup. Fig. 6A). Transverse sections revealed that the PIN3-GFP signal was only present in the presumptive provasculature cells in the carpel walls and was absent in the septum region. This pattern was preserved from gynoecium stage 8 to 12 (Fig. 11c and Sup. Fig. 6A). On the other hand, the PIN3-GFP signal was increased in the *35S::SPT* line since early stages (Fig. 11d and Sup. Fig. 6A), and this pattern was preserved from gynoecium stage 8 to 12 (Sup. Fig. 6A). This data suggest that SPT function is necessary for proper *PIN3* expression in the CMM and septa primordia.

However, these observations raised a key question: Is SPT a central mediator of the cytokinin-*PIN3* induction in the CMM and septa primordia? To test this, *spt-2 PIN3::PIN3:GFP* and wild type inflorescence apices were treated with 100 uM BAP twice in 48 hrs. In treated wild type, PIN3-GFP signal was observed in the septa primordia and the ectopic outgrowths from the medial region (Fig. 11e and Sup. Fig. 4). In contrast, treated *spt-2* PIN3-GFP mutant gynoecia did not show an increased signal, only an increased GFP signal restricted to the presumptive provasculature cells (Fig. 11e and Sup. Fig. 7). Interestingly, BAP treated *35S::SPT PIN3::PIN3:GFP* plants did not show an increased signal compared to untreated *35S::SPT PIN3::PIN3:GFP* (Sup. Fig. 7). This data suggests that SPT connects the cytokinin-signaling pathway with auxin transport at the medial region.

To determine whether SPT may directly mediate *PIN3* expression, we used a transient luciferase reporter assay in tobacco leaves (in collaboration with Patricia Ballester of the laboratory of Dr. Cristina Ferrándiz, CSIC, Valencia, Spain). In the luciferase reporter assay, SPT was able to activate a *PIN3::LUC* reporter (Fig. 11i), suggesting that SPT interacts with the *PIN3* promoter and can activate *PIN3* expression. We then confirmed in *vivo* binding of SPT to *PIN3* promoter sequences by ChIP assays. ChIP assays revealed that SPT is able to

bind to a region in the *PIN3* promoter that contains a G-box motif (Fig. 11j) (In collaboration with Dr. Dario Paolo and Dr. Ignacio Ezquer of the laboratory of Dr. Lucia Colombo, University of Milan, Italy). Together, this indicates that SPT is a direct regulator of *PIN3* expression and links cytokinin signalling to auxin transport in the gynoecium.

To know whether auxin itself can directly influence *PIN3* gene expression, inflorescence apices of *PIN3::PIN3:GFP* plants (Fig. 11g and Sup. Fig. 5) and in the *spt* mutant background (Fig. 11h and Sup. Fig. 7) were treated with 100 µM IAA twice in 48 hrs. In both cases, PIN3 expression was reduced or absent. This data suggests that *PIN3* is sensitive to auxin, but that this is independent of SPT.

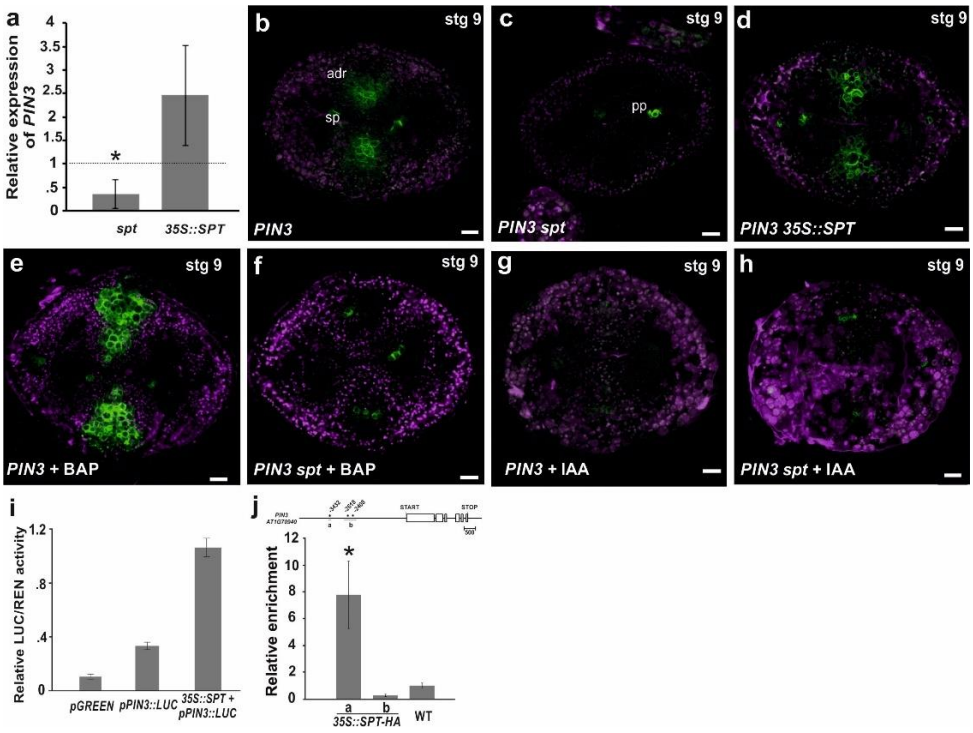


Figure 11. Cytokinin signalling and SPT are necessary for *PIN3* activation in CMM. a, qRT-PCR of *PIN3* in dissected gynoecia from *spt-12* and from *35S::SPT*. **b-h**, Expression of the translational fusion *PIN3::PIN3-GFP* line in wild type (b), in *spt-2* (c), and in *35S::SPT* (d). Wild type gynoecia treated for 48 hours with the synthetic cytokinin 6-benzylaminopurine (BAP) (e), *spt-2* treated for 48 hours with BAP (f). Wild type gynoecia treated for 48 hours with Indole 3-Acetic Acid (IAA) (g), *spt-2* treated for 48 hours with IAA (h). **i**, Ratio of LUC/REN activity in *N. benthamiana* leaves co-transformed with *35S::SPT* and *pPIN3::LUC*. **j**, A ChIP experiment against the *PIN3* promoter region using a *35S::SPT-HA* line. *ACT2/7* served as a negative control. Error bars represent the s.d.

for the LUC assays and for the qRT-PCR analyses based on three biological replicates. The ChIP result of one representative experiment is shown and the error bars represent the s.d. of the technical replica. *P < 0.05 (qRT-PCR and qPCR: ANOVA; LUC: Student-t test). Scale bars: 10 µm (b-h).

AHP6 and ARR16 are negative modulators of cytokinin signalling in the gynoecium

One might still wonder why *TCS* is expressed only in the CMM and septa primordia and not in the valves, and why no outgrowths can be induced from the valves by cytokinin applications. It can be hypothesized that repressors of cytokinin signalling should be present in the valves. The first we identified is the cytokinin signalling repressor *AHP6* (Mahonen *et al.*, 2006), which has been shown to be part of auxin-cytokinin interaction in other tissues (i.e., it is induced by auxin and it represses cytokinin signalling) (Mahonen *et al.*, 2006; Bishopp *et al.*, 2011). Using an *AHP6::GFP* line (Besnard *et al.*, 2014), we observed that *AHP6* is specifically expressed in the valves (or presumptive valves) at gynoecia stages 6-9, and afterwards, in gynoecia stages 10-12, *AHP6* expression is reduced (Fig.12a and Sup. Fig. 3). It has been demonstrated that auxin activates the expression of the *AHP6* in roots and in the SAM (Bishopp *et al.*, 2011; Besnard *et al.*, 2014). To investigate whether auxin also activates the expression of the *AHP6* during early gynoecium development, we treated inflorescence apices of wild type *AHP6::GFP* plants with 100 µM IAA twice in 48 hrs. The *AHP6* expression in the valves was either unchanged or even decreased (Sup. Fig. 5). Next, we evaluated the effect of 100 µM BAP on *AHP6* expression. BAP treated plants showed decreased *AHP6* expression in gynoecia stages 9-12 (Sup. Fig. 4). This means that the relation between auxin-cytokinin and *AHP6* expression is more complex in the gynoecium.

AHP6 functions as a cytokinin signalling repressor in the root and SAM (Mahonen *et al.*, 2006; Besnard *et al.*, 2014). Therefore, we analyzed the effects of *ahp6* in cytokinin signaling. For this, we analyzed *ahp6 TCS::GFP* gynoecia (Besnard *et al.*, 2014). Interestingly, the *ahp6* mutant presented a clear change in the *TCS::GFP* pattern, characterized by a strong TCS::GFP signal in the valves (Fig. 12), coinciding with the idea that AHP6 restricts cytokinin signalling to the medial region. The second repressor

identified is the type-A *ARR16*, which forms a negative feedback to the cytokinin signalling pathway (Schaller *et al.*, 2015). *ARR16::GUS* activity was also observed in the valves (Fig. 12c). Moreover, we monitored *ARR16::GUS* plants treated with 100 µM BAP for 48 hrs. We found that the *ARR16* expression was increased, as expected for a type-A *ARR* gene (Sup. Fig. 4). Finally, to know more about the role of ARR16, we analyzed its expression in *spt-12* and *35S::SPT* plants. The qRT-PCR experiments using gynoecia showed an increase in *ARR16* expression in the *spt* background and a decrease in a *35S::SPT* background (Fig. 12d). These results are surprising, because the opposite is expected. Further investigation should be done at the tissue level by making crosses with the ARR16 marker line to *spt*.

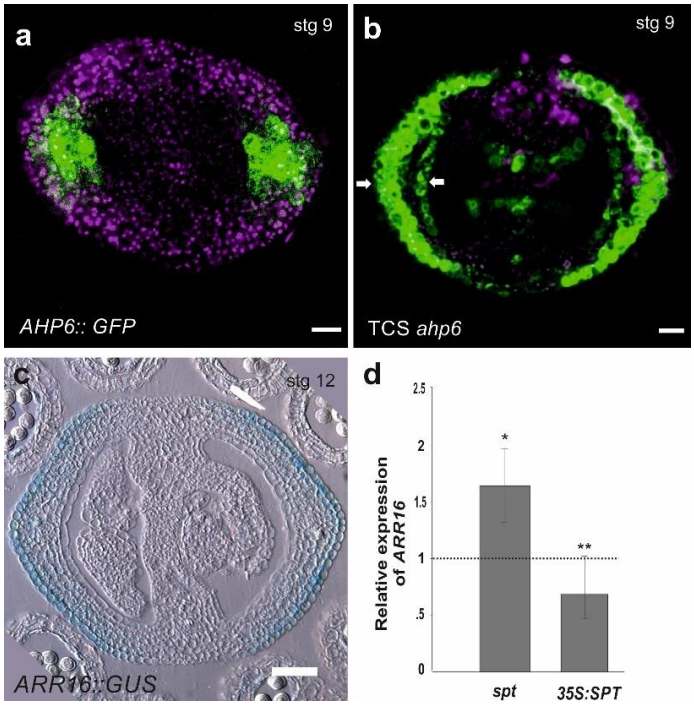


Figure 12. Expression of the cytokinin signalling repressors *AHP6* and *ARR16*. a, Expression of the transcriptional reporter *AHP6::GFP* in a transverse section of a stage 9 gynoecium. **b**, Expression of the transcriptional reporter *TCS::GFP* in a transverse section of a stage 9 gynoecium in an *ahp6-1* background. **c**, Expression of the transcriptional reporter *ARR16::GUS* in a transverse section of a stage 9 gynoecium. **d**, qRT-PCR of *ARR16* in dissected gynoecia from *spt-12* and from *35S::SPT*. *P < 0.05 (qRT-PCR and qPCR: Student-t test) Scale bars: 10 µm (a–c).

7.2 *ETTIN* (*ARF3*) is a repressor of cytokinin signalling in the medial region

ETT is a repressor of cytokinin signaling

In the *ett* mutant, the gynoecium often develops outgrowths from the ovary. These outgrowths exhibit stylar and stigmatic features (Sessions and Zambryski, 1995) (Fig. 13b). Interestingly, these outgrowths strikingly resemble the morphology seen in plants treated with exogenous cytokinin (Marsch-Martinez *et al.*, 2012b) (Fig. 13c). On the other hand, when we treated the overexpression *35S::ETT* line with exogenous cytokinin, the gynoecia frequently (80%, n=12) did not display outgrowths at the ovary region, although it developed ectopic ovules in the stigma region (Fig. 13d). This data suggests a connection between *ETT* and cytokinin.

To explore a possible interaction between *ETT* and cytokinin, the effect of cytokinin on *ETT* expression was monitored using the marker line *ETT::GFP* (Rademacher *et al.*, 2011). In control plants, strong ETT::GFP signal was observed in the valves and the replum, forming a ring during gynoecium development (Fig. 13e and Sup. Fig. 3B). In gynoecia treated with 100 µM BAP for 2 times in 48 hrs. the ETT::GFP signal was decreased in the replum region (Fig. 13f and Sup. Fig. 4), suggesting that cytokinin might negatively regulate *ETT* expression in this region.

The phenotype of *ett* and the response of *ETT::GFP* to cytokinin applications strongly suggest a relationship between *ETT* and cytokinin signaling. To test this further, we crossed the cytokinin reporter *TCS::GFP* line with the *ett-2* mutant. In wild type stage 12 gynoecia, *TCS::GFP* expression is confined to the septum and the valve margin region (Fig. 13g and Sup. Fig. 3A). In contrast, in *ett* mutant gynoecia the TCS::GFP signal was increased in the medial region and moreover, expanded, now also visible in the valves and in the cytokinin-induced ectopic outgrowths produced from the medial region (Fig. 13h and Sup. Fig. 6B). In the mature *ett* gynoecia (stage 12), TCS::GFP signal was not in the valves, but strong TCS::GFP signal was observed in the ectopic outgrowths (Fig. 13h). Since in *ett* gynoecia the *TCS* intensity and pattern were altered, we tested whether the opposite effect occurs in a *35S::ETT* overexpression line. Indeed, the TCS::GFP signal

showed a reduction in the septum and the valve margin region in *35S::ETT* gynoecia compared to the *ett* mutant gynoecia. No TCS::GFP signal in valve tissue in *35S::ETT* gynoecia was observed, as seen in wild type gynoecia (Sup. Fig. 6B). This data indicates that *ETT* is an important repressor of cytokinin signalling in the tissues where it is expressed during early gynoecium development.

ETT is a transcription factor that binds with specificity to AuxREs in the auxin response DR5 reporter, so we analyzed *DR5::GFP* in the *ett* mutant and the *35S::ETT* background. In wild type at stages 8-12, the *DR5* signal is expressed in the presumptive provasculature cells in the medial domain (Fig. 13i and Sup. Fig. 3A). In *35S::ETT* plants the DR5 signal is increased in the provasculature cells of the medial region (Fig. 13j); this pattern continued till stage 12 (Sup. Fig. 6B). Interestingly, we did not observe ectopic expression of the DR5 signal in *35S::ETT* gynoecia, which suggests that the effect of *ETT* is restricted only to the region where it is expressed in the gynoecium. Since flowers of the *ett* mutant are female sterile, pollen from homozygous *ett* plants was used for crosses with a plant homozygous for *DR5::GFP.* This experiment is in progress.

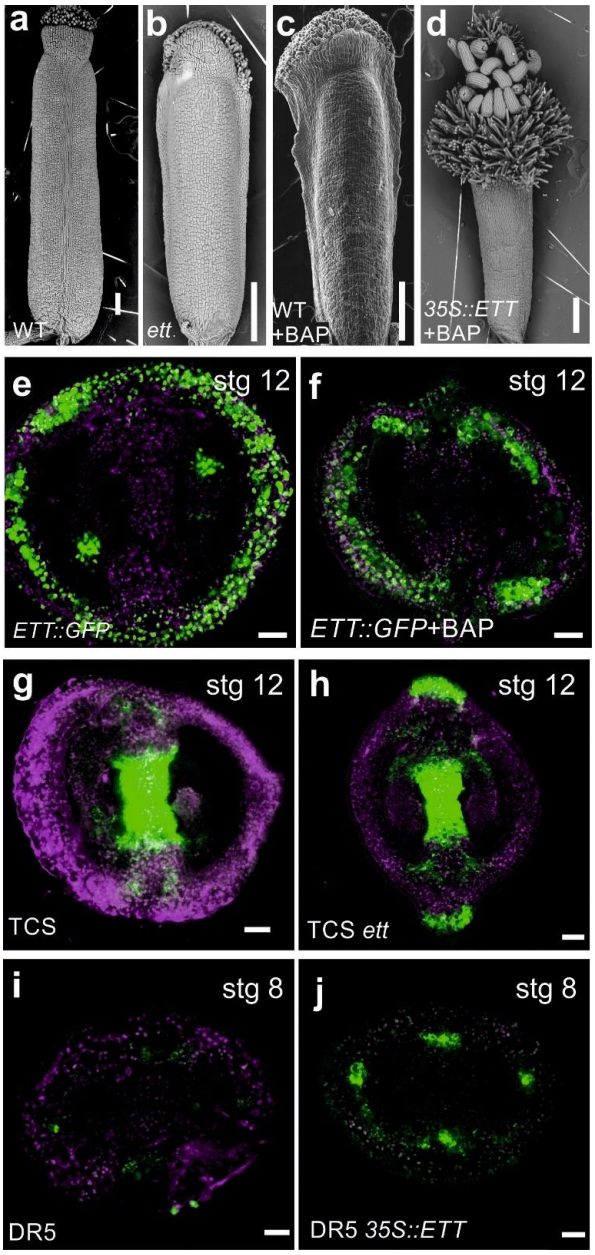


Figure 13. *ett* mutant is affected in cytokinin response. a-d. Scanning electron microscopy image of Phenotypes of the *ett* mutant (b) compared to wild type (c) and *35S::ETT* (d) gynoecia three to four weeks after receiving a BAP treatment for five days. **e,f**, Expression of the transcriptional reporter *ETT::GFP* line in transverse sections of stage 12 wild type gynoecium that either received mock treatment (e) or 48 hours of BAP treatment (f). **g, h.** Expression of the transcriptional reporter *TCS::GFP* in transverse sections of stage 12 gynoecia of wild type (g) and *ett-2* mutant (h). **i-j**, Expression of the transcriptional reporter *DR5::GFP* line in wild type (i), and *ett-2* (j). Scale bars: 100 µm (a-d), 20 µm (e-h), 10 µm (i, j).

SPATULA is a repressor of *ETTIN* expression

SPT is an important gene involved in CMM development (Alvarez and Smyth, 2002), and of cytokinin signalling (according to our results). In other reports, there is evidence of negative regulation of *SPT* by *ETT* (Heisler *et al.*, 2001). Several putative Auxin Response Elements (AuxREs) in conserved regions in the *SPT* promoter have been characterized (Sessions *et al.*, 1997; Groszmann *et al.*, 2010), suggesting a direct regulatory role of ETT over *SPT*. To test this, we will use the luciferase reporter assay in tobacco leaves, using a *35S::ETT* construct and the 2 kb *SPT* promoter that contains three conserved AuxREs.

In this work, it was demonstrated that cytokinin affects the expression of *SPT* (Fig. 8o-p). Thus we tested whether cytokinin induces *SPT* expression in ectopic outgrowths. To test this, we treated inflorescence apices of the *SPT::GUS* line with 100 µM BAP for 5 days. In mock gynoecium, *SPT* expression is limited to septum, transmitting tract and ovules (Sup. Fig. 3A). After BAP treatment, *SPT* expression showed an altered pattern, displaying increased intensity in the transmitting tract and ovules, and notably *SPT* is also expressed in the cytokinin-induced ectopic outgrowths (Fig. 14a), which will be interesting to follow-up. On the other hand, we also hypothesized that SPT may regulate *ETT* expression. For this, we analyzed *ETT* expression in the *spt* mutant and in the *35S::SPT* line. The qRT-PCR results showed that *ETT* expression was modestly induced in the *spt* mutant and repressed in the *35S::SPT* overexpression line (Fig. 14b). To further analyze the spatial and temporal expression of *ETT* in *spt,* a cross was made between the reporter line *ETT::GFP* (Rademacher *et al.*, 2011) and the *spt-12* mutant. Segregating F2 plants will be analyzed by PCR (genotyping) with specific primers for the *spt* mutation (Table 1) and afterwards analyzed by confocal microscopy.

So far, this data suggests that *SPT* negatively regulates *ETT*. To test whether this might be direct, we examined the *ETT* promoter and found one G-box motif in the first intron and one G-box sequence in the promoter region approximately 8257 bp upstream of the ATG. To confirm binding of SPT to the *ETT* promoter we performed a ChIP assay with a monoclonal anti-HA in the *35S::SPT-HA* line (In collaboration with Dr. Ignacio Ezquer of the

laboratory of Dr. Lucia Colombo, University of Milan, Italy). We found enrichment of a region at the *ETT* promoter containing a G-box (Fig. 14c), supporting that SPT can directly regulate *ETT*.

The *ETT* and *SPT* genes are coexpressed in the replum region during early gynoecium development, with *ETT* expressed in the abaxial replum and *SPT* expressed in the whole CMM, septa primordia and replum region (Sup. Fig. 3A, B) (Sessions *et al.*, 1997; Groszmann *et al.*, 2010). The coincident expression patterns of *ETT* and *SPT* in the replum region might suggest a protein-protein interaction. To investigate this hypothesis, a Bimolecular Fluorescence Complementation assay (BiFC) in tobacco leaves using the C-terminal portion of the yellow fluorescent protein (YFP) fused to SPT (SPT-YFPc) and ETT fused to the N terminal portion of the YFP (ETT-YFPn) was performed. Interestingly, the assay resulted in fluorescence signal, but this was observed outside the nucleus, suggesting a negative posttranscriptional regulation between ETT and SPT (Fig. 14d).

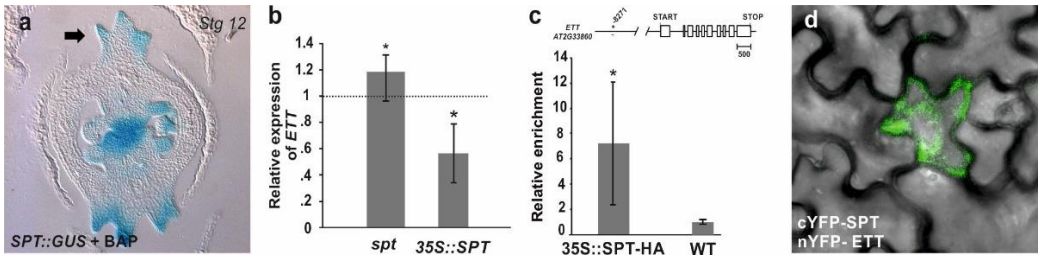


Figure 14. SPT is a repressor of *ETT*. a, Expression of the transcriptional reporter *SPT::GUS* in a transverse section of a BAP-treated gynoecium. **b**, qRT-PCR of *ETT* in dissected gynoecia from *spt-12* and from *35S::SPT.* **c***,* A ChIP experiment against the *ETT* promoter region using a *35S::SPT-HA* line. *ACT2/7* served as a negative control. **d**, Bimolecular fluorescence complementation (BiFC) assay of SPT with ETT in *N. tabacum* leaves, where no interaction in nucleus is detected. Error bars represent the s.d. for the qRT-PCR analyses based on three biological replicates . The ChIP result of one representative experiment is shown and the error bars represent the s.d. of the technical replicates. *P < 0.05 (qRT-PCR and qPCR: ANOVA). Scale bars: 50 µm (a, d).

7.3 New Role of *SHOOT MERISTEMLESS* (*STM*) in Medial Region Development

STM is expressed in cytokinin-induced outgrowths

Previous reports have already demonstrated that prolonged BAP application causes ectopic outgrowths from the repla (Marsch-Martinez *et al.*, 2012b). However, little is known about the molecular mechanisms of this process. The *STM* gene is a transcription factor that plays an essential role in the maintenance and generation of the SAM and the CMM through induction of cytokinin biosynthesis (Jasinski *et al.*, 2005; Yanai *et al.*, 2005; Scofield *et al.*, 2007). The overlapping expression between *STM* and *TCS* in the early gynoecium (Long *et al.*, 1996; Marsch-Martinez *et al.*, 2012b), suggests a connection between *STM* and cytokinin signalling during gynoecium development and the ectopic outgrowth formation.

In order to test whether cytokinin induces *STM* expression in ectopic outgrowths, we treated inflorescence apices of the *STM::GUS* line with 100 µM BAP for 5 days. In mock gynoecia, *STM* expression is limited to the abaxial replum and septum primordia at stage 7-8, but decreases in septum primordia at stage 9, where it is confined to the replum zone (Fig. 15a and Sup. Fig. 3A). After BAP treatment of inflorescence apices for 5 days, *STM* expression showed a different pattern, displaying an increased intensity in the CMM and in the ectopic outgrowths (Fig. 15b and Sup. Fig. 4). *STM* expression in the ectopic outgrowths might suggest a role in the induction of the ectopic outgrowths. To test this, we treated inflorescence apices of the weak *stm-2* allele, which exhibits a weak phenotype. Interestingly, the treated *stm-2* gynoecia did not develop ectopic outgrowths and displayed extensive tissue proliferation at the stigma region (Fig. 15c).

STM expression is independent of SPT activation

As mention above, *SPT* is important for CMM and septum development (Heisler et al., 2001), and for cytokinin-induced ectopic outgrowths (Marsch-Martinez et al., 2012b). *SPT* is expressed in the medial domain, where the septum arises at stage 8-9 and, in stage 10-12, the expression is confined to the transmitting tract and ovule primordia (Heisler *et al.*,

2001) (Fig. 15d and Sup. Fig. 3A). In BAP treated inflorescence apices, *SPT* is expressed in the ectopic outgrowths in a pattern that closely mirrored that of *STM* (Fig. 15e and Sup. Fig. 4). The overlapping expression between *STM* and *SPT* in the CMM and ectopic outgrowths suggests a close connection between these genes. One possibility is that *SPT* could be participating in the transcriptional regulation of *STM* as it does with *ARR1* (Fig. 8i-l). To test this possibility, we performed a qRT-PCR in dissected gynoecia of the *spt-12* mutant and the *35S::SPT* overexpression lines and found that the mRNA level of *STM* was not much affected (Fig. 15g). On the other hand, it is also possible that STM can activate *SPT* expression. To test this, we will characterize *SPT* expression in the STM dexamethasone (DEX)-inducible line (*35S::STM-GR*).

To broaden our understanding in the interaction between STM and SPT, we performed a yeast-two-hybrid (Y2H) assay to test whether a protein–protein interaction occurs. We first tested whether the fusion of STM with the GAL4 binding domain (BD-STM) had autoactivation. A low level of autoactivation was detected (Fig. 15h), which was suppressed with 5 mM of 3-Amino-1, 2, 4-triazole (3-AT). Results of the Y2H assay suggested a weak interaction between STM and SPT, since very faint blue staining (Fig. 15h) was observed. To confirm these results, we performed a bimolecular fluorescence complementation assay (BiFC). The transient co-expression of STM fused to the C-terminal portion of the yellow fluorescent protein (YFP) (STM-YFPc) and SPT fused to the N terminal portion of the YFP (SPT-YFPn) in tobacco epidermal cells did not show fluorescence complementation, inside or outside the nucleus. These results demonstrate that no significant interaction occurs between STM and SPT in *planta* (Fig. 15i).

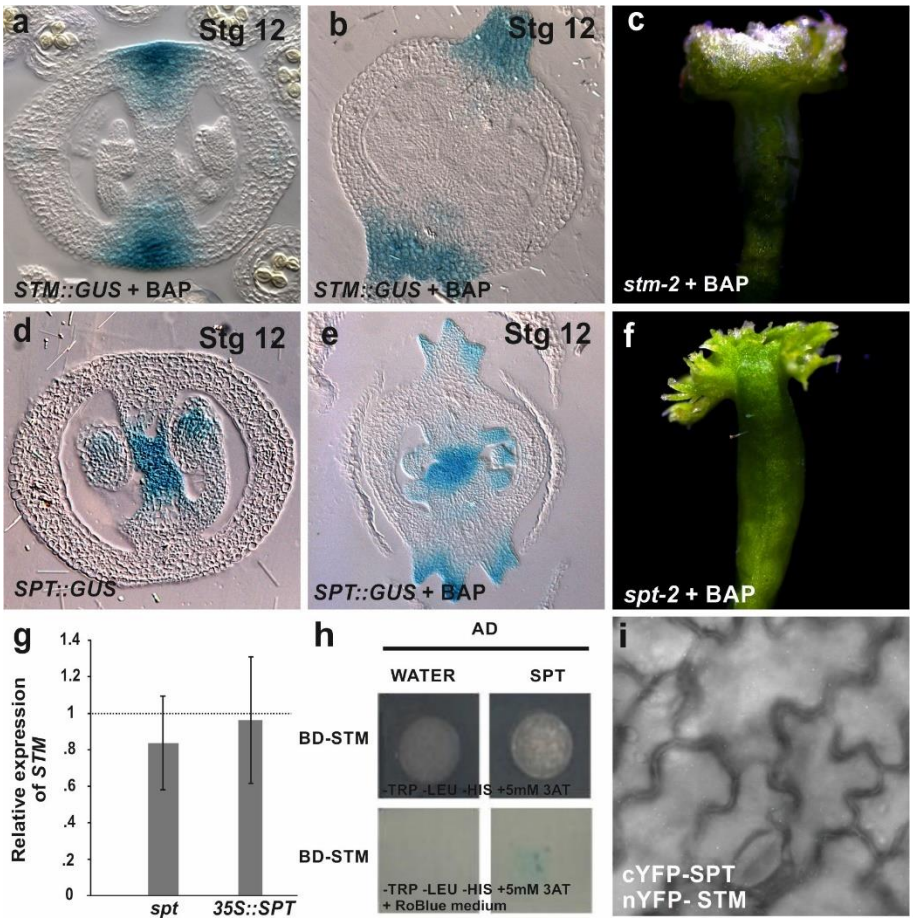


Figure 15. STM and SPT are necessary for cytokinin response. a, b, Expression of the transcriptional reporter *STM::GUS* in transverse sections of mock gynoecia (a) and BAP-treated (b) stage 12 gynoecia. **d-e,** Expression of the transcriptional reporter *SPT::GUS* in transverse sections of ovaries of mock-treated (d) and BAP-treated (e) stage 12 gynoecia. **c, f**; *stm-2* (c) and *spt-2* (f) gynoecia three to four weeks after receiving a BAP treatment for five days. **g**; qRT-PCR of *STM* in dissected gynoecia from *spt-12* and from *35S::SPT*. **h**; Yeast two-hybrid assay with SPT fused to the GAL4 DNA binding domain in combination with STM. **i**, Bimolecular fluorescence complementation (BiFC) assay of SPT with STM in *N. tabacum* leaves, where no interaction in nucleus is detected. Error bars represent the s.d. for the qRT-PCR analyses based on three biological replicates. *P < 0.05 (qRT-PCR and qPCR: ANOVA) Scale bars 5 mm (c, f), 100 µm (a, b, d, e), 50 µm (i).

7.4 *CRC* Regulates Auxin Response in Carpel Medial Region

CRC encodes a transcription factor belonging to the YABBY protein family and mutations in the gene cause severe alterations in carpel development; characterized by a reduced amount of style tissue and loss of PG fusion at the apex due to the unfused septum (Bowman *et al.*, 1999; Bowman and Smyth, 1999). Interestingly, the *crc* mutant has a striking resemblance with the *spt* mutant (Alvarez and Smyth, 2002), suggesting the possibility that the *crc* mutant has also defects in cytokinin signalling, similarly to the *spt* mutant.

First, we evaluated the cytokinin response of the *crc* mutant through treating inflorescence apices with 100 µM BAP for 2 and for 5 days. The newly opened flowers were examined after 3-4 weeks of treatment. Wild type fruits treated for two days did not show apparent phenotypes at the outside (Fig. 16b). However, in cross-sections of these fruits, the septum and transmitting tract region showed a significant widening in these tissues (Fig. 7o). After 5 days of BAP treatment, wild type gynoecia often developed outgrowths from the replum observed 3-4 weeks after the treatment, as previously described (Marsch-Martinez *et al.*, 2012b). In *crc* gynoecia treated for 2 days with 100 µM BAP, partial fusion of the apical region with light suppression of the stylar split phenotype was observed (Fig. 16d, f). Observations of cross-sections of the ovary indicated a significant widening and increase of the septum and transmitting tract tissue (Fig. 16g, h). Remarkably, a partial rescue of the postgenital fusion defect was observed (Fig. 16g, h). Interestingly, *crc* gynoecia treated with 100 µM BAP for 5 days, and observed after 3-4 weeks, developed overproliferation with carpelloid features and with various degrees of fusion, stigmatic papillae and ovule-like structures (Fig. 16i-k). This data suggests a relationship between *CRC* and cytokinin signalling.

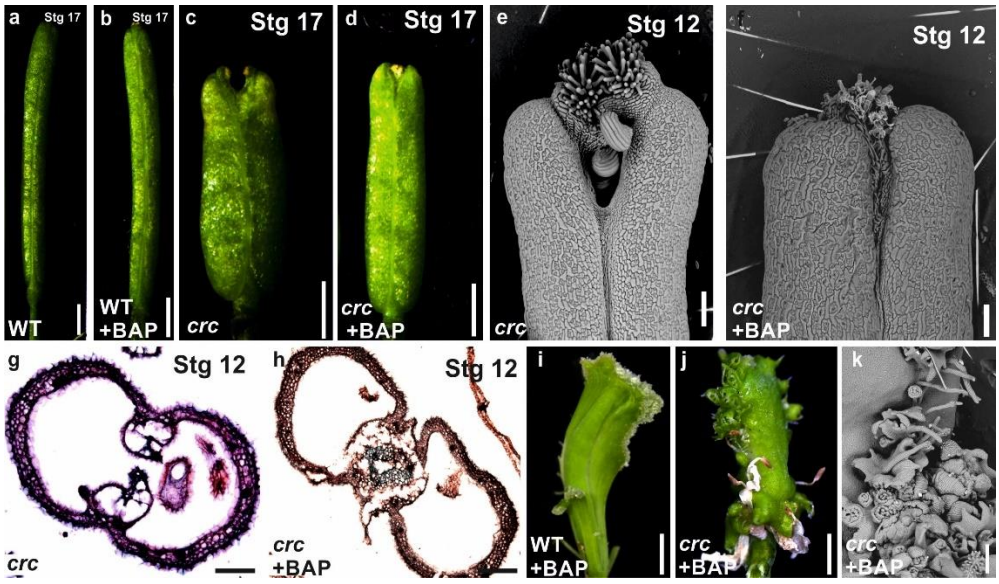


Figure 16. Restoration of style development by exogenous BAP application. a-d, Phenotypes of the wild type and *crc-1* stage 12 gynoecia one day after either receiving mock treatment (a, c) or 48 hours of BAP treatment (b, d). **e, f**, Scanning electron micrographs of mock-treated *crc-1* (e) and *crc -1* treated for 48 hours with BAP (f). **g, h**; Transverse section of the ovary of mock-treated *crc-1* (g) and *crc -1* treated for 48 hours with BAP (h). **i, j**, wild type (i), *crc-1* (j) gynoecia, three to four weeks after receiving BAP treatment for five days. **k**, Scanning electron micrographs of *crc-1* gynoecia after receiving a BAP treatment. Scale bars: 5 mm (a-d, i, j), 100 µm (e-h, k).

To test this, the *TCS::GFP* line was crossed with the *crc* mutant. In wild type at stage 9, the *TCS* signal is expressed in the CMM, specifically in the septum primordia and provasculature cells region, as described above (Fig. 17a). In the provasculature cell region fluorescence is seen as a ring encircling the presumptive provasculature cells (Fig. 17a). In septum primordia the TCS::GFP signal is restricted to the edges of the putative progenitor cells of the septum. This data is consistent with previous analysis (Fig. 17a) (Marsch-Martinez *et al.*, 2012b). At stage 12, TCS::GFP signal is detected in the valve margins, transmitting tract and funiculi (Fig. 17c). In *crc*, the *TCS* did not show changes in the expression pattern in early stage 9 or at late stage 12 as compared to the control. However, in late 12 stage, we observed a moderate increase in the TCS::GFP signal in the valve margins (Fig. 17b, d). The above results indicate that *CRC* function is not necessary to activate the cytokinin signalling pathway in the CMM and septum region.

Another important phytohormone is auxin, which acts either synergistically or antagonistically with cytokinin during the development of the gynoecium. To test whether the *crc* mutant is affected in auxin signalling, we crossed the *DR5::GFP* with the *crc* mutant. The results showed that in wild type at stage 9, the *DR5* signal is detected in the apical tips of the gynoecium. Observations of transverse sections confirmed that the signal is detected in the presumptive provasculature cells of the medial region, as well as in the valves and ovule primordia (Fig. 17e). This pattern persists until late stage 12 (Fig. 17g). In contrast, during early stages of gynoecium development in the *crc* mutant, *DR5::GFP* showed a drastically decreased signal in the presumptive provasculature and cells of the CMM and valves (Fig. 17f, h). The decreased signal of *DR5::GFP* in *crc* suggests that *CRC* has a role in auxin homeostasis.

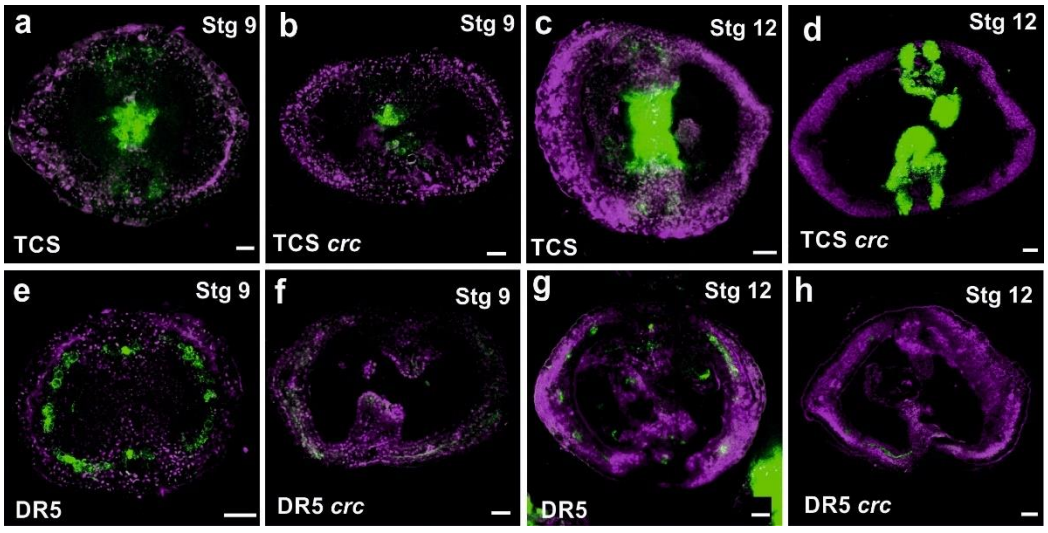


Figure 17. *CRC* expression mediates auxin response at the CMM. a-d, Expression of the transcriptional reporter *TCS::GFP* in transverse sections of stage 9 gynoecia of wild type (a), *crc-1* (b), and stage 12 gynoecia of *TCS::GFP* wild type (c), *crc-1* (d). **e-h**, Expression of the transcriptional reporter *DR5::GFP* in transverse sections of stage 9 gynoecia of wild type (e), *crc-1* (e), and stage 12 gynoecia of *TCS::GFP* wild type (g), *crc-1* (h). Scale bars: 20 µm (c, d, g, h), 10 µm (a, b, e, f).

PIN proteins play an important role in the establishment of auxin homeostasis (Blilou *et al.*, 2005; Grieneisen *et al.*, 2007). In the gynoecium, *PIN1* and *PIN3* are expressed in the CMM and derived tissues (this thesis). We tested whether the defects in septum growth

was due to PIN expression alterations. To test this, *PIN1::PIN1:GFP* and *PIN3::PIN3:GFP* lines were crossed with the *crc-2* mutant. The results of both crosses showed the typical pattern of expression of *PIN1* and *PIN3* expression pattern compared to wild type (Fig. 18a-f). On the other hand, PIN1/PIN3 proteins are important for the auxin-signalling at the style-stigma region, presumably by directing the flow of auxin to the apex (Larsson et al., 2014; Moubayidin and Ostergaard, 2014). Thus, we analyzed the cross of *pin3-4* with the *crc* mutant. Surprisingly, the *pin3 crc* double mutant partially restored the apical carpel fusion defect observed in the single *crc-1* mutant (Fig. 18i). However, other characteristics such as septum fusion defects and shorter gynoecium are unchanged (Fig. 18l). This suggests that *CRC* plays a role in auxin homeostasis, maybe through the regulation of auxin signalling.

ARF (Auxin Response Factor) proteins are important transcription factors involved in auxin response. They promote the activation or the repression of auxin-responsive genes (Teale *et al.*, 2006). *ETT* is an important ARF (ARF3) involved in auxin response in the gynoecium, which shows an abaxial expression pattern similar to *CRC* (Bowman and Smyth, 1999) Moreover, *CRC* and *ETT* acted together to specify the gynoecium abaxial-identity (Eshed *et al.*, 1999). These observations and the observed *DR5* response defects in *crc* suggest that *CRC* has an important role in auxin signalling, possibly through *ETT* activation.

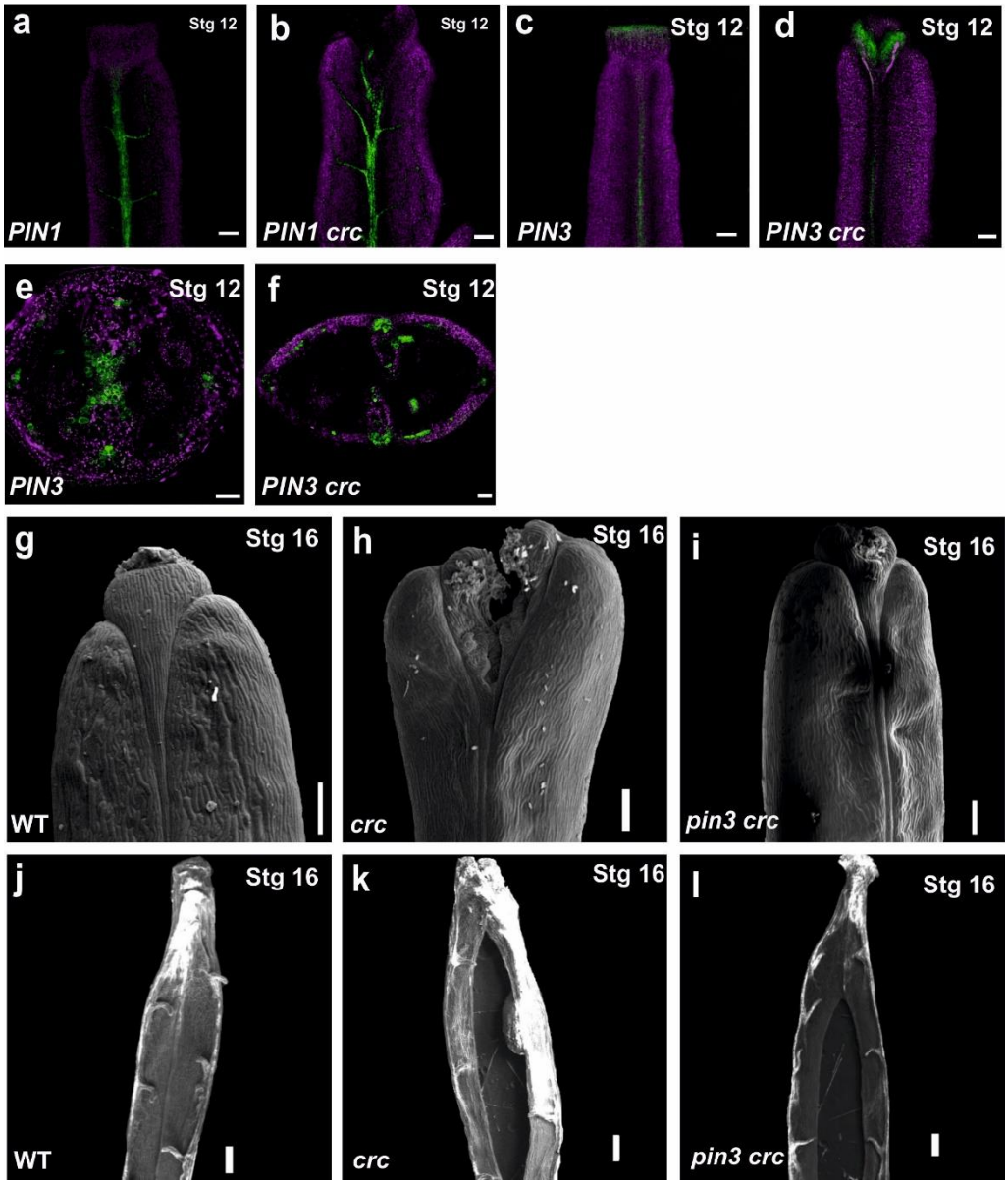


Figure 18. *CRC* expression is not necessary for activation of PIN proteins in the CMM. a-b, Expression of the translational fusion *PIN1::PIN1-GFP* line in a wild type (a) and *crc-1* (b) stage 12 gynoecium. **c-f**, Expression of the translational fusion *PIN3::PIN3-GFP* line in a wild type (c, e) and *crc-1* (d, f) stage 12 gynoecium. **g-l,** Scanning electron microscopy image of phenotypes of wild type (g, j), *crc* mutant (h, k), and *crc pin3* double mutant fruits (i, l). Scale bars: 20 µm (a-f), 200 µm (g-l).

7.5 ABAXIAL-ADAXIAL Gynoecium Identity

The adaxial replum (adr) encompasses the CMM that gives rise to the reproductive organs such as ovules (Roeder and Yanofsky, 2006; Alvarez-Buylla *et al.*, 2010; Reyes-Olalde *et al.*, 2013). Many genes are involved in abaxial-adaxial identity (Eshed *et al.*, 2001), for example *CRC, KAN1/2, ETT* and *miR166/165* family in abaxial-identity, and on the other hand, *REV*, *PHV* and *PHB* in adaxial identity.

We analyzed the expression of *CRC, KAN1, KAN2, ETT* and *miR166/165* genes in stages 8, 9 and 12 gynoecia. The translational *CRC::CRC-GFP* fusion line was used to analyze the expression of *CRC* (this line is obtained from Dr. Charlie Scutt, France). At stage 8, *CRC* is expressed in the outer epidermal layer of the valves and the medial domain fully around its circumference (Fig. 19a). At stage 9 this expression declines in the valves and the medial domain and begins to have a CRC-GFP signal in the ovules (Sup. Fig. 3B) and at stage 12, the GFP signal is detected in ovules and is absent in the valves (Sup. Fig. 3B).

For *KAN2* expression analysis, we used the enhancer trap line *E2023* (Gillmor *et al.*, 2010). At stage 8, the GFP signal is detected in two distinct domains in the abaxial region and in the adaxial margin. In the abaxial region of the gynoecium, the GFP signal was mostly observed in the abaxial margin and abaxial valve regions, where expression was mainly in the mesocarp and to a lesser degree in the outer epidermal layers (Fig. 19b). In the adaxial margin region, the GFP signal was specifically detected in septum primordia in only 2 or 3 cells. At stage 9, the GFP pattern persisted at the abaxial margin and abaxial valve region forming a circle. However, the GFP expression declined in the presumptive valve margins. At stage 12, the GFP signal disappeared in the valve margin and started to increase in the septum and ovules (Sup. Fig. 3B).

ETT expression was analyzed using the *pETT::SV40-3xeGFP* marker line (Rademacher *et al.*, 2011). During stage 8, the GFP signal was detected in the valves and in the medial region. Interestingly, the GFP signal was undetected in the adaxial valve, adaxial replum, and the CMM region and septum primordia. At stage 9, the CMM gives rise to ovule primordia and septum primordia. In this stage, the maximum GFP signal was observed in the valves and

abaxial margin. At stage 12, GFP signal was detected in the ovules and integument tissue (Fig. 19c and Sup. Fig. 3B).

Another important group of genes are the *miR166/165*. To analyze their expression, we used the *pmiR165A::GFP* and *pmiR166B::GFP* marker lines (Carlsbecker *et al.*, 2010). *MIR165A* and *MIR166* have the same expression pattern; expression was detected abaxially in the outer epidermal layer of the valves and the medial domain forming the circle. However, during stage 12 the GFP signal disappeared in the epidermal layer and started to show signals in the transmitting tract cells (Fig. 19d and Sup. Fig. 3B).

Finally, REVOLUTA (REV) expression was analyzed using the translational *REV::REV:VENUS* fusion line (Gordon *et al.*, 2007). At gynoecia stages 8 and 9, the REV-VENUS signal was mainly detected at the adaxial valve. Interestingly, the REV-VENUS signal was not detected in the outer epidermal layer of the valves, where *MIR165A* and *miR166B* are expressed (Fig. 19e). At stage 12, REV expression decreased considerably in the adaxial valve and was expressed in the vasculature of the valves (Sup. Fig. 3B).

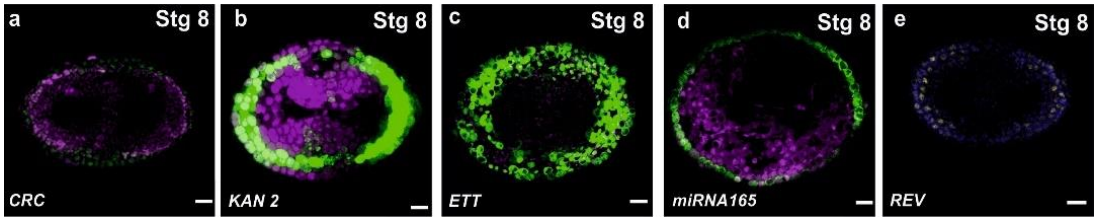


Figure 19. Expression pattern of adaxial-abaxial identity genes. a, Expression of the translational fusion *CRC::CRC-GFP* at stage 8 gynoecium. **b,** Expression of the Enhancer trap line *E2023* at stage 8 gynoecium. **c,** Expression of the transcriptional reporter *pETT::GFP* at stage 8 gynoecium. **d,** Expression of the transcriptional reporter *pmiR166B::GFP* at stage 8 gynoecium. **e,** Expression of the translational fusion *REV::REV-GFP* at stage 8 gynoecium. Scale bars: 10 µm (a-e).

7.6 Other Genes Involved in Gynoecium Development

We analyzed the expression patterns of the *MONOPTEROS* (*MP*), *PINOID* (*PID*), and *miR164* genes. *MP* is an important ARF that has been implicated in gynoecium and ovule development (Galbiati *et al.*, 2013). To analyze the *MP* expression we used the *pMP::SV40-3xeGFP* marker line (Rademacher *et al.*, 2011). At late stage 9, *MP* expression was detected in two distinct domains, in the adaxial valve and adaxial replum and the epidermis of the replum and CMM. In the valves, GFP was present in inner epidermis of the endocarp and second endocarp layer. In the medial region, GFP was present in epidermal layer of the CMM and placenta primordia. This pattern continued during stage 12 in the ovules and funiculi (Fig. 20a and Sup. Fig. 3C).

PINOID (*PID*) is an important gene implicated in the localization of *PIN*. Moreover, *pid* mutants show severe carpel patterning phenotypes similar to those found in *pin1* or *pin3 pin7* mutants (Okada *et al.*, 1991; Benkova *et al.*, 2003). To analyze the *PID* expression, we used the *PID::PID:GFP* marker line (Martinez-Fernandez *et al.*, 2014). In gynoecia, *PID* was expressed only in the epidermis of the valves and the endodermis of the medial region in a similar manner as *CRC* and *MP* (Fig. 20b and Sup. Fig. 3C), suggesting a possible interaction.

Finally, we analyzed the *microRNA164* family genes of group a redundant microRNA involved in the regulation of CUC expressions. To analyze the *miR164* expression we used the *miR164c::VENUS* marker line (Sieber *et al.*, 2007). At gynoecium *miR164* was expressed in the septa primordia and the provasculature cells in the medial region (Fig. 20c and Sup. Fig. 3B).

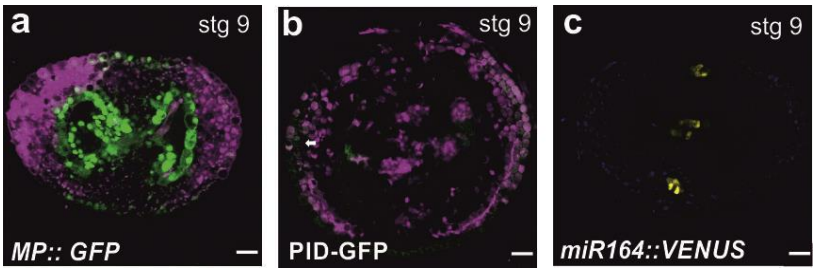


Figure 20. Expression of several transcriptional and translational fusion lines in stage 9 gynoecia: **a,** *pMP::SV40-3xeGFP*, **b,** *PID::PID-GFP,* and **c,** *miR164C::VENUS.*

8 DISCUSSION

Based on the expression pattern of the hormone response markers *DR5* (auxin) and *TCS* (cytokinin) and the expression patterns of several transcription factors, we laid out a regulatory framework for early gynoecium development (Fig. 21a-c). The mechanism proposed here, describes that the mutual inhibition between domains of high auxin and high cytokinin signaling, necessary to control CMM development and medial-lateral axis formation. Based on the results presented in this study and previous work, we suggest the following model: The presence of strong cytokinin signalling (*TCS*) is necessary to provide meristematic properties to the CMM, and later growth and differentiation of transmitting tract tissues derived from the CMM. In our model, strong cytokinin signalling is directed by SPT via activation of the type-B *ARR1*, and maintained by a positive feedback loop *SPT-ARR1*. This network is necessary to maintain and restrict the cytokinin signaling in the medial domain, ensuring a balance between cell division and cell differentiation. This mechanism ensures the continuity of meristematic activity at the CMM without the need of expression of *WUS*. On the other hand, the combined action of *SPT* and *ARR1* also regulate components of the auxin pathway (*TAA1* and *PIN3)* in these tissues, probably causing a PIN3-dependent auxin drainage, ensuring that no accumulation of auxin occurs in the CMM, but that auxin is directed towards the abaxial repla and the lateral domain. The recent demonstration that HEC contributes to SAM function by promoting meristematic activity and the activation of the expression of *PIN1* and *PIN3* in the gynoecium (Schuster *et al.*, 2014; Schuster *et al.*, 2015), suggests that also *HEC* may be involved in cytokinin signalling through the interaction with *SPT*, probably as a protein-protein complex (Gremski *et al.*, 2007). The high auxin response and low cytokinin signalling at the valves may promote the activation of several genes involved in lateral tissue differentiation such as *AHP6* and *FUL* (Ferrandiz et al., 2000; Besnard et al., 2014). *AHP6* together with *ARR16* restrict the high cytokinin signaling output, while *FUL* promotes the activation of valve identity genes. Finally, these findings provide a model in which the balance between proliferation and differentiation is achieved by asymmetrical auxin-cytokinin distribution.

Cytokinin and *SPT* Interact to Control Medial Region and Septum Development

Although, we previously demonstrated that cytokinin plays an important role in cell proliferation at the medial region (Marsch-Martinez *et al.*, 2012b), the mechanism of how cytokinin is involved in this process remains unclear. In this way, we first analyzed the role of cytokinin in gynoecium development and medial-lateral axis (Fig. 21a). In *Arabidopsis,* cytokinin signalling requires the expression of the cytokinin receptors (AHK2, AHK3, and AHK4) (Hwang and Sheen, 2001; Muller and Sheen, 2007). According to this, we detected *AHK2, AHK3, and AHK4* in the gynoecium and in the medial region. Moreover, the exogenous cytokinin application to *ahk2 ahk4*, *ahk3 ahk4* and *ahk2 ahk3* double mutants resulted in a noticeable reduction of outgrowths from the medial region (Fig. 6). Interestingly, the *ahk2 ahk4* double mutant showed a significant reduction of outgrowths after cytokinin treatment. Recently, Gordon *et al.* 2009, reported that cytokinin effects in the SAM are mediated primarily through the *AHK2* and *AHK4* receptors (Gordon *et al.*, 2009). This suggests that *AHK2* and *AHK4* (Fig. 6) have a similar role in modulating the sensitivity to cytokinin in the gynoecium.

We propose that the high levels of cytokinin signalling (*TCS::GFP*) are required for meristematic activity of the CMM. Therefore, any alteration in cytokinin homeostasis causes defects in CMM development and tissues that arise from it. Those defects are seen in the cytokinin receptor double mutants and *ahk2 ahk3 ahk4* and *arr1, 10, 12* triple mutants. In these mutants, we observed defects in septum and transmitting tract development. Also a drastic reduction in repla width and ovule number was observed (Fig. 1a-c, Fig. 8d-e and Sup. Fig. 1b) (Bencivenga *et al.*, 2012). On the other hand, previously it was reported that when internal cytokinin levels are increased the replum width was increased (Marsch-Martinez *et al.*, 2012b). Furthermore, the increased cytokinin level observed in the *ckx3 ckx5* double mutant resulted in an increased gynoecia size and ovule number (Bartrina *et al.*, 2011). In summary, these results show that cytokinin is necessary for growth and differentiation of the medial region (Fig. 21a). The role of cytokinin in the CMM may be explained in a similar manner as in the SAM, where cytokinin promotes cell division activity (Riou-Khamlichi *et al.*, 1999).

In addition, our study explored the molecular mechanisms that control asymmetric cytokinin distribution in the gynoecium. The mechanism proposed here, describes a positive feedback loop between cytokinin signalling and the *SPT* expression, which is necessary for the meristematic activity of the CMM. In our model, the meristematic activity and cell number in the CMM is controlled by the *SPT*-cytokinin loop. Thus, alteration in the *SPT*-cytokinin loop will cause defects in the medial region and their derivatives such as septum or transmitting tract. The importance of this *SPT*-cytokinin loop is seen in the *spt* and *arr1, 10, 12* mutants that display severe defects in septum and transmitting tract development (Fig. 8e and 9q). Moreover, it has been reported that the *spt* mutant has defects in cell number in the medial region (Alvarez and Smyth, 2002), which is consistent with the idea that *SPT* stimulates directly or indirectly cell proliferation. Furthermore, it has been reported that cytokinin promotes cell division through positive regulation of *CYCLIN D-3* (*CYCD3*) (Riou-Khamlichi *et al.*, 1999). Based on *SPT* and the cytokinin role in meristematic activity of the CMM, we hypothesize that *SPT* might regulate the cell cycle either directly by controlling expression of cyclins or through cytokinin signalling stimulation.

Interestingly, a function for the *HEC* genes and for *SPT* in SAM function was recently reported: HECs were shown to stimulate stem cell proliferation in a *SPT*-dependent manner, suggesting that the relative levels of these transcription factors dictate the proliferative potential of stem cells (Schuster *et al.*, 2014). A reduced SAM size was observed in *spt* mutant plants (Schuster *et al.*, 2014), which suggests that *SPT* function is also likely necessary for a positive cytokinin signalling output in the SAM. It would be interesting to explore other elements participating in the regulatory network in early gynoecium development, including the HEC genes, whose triple mutant has similar developmental defects in medial tissues to those observed in the *spt* mutant (Schuster *et al.*, 2015), which strongly indicates that *SPT* and *HEC* could also be also involved during gynoecium development. One possible scenario to explain this regulation could be that SPT and HEC work in a protein complex during the activation of cytokinin signalling.

Our results demonstrate that *SPT* positively controls the cytokinin signaling output through type-B *ARR* activation, at least via *ARR1*. The significant overlap between their spatiotemporal expression pattern in roots (Dello Ioio *et al.*, 2007; Wendrich *et al.*, 2015) and root meristem phenotypes of *spt* and *arr1* (Dello Ioio *et al.*, 2007; Makkena and Lamb, 2013), suggests that *SPT-ARR1* interact also during other plant development processes such as RAM regulation. Furthermore, recently it has been demonstrated that the DELLA protein is recruited by ARR1 and SPT (Josse *et al.*, 2011; Marín-de la Rosa *et al.*, 2015), reinforcing the idea that *SPT* and *ARR1* could be cooperatively work. Although, direct protein–protein interaction was not detected between SPT and ARR1 (Sup. Fig. 10 A, B), it is possible that the DELLA protein is necessary to mediate SPT-ARR1 interaction. In addition, this data suggests that GA signalling and cytokinin signalling can act together to regulate different developmental processes.

In this work we also studied the relation between *STM* and cytokinin during gynoecium development. Little is currently known about interactions between *STM* and cytokinin, but it is generally accepted that a positive feedback loop exists between *STM* and cytokinin signaling, because the cytokinin overproduction triggers a rapid increase in mRNA levels of the *STM* (Jasinski *et al.*, 2005), while *STM* induces cytokinin biosynthesis through activation of *IPT* genes (Jasinski *et al.*, 2005; Yanai *et al.*, 2005). Here, we found coexpression of *IPT1* and *STM* during gynoecium development (Sup. Fig. 3C), suggesting that a similar mechanism that happens in the SAM*,* may also occur in the CMM. The direct activation of *STM* by type-B ARRs has not been tested. However, it is possible that *type-B ARRs* could be positively regulating *STM* expression in the CMM (Fig. 21 b), since we found that cytokinin application increases *STM* expression in the medial region (Fig. 15b and Sup. Fig. 4). Interestingly, it is likely that *STM* activation is independent of *SPT,* since mRNA levels of *STM* were not much affected in the *spt* mutant or in the *35::SPT* overexpression line (Fig. 15 g). Alternatively, *STM* could be positively regulating type-B *ARRs* or *SPT* expression, reinforcing cytokinin signalling in the CMM and medial region (Fig. 21b)

SPT-cytokinin Signaling Regulates Auxin Synthesis and Auxin Transport

It has been previously shown that cytokinin and *SPT* can interact with elements of the auxin pathway (Dello Ioio *et al.*, 2007; Dello Ioio *et al.*, 2008; Pernisova *et al.*, 2009; Bishopp *et al.*, 2011; Moubayidin and Ostergaard, 2014). However, the molecular mechanisms of this regulation remain unclear. Here we demonstrated that the *SPT*-cytokinin loop is required for auxin biosynthesis, as well as *PIN3* expression in the CMM. Based on our findings, we propose a mechanism where *SPT*-cytokinin signalling causes the activation of *TAA1,* an auxin biosynthetic gene, resulting in auxin accumulation in the CMM. In turn, this auxin is fluxed away (auxin drainage) from the gynoecium center towards the repla and the valves in a PIN3-dependent mode. This results in the formation of two signalling domains: high auxin and high cytokinin signalling domains. A similar mechanism is observed in vascular pattern formation (Bishopp *et al.*, 2011), supporting the notion that PIN regulation by cytokinin is a common mechanism in organ formation. Alternatively, *SPT*-cytokinin signalling might be required as well for *PIN1* expression. According with this, we previously reported that the *PIN1* expression is also induced by cytokinin (Zuñiga-Mayo *et al.*, 2014).

On the other hand, we detected a positive feedback between cytokinin and auxin synthesis during media-lateral formation (Fig. 20a). Our data indicate that cytokinin, through *ARR1*, can activate directly *TAA1* expression at the medial region of the ovary. *SPT* is also required for this activation and can also bind and activate the *TAA1* promoter independently of *ARR1*. Support for this is the observation that SPT and ARR1 bind to the same fragments of the *TAA1* promoter in ChIP assays. Therefore, *ARR1* and *SPT* can integrate the cytokinin signalling pathway and auxin biosynthesis in the medial region (Fig. 9j-m). Interestingly, similar mechanisms are observed in roots and style-stigma development. In style-stigma region, *HEC1* modulates the auxin biosynthesis through activation of *YUC4*, an auxin biosynthetic gene (Schuster *et al.*, 2015). Furthermore, in root meristem development has also linked ARR1 to auxin biosynthesis activation (Moubayidin *et al.*, 2013).

At stage 7-8, the *PIN1* and *PIN3* genes are the main PINs expressed in the gynoecium. At these stages, *PIN1* and *PIN3* show different subcellular polar localization. In ovules and in the inner epidermis of the CMM, *PIN1* and *PIN3* display a pronounced polarity (Sup. Fig. 3A), while, in the CMM and septum primordia *PIN1* and *PIN3* are expressed without pronounced polarity (Sup. Fig. 3A). The polarity pattern is important in organ initiation, since it allows the formation of high auxin signaling (Reinhardt *et al.*, 2003; Yoshida *et al.*, 2011). Interestingly, the polar orientation of *PIN1* and *PIN3* coincide with the *PINOID* (*PID*) expression (Sup. Fig. 3B), which is an important gene implicated in PIN polar localization (Friml *et al.*, 2004). On the other hand, the no-polar pattern of PIN proteins in the CMM coincide with the expression of *SPT* and *IND* (Sup. Fig. 3A) (Heisler *et al.*, 2001; Girin *et al.*, 2011), which are two genes involved in the direct repression of *PID* (Sorefan *et al.*, 2009; Girin *et al.*, 2011). Altogether, these data suggest that the *PID* expression is necessary for *PIN1* and *PIN3* polarity. Future experiments are necessary to demonstrate the relevance of *PID* in PIN polarization during CMM development.

Moreover, the striking resemblance between the phenotype of the *pin1* and *pid* mutant, and between wild type plants treated with BAP (Zuñiga-Mayo *et al.*, 2014), strongly suggests that regulation of auxin distribution by cytokinin signalling plays an important role in medial-lateral axis specification. These results indicate that cytokinin regulates the distribution of auxin, directly through transcriptional activating of *PIN* (Fig. 21a). Alternatively, it could also be that cytokinin regulates polar localization of PIN proteins through the transcriptional regulation of *PID.*

Cytokinin Repression in the Valves

In our model, the auxin flux away from the gynoecium medial region towards the repla and the lateral region (valves), causes a high auxin response in these tissues. The high auxin response promotes the expression of *AHP6,* a gene involved in cytokinin signaling inhibition (Mahonen *et al.*, 2006). The expression of *AHP6* represses the high cytokinin signaling at valves, thus concentrating cytokinin signalling to the medial domain. Support for this is the observation that the *ahp6* mutant gynoecia show *TCS* expression in the

valves and appear to be more sensitive to cytokinin applications compared to the wild-type, although, the non-treated gynoecia of the mutant seem normal, suggesting redundancy of this cytokinin restriction function. Other important genes involved in cytokinin signaling repression are the type-A *ARR* genes. We observed that *ARR16::GUS* is also expressed in the valves, suggesting that *ARR16* and *AHP6* repress together cytokinin signaling in the valves (Fig. 21c). Interestingly, the A-type *ARR* genes are direct targets of the B-type ARR transcription factors (Taniguchi *et al.*, 2007). However, the *ARR16* is not expressed in septum or CMM region where high cytokinin signalling is detected, which suggests that *ARR16* is regulated by other genes or by a non-cell autonomous mode of type-B ARR proteins.

High auxin output in the valves could also promote the activation of several genes involved in lateral tissue differentiation such as *FRUITFULL* (*FUL*), which is involved in valve determination. The loss-of-function mutations in *FUL* result in several defects in valve differentiation (Ferrandiz *et al.*, 2000). Interestingly, we previously discovered that the *ful* mutant shows high cytokinin signalling (*TCS*) in the valves (Marsch-Martinez *et al.*, 2012b), which closely resembles that of the *ahp6* mutant. *FUL* is a transcription factor that represses SHP1/2, *IND* and other genes involved in valve margin identity (Ferrandiz *et al.*, 2000; Liljegren *et al.*, 2004). The absence of *FUL* results in conversion of valve cells into valve margin cells, which could suggest that cell identity changes are caused by the presence of *TCS* in *ful* mutant valve tissue. An alternative explanation is that *FUL* may restrict *TCS* signaling through the regulation of *AHP6* or repression of *SPT* expression (Fig. 21c).

The Auxin-Cytokinin Interaction during Gynoecium Development

Cytokinin and auxin act together to regulate different developmental processes, thus, many genes are convergently regulated by both hormones (Su *et al.*, 2011). In this sense, our results show a putative feedback loop between auxin-cytokinin signalling during gynoecium development (Fig. 20c). We demonstrated that the expression of *ETT* is necessary to maintain cytokinin signaling within the medial region (Fig. 13), because in the

ett mutant *TCS* signal is ectopically found (Fig. 13) and in *35S::ETT* the *TCS* signal is reduced in the medial region (Fig. 13). A possible mechanism for this repression is that *ETT* mediates the repression of cytokinin signalling through the direct repression of *SPT*. This hypothesis is supported by the evidence of ectopic expression of *SPT* in *ett* mutant gynoecia and the presence of several AuxRE-like elements in the *SPT* promoter sequence (Sessions *et al.*, 1997). An alternative mechanism is that *ETT* could directly regulate cytokinin signalling. Moreover, we found that *SPT* directly represses the expression of *ETT*, ensuring that auxin signalling output occurs only at the abaxial replum region (Fig. 21b).

In this thesis, we demonstrated that auxin application to *spt TCS::GFP* caused an even stronger repression of cytokinin signaling, even at late stages (Fig. 7 and Sup. Fig. 7). A way to explain this is that in the absence of *SPT* auxin signalling increases due to an increase in *ETT* expression, causing a complete loss in cytokinin signaling in the *spt* mutant.

On the other hand, in this thesis I demonstrated that auxin applications increase cytokinin signaling at the CMM (Fig. 7 and Sup. Fig. 5). This suggests that auxin affects cytokinin signaling. However, the mechanism that controls this is not fully elucidated. Therefore, it is necessary to perform more experiments to demonstrate the effect of auxin on cytokinin signalling such as the effect of NPA (a polar auxin inhibitor) in *DR5* and *TCS* lines, to analyze the effects of auxin application on the expression of important genes involved in cytokinin signaling such as *SPT* and *ARR1.* Furthermore, also test whether the expression of the auxin biosynthetic gene *iaaM* under the control of the *SPT* or *PIN3* promoter could affect the *DR5* and *TCS* pattern.

It is generally accepted that auxins stimulate PIN expression (Vieten *et al.*, 2005). However, our data shows that *PIN3* expression is slightly decreased in wild type and *spt* mutant gynoecia after application with the auxin indole-3-acetic acid (IAA) (Fig. 11g, h). In order to understand this phenomenon better, it would be necessary to test whether auxin application also negatively affects the expression of other genes implicated in PIN3 regulation such as *SPT* and *ARR1.*

crabs claw (*CRC*) Mutant Affected in Auxin Response

The adaxial-abaxial axis is established during auxin-cytokinin pattern formation and depends on the activation of several genes such as *CRC*. *CRC* is a transcription factor belonging to YABBY family and is expressed in the abaxial side of lateral organs and it has been demonstrated that it is involved in abaxial-identity (Eshed *et al.*, 2001). Despite the fact that *CRC* is expressed in the abaxial side of the gynoecium (Fig. 20e and Sup. Fig. 3B), the *crc* mutant does not display dramatic abaxial defects (Alvarez and Smyth, 2002). On the other hand, the *crc* mutant shows a similar phenotype as seen in the *spt* mutant, characterized by apical defects and septum fusion defects (Alvarez and Smyth, 1999). Our data demonstrates that unlike the *spt* mutant, the *crc* mutant does not display dramatic alterations in cytokinin signalling (*TCS*) (Fig. 7 and Fig. 17). However, the *crc* mutant presents defects in auxin response (*DR5*) (Fig. 17e-h). Moreover, *PIN1* and *PIN3* expression is still detected in the *crc* mutant, suggesting that auxin transport is not responsible of the altered auxin response in the *crc* mutant. Another important fact is that the overexpression of *STY1*, an auxin biosynthesis regulator gene, often develops polar defects and can only partially restore style defects in the *crc* mutant when overexpressed (Staldal *et al.*, 2008). Altogether, this strongly suggests that *CRC* is directly involved in the regulation of auxin signalling, perhaps through the modulation of *ARF* genes such as *ETT* (Fig. 21b). Therefore, one mechanism underlying these effects could be the imbalance between cell division (cytokinin) and differentiation (auxin) due to increased responses to cytokinin and decreased responses to auxin in the *crc* mutant.

Abaxial-Adaxial Gynoecium Identity is determined by Auxin Response

The adaxial-abaxial axis is established during auxin-cytokinin signalling domain formation and depends on the activation of several genes such as *CRC*, *ETT* and *KAN*. Recent studies have shown that during the formation of the adaxial-abaxial axis in leaves, auxin accumulation plays an important role (Qi *et al.*, 2014). However, it remains unknown what the role of auxin is in the adaxial-abaxial axis formation in the gynoecium. Therefore, it is still necessary to evaluate the effect of auxin in this process. For this, we propose the use

of *DII-VENUS* (Brunoud *et al.*, 2012), an auxin signalling sensor, to analyze the distribution of auxin concentration during abaxial-adaxial axis formation. On the other hand, the abaxial expression of *ETT*, makes it an excellent candidate to study in adaxial-abaxial polarity and auxin signalling. However, *ETT* has limited interactions with the auxin negative regulators AUX/IAA proteins (Qi *et al.*, 2014), which suggests that *ETT* is mainly transcriptionally regulated. Our results, suggest the possibility that CRC is important in regulating *ETT* expression, and thus CRC and ETT may modulate auxin signalling in the abaxial side (Fig. 21d). This agrees with previous reports where it has been proposed that *CRC*, *ETT* and *KAN* operate together in the establishment of abaxial polarity (Eshed *et al.*, 1999; Pekker *et al.*, 2005). We observed a direct protein-protein interaction between CRC and KAN2 proteins (Sup. Fig. 11). On the other hand, we did not detect a direct protein-protein interaction either between ETT-CRC or between ETT-KAN2 (Sup. Fig. 11). However, we cannot rule out the existence of a higher-order complex where CRC-ETT-KAN2 factors are present. Alternatively, the interaction of CRC-ETT-KAN2 activates the expression of several abaxial polarity genes such as of the *miR166/165* group (Fig. 21d). It would be interesting to test the effects of elevated or decreased auxin levels as well as altered cytokinin signalling on *CRC*, *ETT*, *KAN* and *miR166/165* expression. This could be done by artificially expressing the auxin biosynthetic gene *iaaM* or the cytokinin biosynthetic gene *IPT7* under the control of the miR166/165 promoter.

Adaxial-identity is determined by the expression of three HD-ZIP III transcription factors *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*) and *REVOLUTA* (*REV*). In order to further study their role in gynoecium development we analyzed the expression of *REV*. The expression of *REV* in early stages is confined to the adaxial valve, suggesting a role in valve determination and auxin response. Further evidence for the importance of *REV* in auxin transport comes from the observation of defects in medial domain development in the *rev* mutants, which are increased after NPA treatment (Nole-Wilson *et al.*, 2010).

CMM growth and differentiation

The plant hormone auxin is the main positional cue for the establishment of the ovule primordia and ovule development (Pagnussat *et al.*, 2009; Bencivenga *et al.*, 2012; Galbiati *et al.*, 2013). In our data, *DR5* expression is detected in ovule primordia at early stages (stage 7). Interestingly, *DR5* signal is not affected in ovule primordia in the *spt* mutant. The high auxin responses at placenta primordia are established by the expression of *PIN1* and *PIN3*. However, despite the fact that SPT-ARR1 is necessary for the expression of *PIN3* in the CMM and septum primordia, *PIN3* expression is still detected in placenta primordia in the *spt* mutant (Sup. Fig. 3A). This suggests that the SPT-ARR1 module may not be necessary to activate PIN expression in ovule primordia and the ovules and additional regulators might contribute to *PIN1* and *PIN3* expression. Compelling reasons came from a new study in which MP is an important regulator of ovule development through direct activation of *CUC* genes, which are required for both correct PIN1 expression and localization (Fig. 21a) (Galbiati *et al.*, 2013).

Septum and transmitting tract tissues originate from cells that divide a number of times in the CMM, in which any alteration in the cell number or division causes septum or transmitting tract defects. In our model, the number of cells is controlled by the *SPT-ARR1* network. The transmitting tract formation commences when postgenital fusion is established at late stage 9 and continues growing to stage 12 (Crawford *et al.*, 2007). During stage 9, *TCS* signal was found within the central region where the transmitting tract will form, this pattern continues throughout stage 12 (Sup. Fig. 3A). On the other hand, *TCS* signal is not detected in the *spt* mutant at stage 9, however at stage 12, *TCS* signal was detected, but did not have a strong expression compared to the wild type. Taken together, these results, point to the possibility that *TCS* activation is independent of *SPT* gene expression at late stages, and perhaps genes such *as INDEHISCENT (IND)* (Girin *et al.*, 2011), *HECATE (HEC)* (Gremski *et al.*, 2007), *HALF FILLED (HAF)* (Crawford and Yanofsky, 2011), or *ALCATRAZ (ALC)* (Groszmann *et al.*, 2011) could fulfill the function of *SPT.* However, despite the fact of *TCS* reactivation, the transmitting tract tissue continues to be severely affected in the *spt* mutant*,* which suggests that *SPT* is required for the

transmitting tract development. One possible explanation is that SPT may form a protein-protein complex or interacts with proteins required in this tissue development.

The gynoecium and the CMM are components of the success of the angiosperms (Endress and Igersheim, 2000), which comprise over 300,000 species on earth. Here we showed that cytokinin signaling is necessary for their correct development and, therefore, for the reproductive competence. Interestingly, the presence of the bHLH transcription factor SPT, cytokinin signaling, auxin biosynthesis, and PIN3 orthologs in basal angiosperms (Pils and Heyl, 2009; Reymond *et al.*, 2012; Pabon-Mora *et al.*, 2014), suggests that these genes already had a function in gynoecium development in early flowering plants. Future work should shed light on how this network emerged in ancestral flowers.

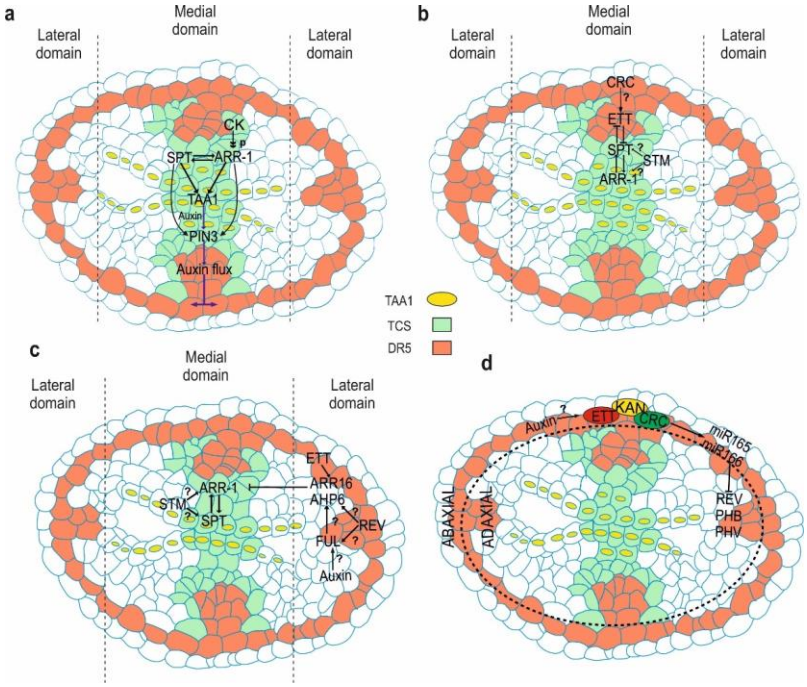


Figure 21. Models of regulatory networks in early gynoecium development. a-c, Models of the Medial-lateral axis establishment in at stage 6-8 gynoecium development. d, Model of the regulatory network in abaxial-adaxial axis establishment at stage 5-7 gynoecium development. Solid black arrows indicate a positive regulation and a T-bar indicates a repression function, a broken black arrow indicates possible positive regulation by auxin, a double arrowhead indicates phosphorylation, purple arrows indicate possible auxin flow; CK, cytokinin; P, phosphate group. Scale bars: 10 µm (a–d).

9 CONCLUSIONS

The communication between auxin-cytokinin signaling pathways has been the subject of intense research. However, few studies have focused specifically on the development of the early gynoecium. Here we postulate the first model for early gynoecium development indicating the role of auxin and cytokinin (Fig. 21a-d). In this model, cytokinin signaling is required for cell cycle divisions and stem cell capacity of the CMM, while auxin signaling promotes cell differentiation and organ growth. Thus, auxin and cytokinin antagonism creates a feedback loop necessary to maintain a balance between stem cell number and cell differentiation. We demonstrated that *SPT-ARR1* plays an important role in the regulation of cytokinin and auxin response. The *SPT-ARR1* network ensures the continuity of cytokinin signaling in the gynoecium. Moreover, the *SPT-ARR1* module activates auxin biosynthesis by activating the expression of *TAA1*. Next, the synthesized auxin is transported away from the gynoecium center by the auxin transporter PIN3 protein. The auxin flux away from the gynoecium medial region towards the valves causes a high auxin response and the activation of several genes such as *AHP6*. The expression of cytokinin signalling repressors *AHP6* and *ARR16* in the valves restricts the cytokinin signalling output to the CMM region. On the other hand, the restriction of cytokinin signalling output to the CMM region is further ensured by the negative feedback loop between *ETT-SPT*. Furthermore, *CRC* expression is necessary to control the auxin signalling in the CMM, probably through *ETT* activation.

10. PERSPECTIVES

The cross-talk between auxin and cytokinin is important for many developmental processes (Zhao et al., 2010; Bishopp et al., 2011; Marsch-Martinez et al., 2012b). Here, we found that cytokinins affect the auxin homeostasis through its synthesis and transport. However, the effects of the auxins on cytokinins homeostasis are not well characterizes. Inversely, future experiments should further analyze the effects of the auxin on cytokinin homeostasis regulation. This could be tested by analyzing the effect of auxin on cytokinin synthesis genes such as *LOG* and *IPT* or cytokinin signalling genes for example: cytokinin receptors, type-B *ARRs* and type-A *ARRs*.

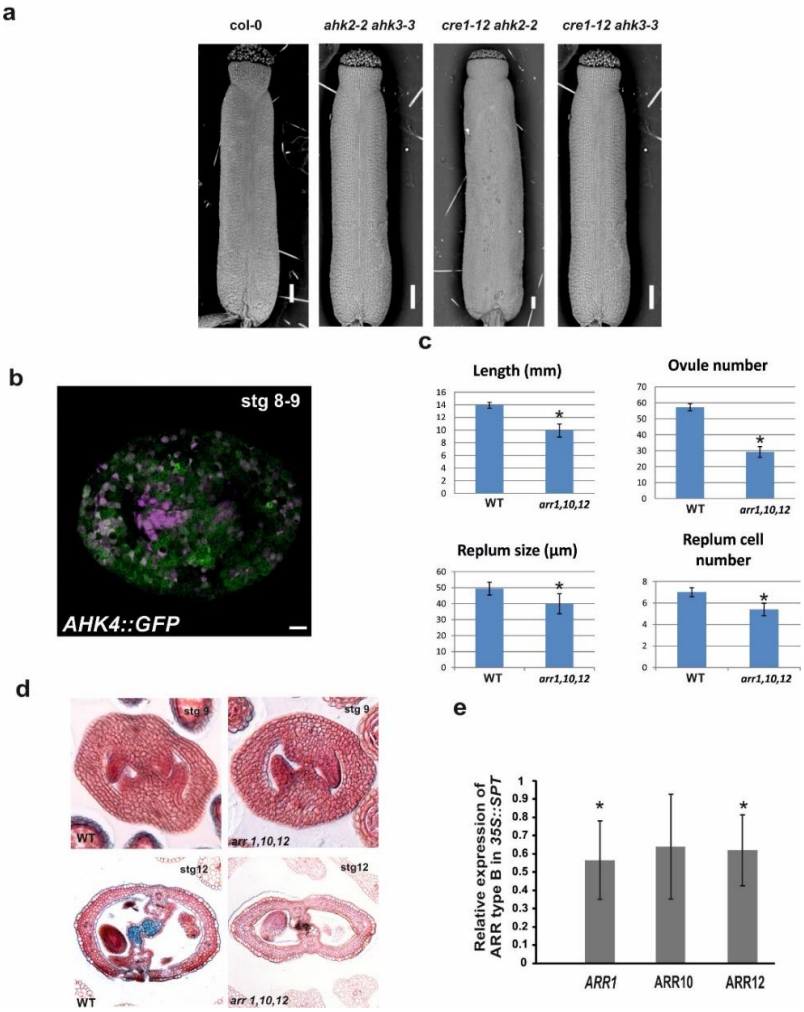
Moreover, cytokinin have an opposite role on the SAM and RAM meristems, while in SAM it stimulates the meristematic activity, in RAM it is associated with a loss of meristematic activity. Future experiments should be conducted to investigate the probable effect of *STP-ARR1-PIN3* in the loss of meristematic activity in the RAM.

On the other hand, based on our results, we suggest the following perspectives:

1. To analyze the role of others marker lines in the *ett* mutant such as *PIN3::PIN3:GFP* and *DR5::GFP.*
2. To analyze the functional redundancy of *SPT* with others bHLH such as *ALC, IND* and *HEC,* during gynoecium development.
3. To analyze the effects of elevated auxin levels in carpel development, through the application of Indole-3-Acetic Acid (IAA) or synthetic auxin 2,4-Dichlorophenoxyacetic acid (2,4-D).
4. To analyze the effects of IAA applications in *spt DR5:GFP, spt TCS:GFP, spt PIN3::PIN3:GFP, pETT:GFP, SPT::GUS, pCRC::CRC:GFP, AHP6::GFP*, lines *pmiR165A::GFP* and *pmiR166B::GFP* and *REV::REV:VENUS*.
5. To genotype and analyze the segregating F2 plants of *spt* pETT:GFP, *ett DR5::GFP*, *ett TCS:GFP*, *ett PIN3::PIN3:GFP*, *35S::SPT 35S::ARR1ΔDDK:GR* and *spt 35S::ARR1ΔDDK:GR*.

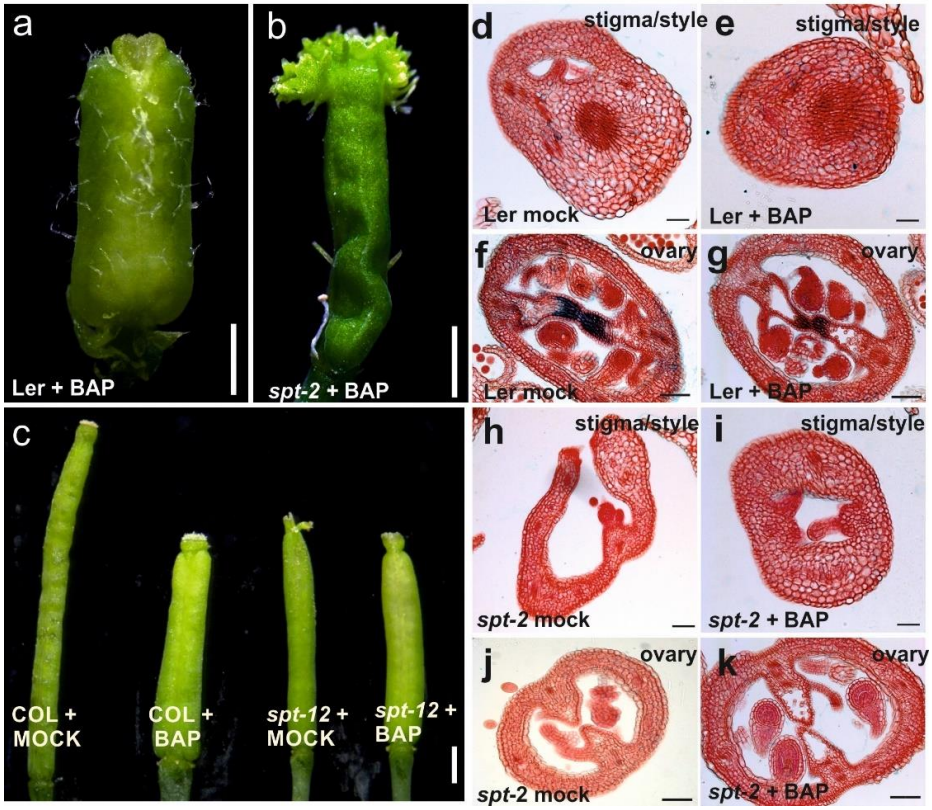
11 SUPPLEMENTARY FIGURES (Sup. Fig.)

Supplementary Figure 1 (SF1)

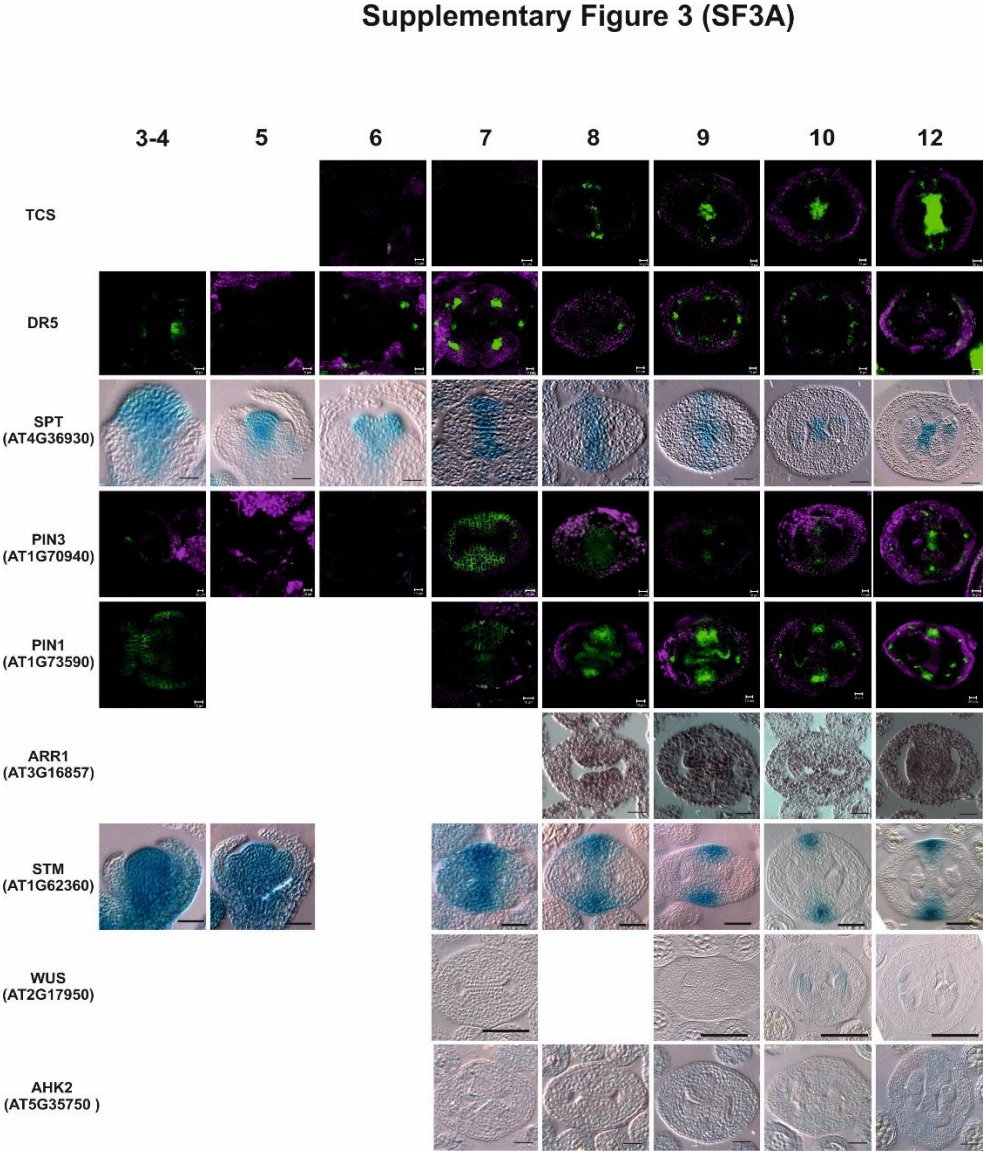


Supplementary Figure 1. Phenotypes of the cytokinin receptor and type-B *arr1 arr10 arr12* triple mutants. **a;** Scanning electron micrographs of wild type and the double mutants *ahk4 ahk2*, *ahk4 ahk3*, *ahk2 ahk3*. **b**, GFP reporter lines AHK4:AHK4::GFP at stage 8-9 gynoecia. **c-d**; Phenotypes of the type-B *arr1 arr10 arr12* triple mutant compared to wild type (WT): mature gynoecium size, fruit size, mature gynoecium length, ovule number, replum width, and replum cell number (b) and transverse sections of stage 12 gynoecia (c). **e**; qRT-PCR of *ARR1, ARR10* and *ARR12* in dissected gynoecia from *35S::SPT*. Error bars represent the s.d. for the qRT-PCR analyses based on three biological replicates. Sample numbers: (f-g) WT, n= 14 and *arr1 arr10 arr12*, n=19; (h-i) WT, n= 20 and *arr1 arr10 arr12*, n=19. Scale bars: 100 µm (a,c).

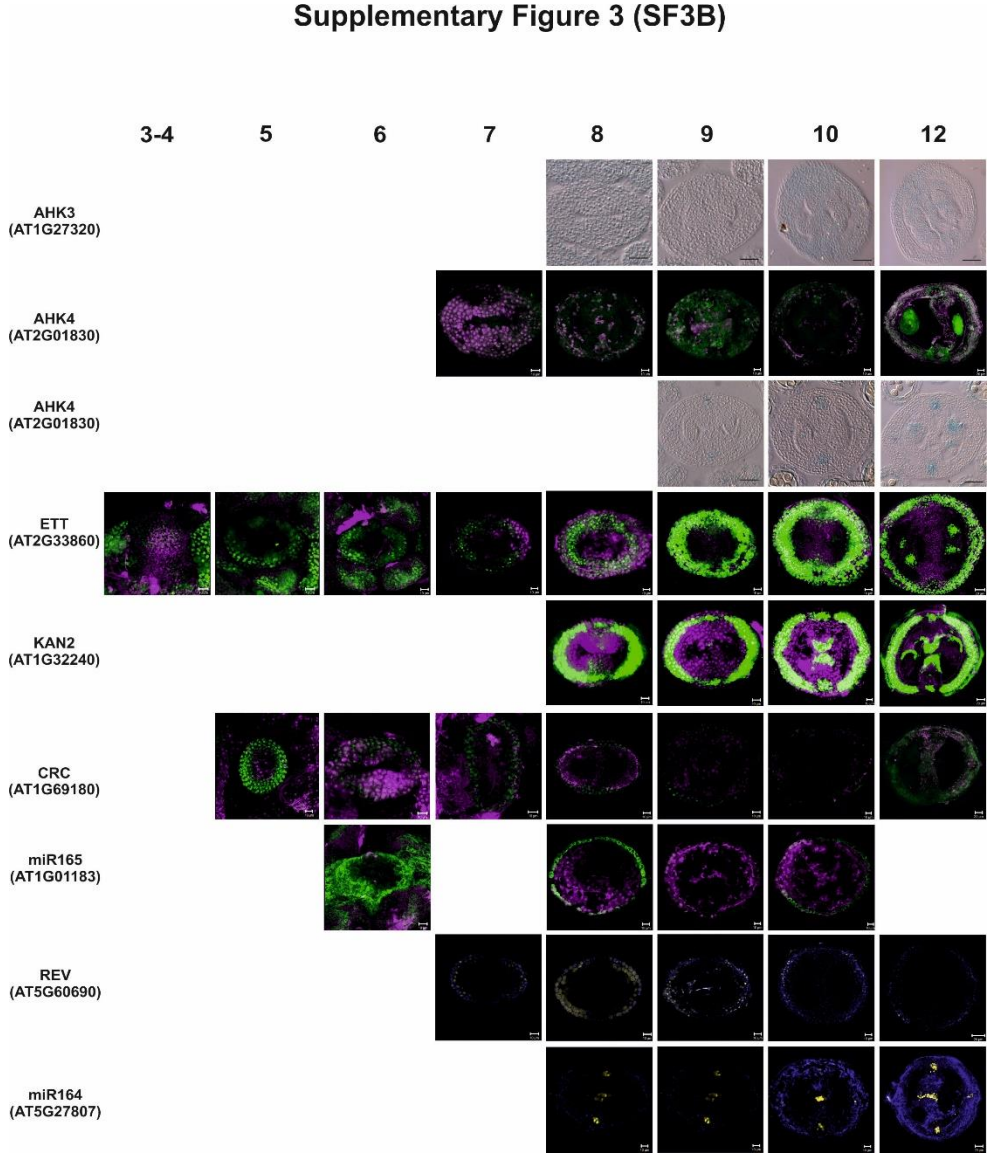
Supplementary Figure 2 (SF2)



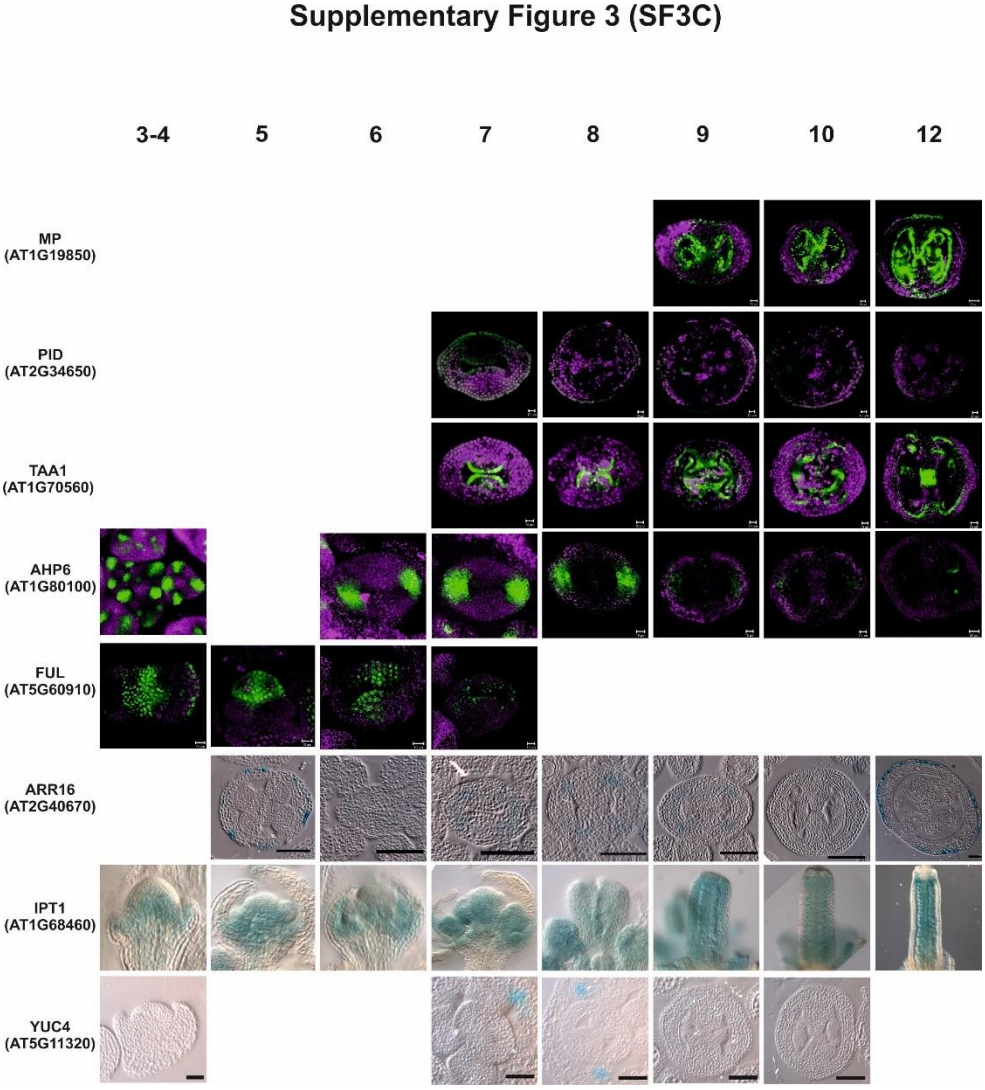
Supplementary Figure 2. SPT enables cytokinin signalling during gynoecium development. a, b, Wild type L*er* (a) and *spt-2* (b) gynoecia treated with BAP for 5 days, photos taken 3-4 weeks after. In (b) is an example of a *spt-2* gynoecium presenting a minor effect to BAP (only in 12.5% of the cases). **c,** Wild type Col-0 (left) and of *spt-12* (right) gynoecia treated with BAP for 48 hours, photos taken 1 day after. **d, e, h, i,** Transverse sections of stigma/style region of gynoecia of wild type (mock) L*er* (d) and *spt-2* (mock) (h), and of 48 hours BAP-treated gynoecia of wild type L*er* (e) and of *spt-2* (i). **f, g, j, k,** Transverse sections of the ovary region of gynoecia of wild type (mock) L*er* (f) and *spt-2* (mock) (j), and of 48 hours BAP-treated gynoecia of wild type L*er* (g) and of *spt-2* (k). Scale bars: 10 mm (a, b, c), 150 µm (d-k).



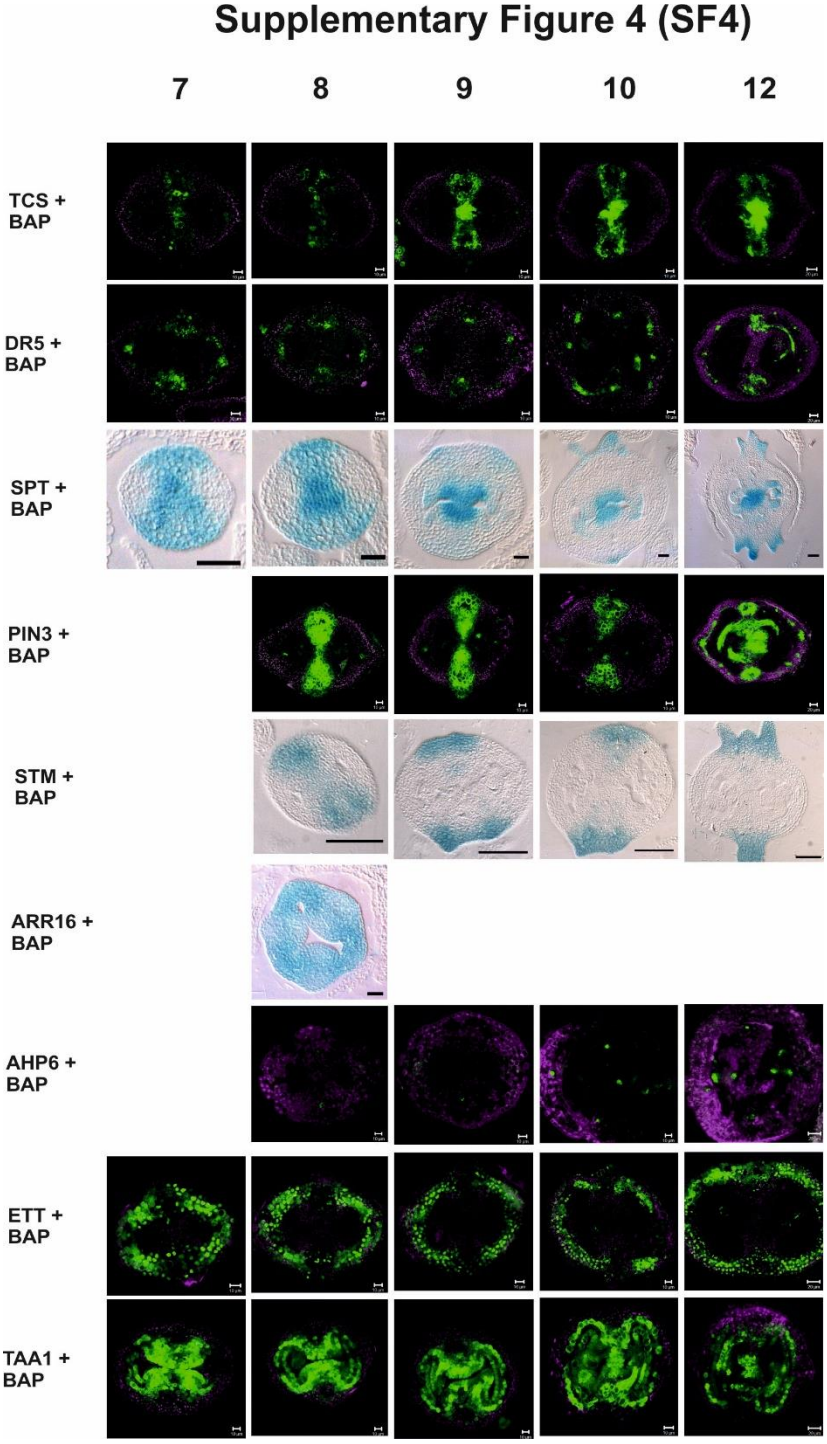
Supplementary Figure 3. Expression of several genes involved in *Arabidopsis* gynoecium development. **A**, Expression of several transcriptional and translational fusion lines in transverse sections of stage 3 to 12 gynoecia: *TCS::GFP, DR5::GFP, SPT::GUS, PIN3::PIN3-GFP, PIN1::PIN1-GFP, STM::GUS, WUS::GUS, AHK2::GUS*.



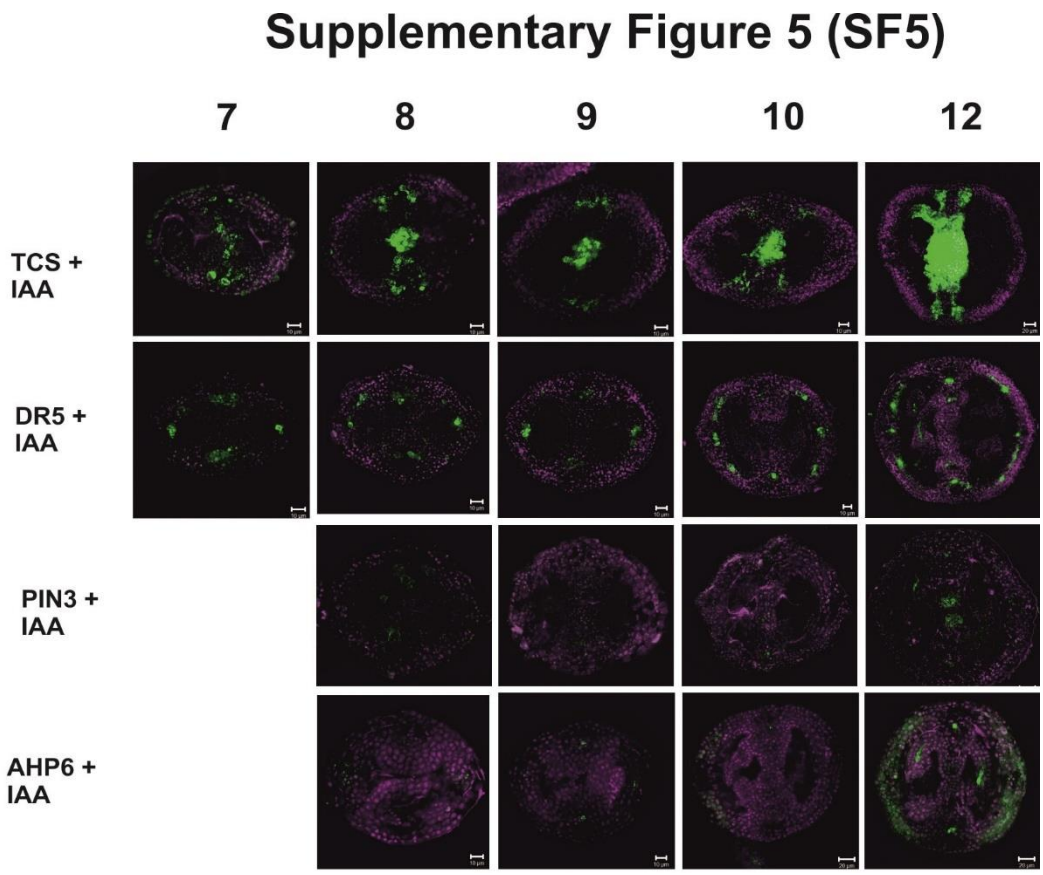
Supplementary Figure 3. Expression of several genes involved in *Arabidopsis* gynoecium development. **B**, Expression of several transcriptional and translational fusion lines in transverse sections of stage 3 to 12 gynoecia: *AHK3::GUS, AHK4:AHK4-GFP, AHK4::GUS, pETT::SV40-3xeGFP, E2023* line, *CRC::CRC-GFP, miR165a::GFP, REV::REV-VENUS, miR164C::VENUS*.



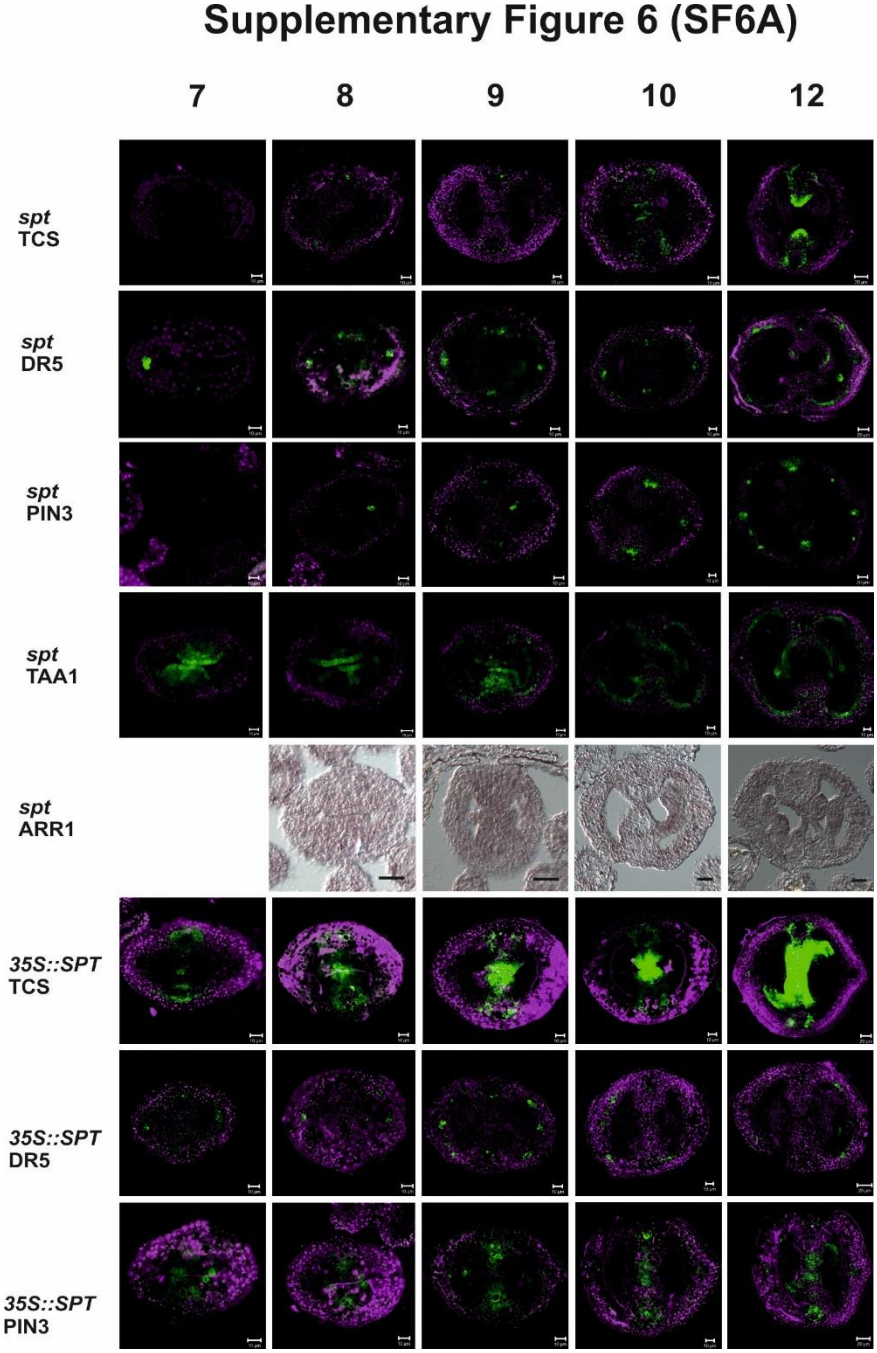
Supplementary Figure 3. Expression of several genes involved in *Arabidopsis* gynoecium development C; Expression of several transcriptional and translational fusion lines in transverse sections of stage 3 to 12 gynoecia: pMP::SV40-3xeGFP, The *PID::PID-GFP, TAA1::GFP-TAA1, AHP6::GFP, FUL::FUL-GFP, ARR16::GUS, IPT1::GUS, YUCCA4::GUS*



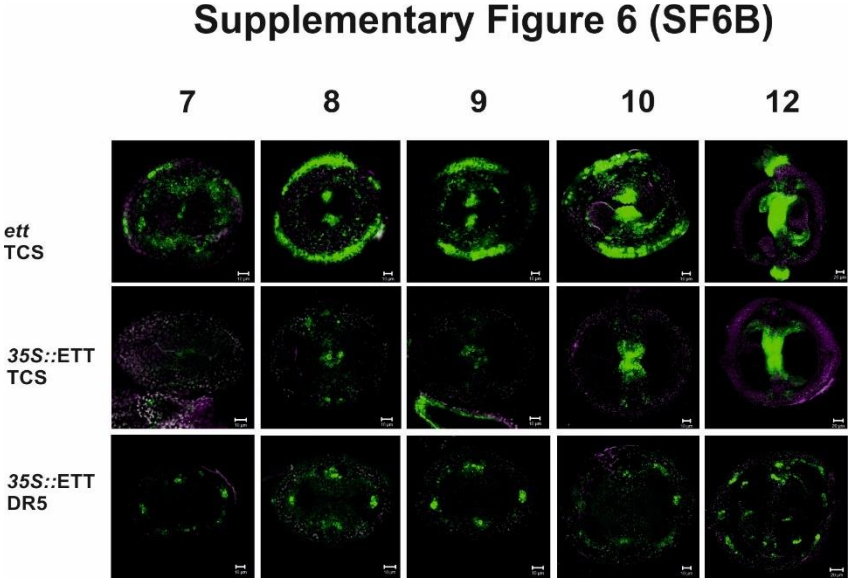
Supplementary Figure 4. Expression of several genes involved in *Arabidopsis* gynoecium development upon cytokinin treatment. Expression of several transcriptional and translational fusion lines in transverse sections of stage 7-12 gynoecia after 48 hours or 5 days of BAP treatment: *TCS::GFP, DR5::GFP*, *PIN3::PIN3-GFP, PIN1::PIN1-GFP, pETT::SV40-3xeGFP, AHP6::GFP, TAA1::GFP-TAA1* after 48 hours and *SPT::GUS, STM::GUS* and *ARR16::GUS* after 5 days.



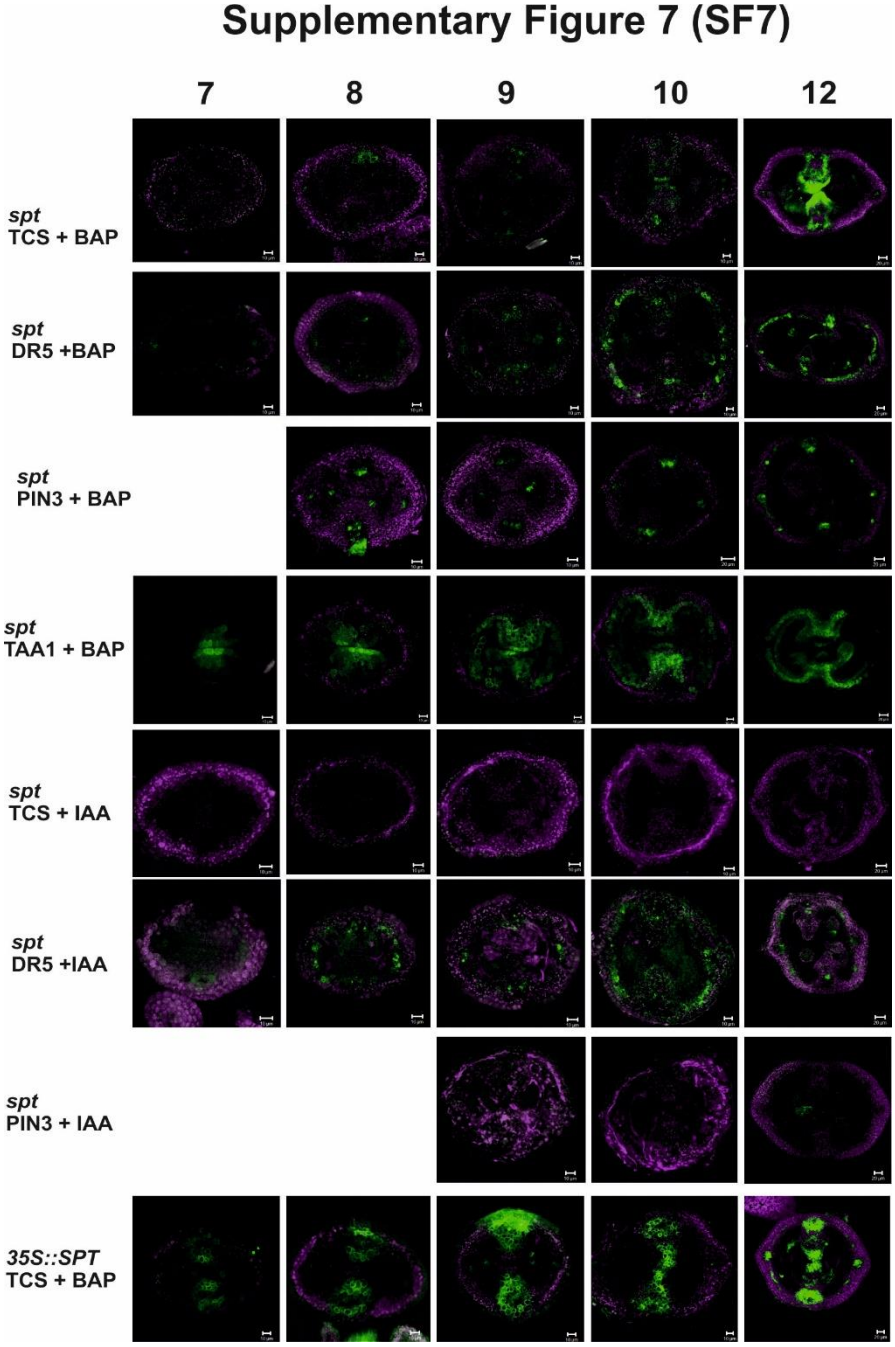
Supplementary Figure 5. Expression of several genes involved in *Arabidopsis* gynoecium development upon IAA treatment. Expression of several transcriptional and translational fusion lines in transverse sections of stage 7-12 gynoecia after 48 hours of IAA treatment: *TCS::GFP, DR5::GFP, PIN3::PIN3-GFP, AHP6::GFP*.



Supplementary Figure 6. Expression of several genes involved in gynoecium development different backgrounds. A) Expression of several transcriptional and translational fusion lines in transverse sections of stage 7-12 gynoecia at *spt-2* and *35S::SPT* backgrounds (continues…..).

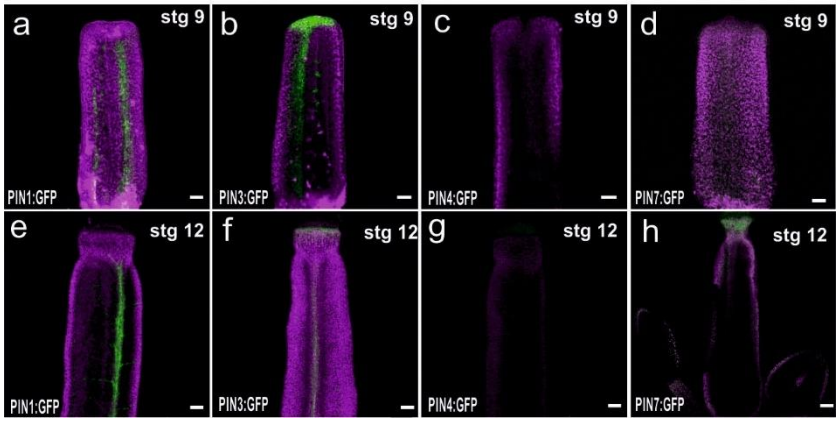


Supplementary Figure 6. Expression of several genes involved in gynoecium development different backgrounds. B) Expression of several transcriptional and translational fusion lines in transverse sections of stage 7-12 gynoecia at *ett* and *35S::ETT* backgrounds.



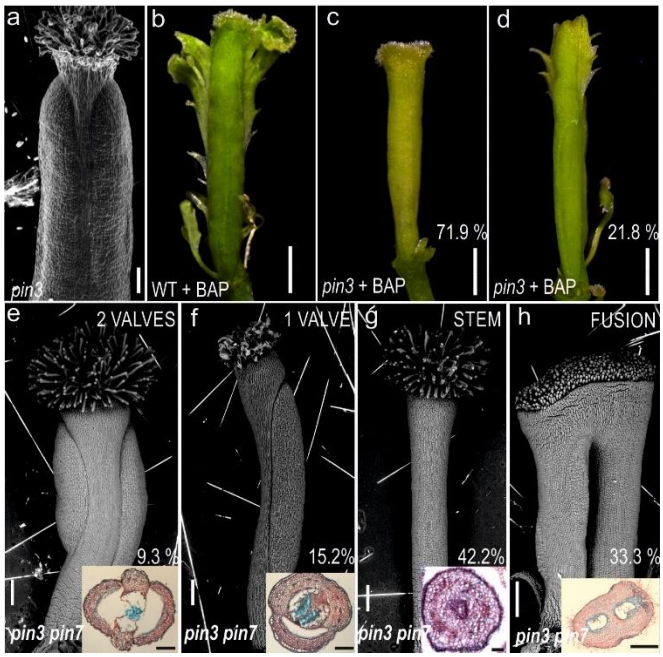
Supplementary Figure 7. Expression of several genes involved in gynoecium development in different backgrounds and upon cytokinin and auxin treatment. Expression of several transcriptional and translational fusion lines in transverse sections of stage 7-12 gynoecia at *spt-2* and *35S::SPT* backgrounds after 48 hours of BAP or IAA treatment.

Supplementary Figure 8 (SF8)



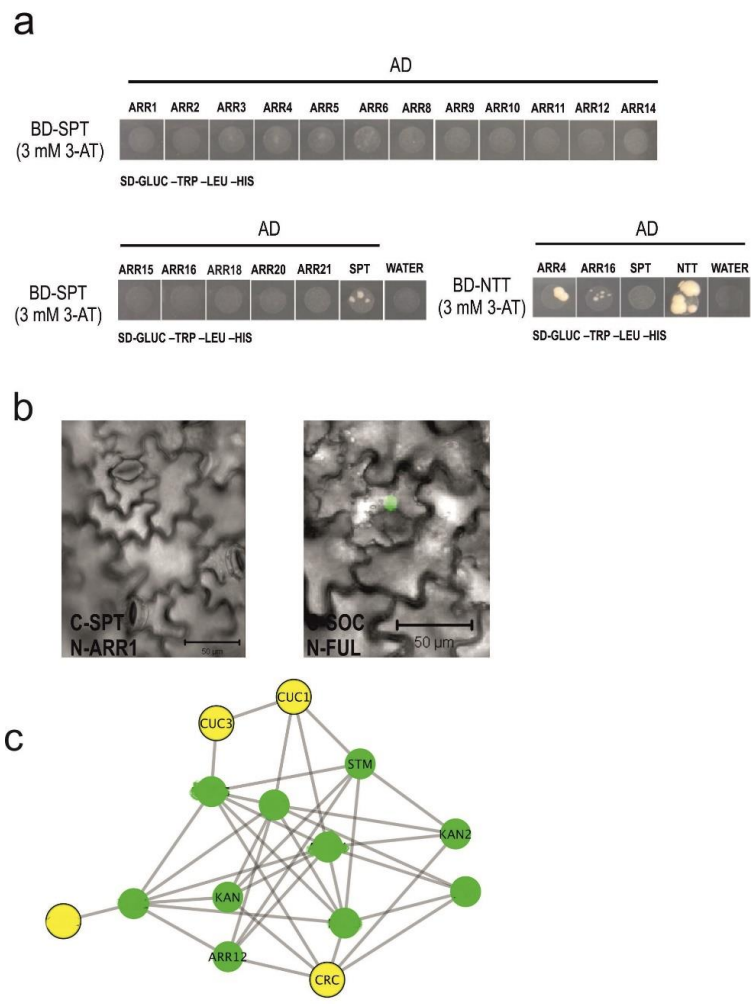
Supplementary Figure 8. Expression of auxin efflux PIN transporters in the gynoecium. Expression of PIN translational GFP fusions in stage 9 and 12 gynoecia: *PIN1::PIN1-GFP* (e , i), *PIN3::PIN3-GFP* (f, j), *PIN4::PIN4-GFP* (g, k), and *PIN7::PIN7-GFP* (h, l). Scale bars: 10 µm (a-c), 20 µm (d-h), 50 µm (i-l).

Supplementary Figure 9 (SF9)



Supplementary Figure 9. *PIN3* is necessary for a cytokinin signalling and with *PIN7* for correct gynoecium development. a, Scanning electron microscopy image of *a pin3-4* mutant gynoecium. b-d, 5 days BAP-treated gynoecia (photos taken 3-4 weeks after) of wild type with the typical proliferating tissue from the repla (b), of *pin3-4* lacking the proliferating tissue from the repla (c), and of *pin3-4* presenting a minor phenotype in 21.8% of the cases (n = 330) (d). e-h, Observed gynoecia phenotypes in the *pin3 pin7* double mutant (non-treated plants; n = 277); Insets show a transverse section at the middle of the ovary structure. Scale bars: 100 µm (a, e-h), 10 mm (b-d).

Supplementary Figure 10 (SF10)



Supplementary Figure 10. Protein-protein interaction assays of SPT with ARR proteins. a, Yeast two-hybrid assay with SPT fused to the GAL4 DNA binding domain in combination with itself (homo-dimerization detection) and with 9 type-B ARR proteins (ARR1, ARR2, ARR10, ARR11, ARR12, ARR14, ARR18, ARR20, and ARR21) and 8 type-A ARR proteins (ARR3, ARR4, ARR5, ARR6, ARR8, ARR9, ARR15, and ARR16), all fused to the GAL4 activation domain. Control reactions, NO TRANSMITTING TRACT (NTT) fused to the GAL4 DNA binding domain in combination with itself (homo-dimerization detection) and with ARR4, ARR16, and SPT fused to the GAL4 activation domain. No interaction is observed between SPT and ARR proteins. b, Bimolecular fluorescence complementation (BiFC) assay of SPT with ARR1 in *N. tabacum* leaves, where no interaction (no fluorescence) is detected and positive BiFC assay of SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1) with FRUITFULL (FUL). c, Yeast two-hybrid assay with CRC fused to the GAL4 DNA binding domain in combination with REGIA matrix.

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Imaging Early Stages of the Female Reproductive Structure of Arabidopsis by Confocal Laser Scanning Microscopy

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Background: **The gynoecium is the female reproductive structure and probably the most complex plant structure. During its development, different internal tissues and structures are formed. Insights in gene expression or hormone localization patterns are key to understanding gynoecium development from a molecular biology point of view.** **Results:** **Imaging with a confocal laser scanning microscope (CLSM) is a widely used strategy; however, visualization of internal developmental expression patterns in the Arabidopsis gynoecium can be technically challenging. Here, we present a detailed protocol that allows the visualization of internal expression patterns at high resolution during gynoecium development. We demonstrate the applicability using a cytokinin response marker (*TCS::GFP*), an auxin response marker (*DR5::VENUS*), and a SEPALLATA3 marker (*SEP3::SEP3:GFP*).** **Conclusions:** **The detailed protocol presented here allows the visualization of fluorescence signals in internal structures during Arabidopsis gynoecium development. This protocol may also be adapted for imaging other challenging plant structures or organs.** ***Developmental Dynamics 244:1286–1290, 2015*****. © 2015 Wiley Periodicals, Inc.**

Key words: Gynoecium; Arabidopsis; confocal laser scanning microscopy; fluorescence signal; expression pattern; cytokinin; auxin; SEPALLATA3

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Introduction

Confocal laser scanning microscopy (CLSM) is a technique for obtaining high-resolution optical images at the cellular level of the sample of interest. CLSM imaging together with the use of a fluorescence protein like Green Fluorescent Protein (GFP) (Chalfie et al., 1994) is a most powerful tool to study cell and developmental biology in plants and animals (e.g., Tsien, 1998; Haseloff and Siemering, 2006; Reddy et al., 2007; Chudakov et al., 2010). In plants, especially in Arabidopsis, CLSM and the use of GFP has become an essential tool to study developmental processes such as root, embryo, and shoot apical meristem (SAM) development (e.g., Benkova et al., 2003; Blilou et al., 2005; Gordon et al., 2009).

The female reproductive structure of the flower is called the gynoecium and is a highly complex organ with a great diversity of forms (Endress and Igersheim, 2000; Endress, 2006). The Arabidopsis gynoecium is a complex syncarpic structure composed of two congenitally fused carpels that arise from a single primordium at the center of the flower. When mature, it consists of an apical stigma, a short style, an ovary, and a basal gynophore (Bowman et al., 1999; Alvarez-Buylla et al., 2010; Ferrándiz et al., 2010; Reyes-Olalde et al., 2013) (Fig. 1). Many patterning and identity genes have been identified during gynoecium development (Roeder and Yanofsky, 2006; Reyes-Olalde et al., 2013). The correct spatial and temporal expression of these genes is essential for proper gynoecium development.

Recently, there is increased interest in gynoecium development, especially in the characterization of GFP fluorescence of gene fusions or marker lines (e.g., Girin et al., 2011; Marsch-Martinez et al., 2012; Larsson et al., 2014; Martinez-Fernandez et al., 2014; Moubayidin and Ostergaard, 2014; Zuñiga-Mayo et al., 2014). However, fluorescence signal observation in tissues located internally in the ovary part of the gynoecium, e.g., the carpel margin meristem (CMM), can still be challenging. Therefore, we describe here a detailed protocol that allows the visualization of internal expression patterns at high resolution during gynoecium development using CLSM. We demonstrate its applicability using a cytokinin response marker (*TCS::GFP*), an auxin response marker (*DR5::VENUS*), and a SEPALLATA3 marker (*SEP3::SEP3:GFP*).

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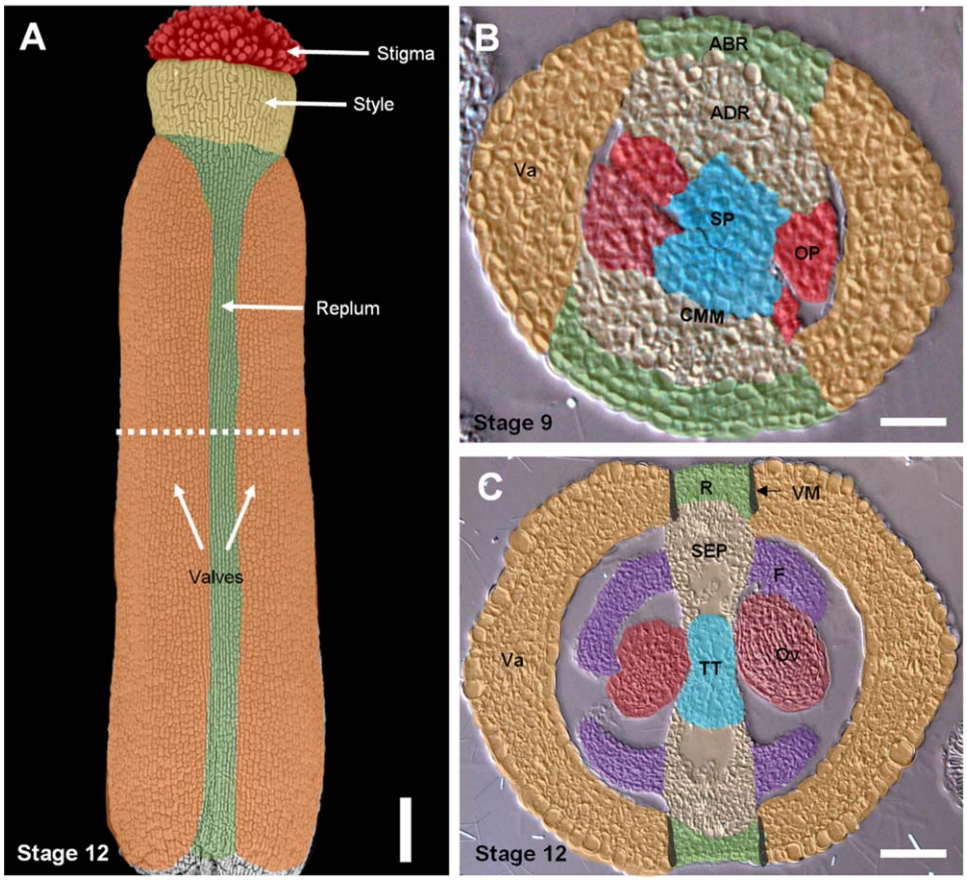

Fig. 1. Overview of *Arabidopsis thaliana* (accession: Col-0) gynoecium development. **A:** False-colored longitudinal scanning electron microscope image of a stage-12 gynoecium. The dotted line indicates where normally a transverse cut is made to obtain a transverse section of the ovary for imaging. False-colored transverse ovary section of a stage-9 (**B**) and a stage-12 (**C**) gynoecium. CMM, carpel margin meristem; OP, Ovule primordium; SP, septum primordium; SEP, Septum; Ov, Ovule; F, Funiculus; TT, Transmitting tract; Va, Valve; VM, Valve margin; R, Replum; ABR, Abaxial replum; ADR, Adaxial replum. Scale bars = 100 μM (A, C), 10 μM (B).

Results and Discusion

In this work, we developed an easy and sensitive protocol that allows the visualization of internal expression patterns at high resolution during gynoecium development. For this we used a cytokinin response marker *TCS::GFP* (Muller and Sheen, 2008), an auxin response marker *DR5::VENUS* (Heisler et al., 2005), and a SEPALLATA3 (SEP3) marker *SEP3::SEP3:GFP*, where GFP is translationally fused with the floral organ identity MADS domain protein SEP3 (de Folter et al., 2007; Urbanus et al., 2009).

We are interested in observing gene expression or expression of marker lines during early gynoecium development in *Arabidopsis thaliana* plants (Fig. 1), i.e., floral stages 5 to 12 (Smyth et al., 1990). A confocal laser scanning microscope (CLSM) allows the visualization of fluorescence expression in tissue and organs. Observing fluorescence signal from the outside of the organ, like a gynoecium, is relatively easy. However, it is more challenging to observe fluorescence signal in the tissue inside the gynoecium, i.e., inside the ovary, at a high cellular resolution.

Basically, the tissue can be mounted in agar or in glycerol, with the latter allowing the visualization of higher intensity fluorescence signal.

One possibility is to mount the tissue in agar, and thus prevent the tissue from moving. Figure 2A shows the TCS::GFP fluorescence signal (in green) from a longitudinal image of a stage-12 gynoecium. In Figure 2C and E, fluorescence signal is observed in images of transversely cut gynoecia (in the ovary region) mounted in agar. The TCS::GFP signal is observed in the carpel margin meristems (CMM) and septa primordia in the medial domain of stage 9 gynoecia (Fig. 2C) and in stage-12 gynoecia in the septum, funiculi, and the valve margins (Fig. 2E), as we have observed before with this method (Marsch-Martinez et al., 2012). In the Experimental Procedures section, a detailed description is given on how to obtain a good transverse-sectioned gynoecium.

Alternatively, glycerol instead of agar can be used as mounting medium, which we found to allow for the visualization of increased intensities of fluorescence signal, as we have observed before with this method (Zuñiga-Mayo et al., 2014). In Figure 2B, the TCS::GFP fluorescence signal (in green) in a longitudinal image of a stage-12 gynoecium can be observed, now mounted in glycerol allowing a better visualization of the signal. Clear GFP signal from the internal medial tissues (e.g., funuculi) can be observed also from the outside, together with the signal from the valve margins that are located in the external part of the gynoecium. In Figure 2D and F, the gynoecia are mounted in glycerol and compared to Figure 2C and E (in agar), the TCS::GFP fluorescence signal (in green) is much more intense. Less intensely expressed genes or reporter lines might not be visible when agar is used as mounting medium.

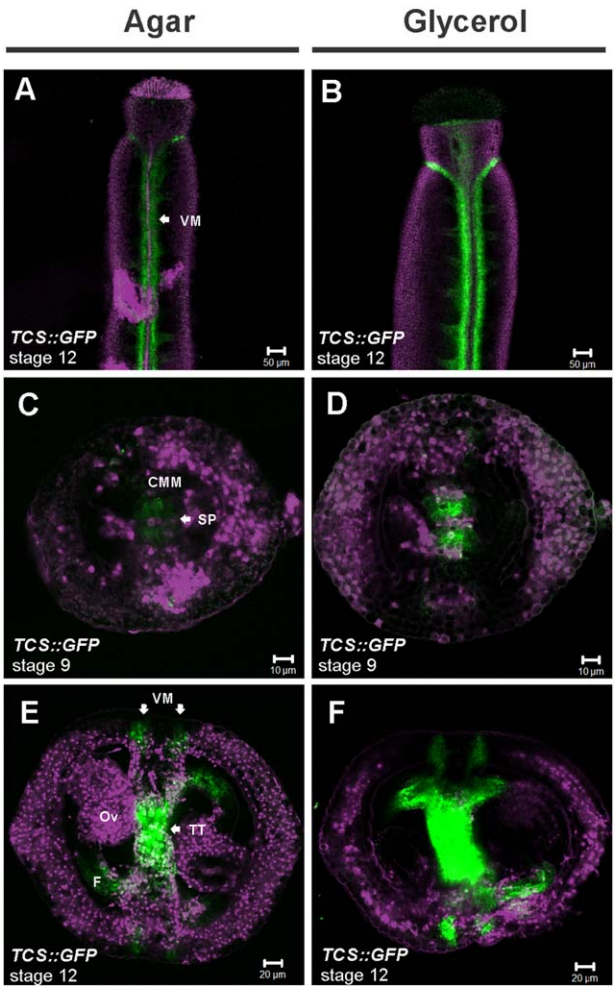

Fig. 2. CLSM imaging of Arabidopsis gynoecia. Longitudinal image of a *TCS::GFP* stage-12 gynoecium mounted in agar (**A**) or mounted in glycerol (**B**). Image of a transverse section of a *TCS::GFP* gynoecium mounted in agar, stage 9 (**C**), and stage 12 (**E**), or mounted in glycerol, stage 9 (D), and stage 12 (**F**). GFP signal in green and PI signal in red. CMM, carpel margin meristem; SP, septum primordium; Ov, Ovule; F, Funiculus, VM, valve margin; TT, transmitting tract.

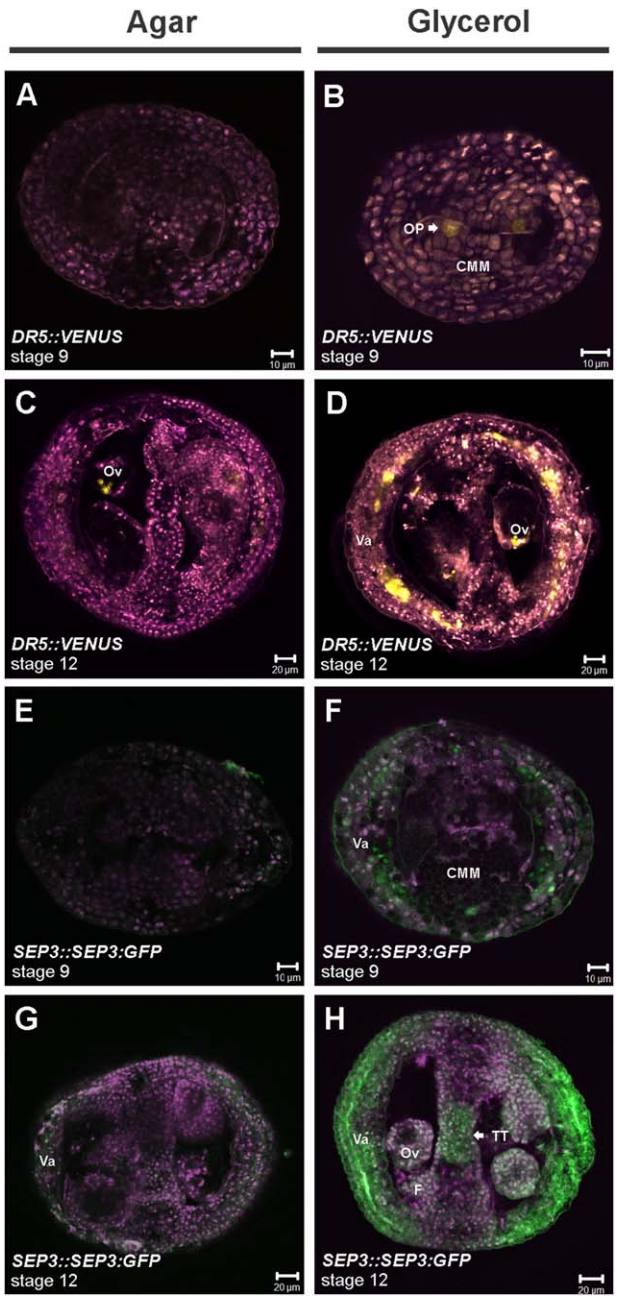

Fig. 3. CLSM imaging of a transverse section of an Arabidopsis gynoecium. Image of a transverse section of a *DR5::VENUS* gynoecium mounted in agar, stage 9 (**A**), and stage 12 (**C**), or mounted in glycerol, stage 9 (**B**), and stage 12 (**D**). Image of a transverse section of a *SEP3::SEP3:GFP* gynoecium mounted in agar, stage 9 (**E**), and stage 12 (**G**), or mounted in glycerol, stage 9 (**F**), and stage 12 (**H**). VENUS signal in yellow, GFP signal in green, and PI signal in red. CMM, carpel margin meristem; SP, septum primordium; OP, ovule primordium; Ov, ovule; F, funiculus; TT, transmitting tract; Va, Valve.

Results presented in Figure 3 show the limitations when using agar as mounting medium for the reporter line *DR5::VENUS* (Fig. 3A and C) and for the translational fusion *SEP3::SEP3:GFP* (Fig. 3E and G). The fluorescence signal cannot easily be detected in transverse ovary sections of stage-9 or -12 gynoecia. In contrast, with glycerol as mounting medium, clear DR5::VENUS signal can be observed in the initiating ovule primordia (Fig. 3B) and during later development in the ovule and in the vasculature present in the valves (Fig. 3D). Notably, the method also works well when using VENUS as a fluorophore. The SEP3::SEP3:GFP fluorescence signal can be observed in the valves at stage 9 and stage 12, when mounted in glycerol (Fig. 3F and H), and at stage 12 also in the transmitting tract, and low expression seems to be present in the funiculi and in ovules (Fig. 3H).

Improving the final image can be accomplished in various ways (e.g., Pawley, 2006), in addition to the type of mounting medium used or increasing the laser power to some extent or changing the pinhole; the use of objectives with higher numerical aperture (NA), the use of immersion objectives, or the type of coverslips used can have a big effect on the final result. Another option is taking more images of the same confocal plane or taking z-stack images and then creating a 3D projection. Creating a 3D projection can give a good impression of the expression of, e.g., a marker line in a complete organ, which can give a better understanding than just having an image of a single optical section.

In summary, the method presented here allows the visualization of fluorescence signals in internal structures, directly through the ovary wall or in a transversely sectioned ovary, during Arabidopsis gynoecium development. This protocol may also be adapted for imaging other challenging plant tissues or organs.

Experimental Procedures

Plant Material and Growth Conditions

Plants were grown in soil in a greenhouse at 22–25°C under natural light (long day conditions; around 16 hr light/8 hr dark). The *TCS::GFP* (Muller and Sheen, 2008), *DR5rev::*3x*VENUS-N7* (Heisler et al., 2005), and the *SEP3::SEP3:GFP* (de Folter et al., 2007; Urbanus et al., 2009) lines are in the Col background. Notably, a decreased light condition may have a negative effect on the fluorescence signal intensity.

Equipment and materials for sample preparation

- Dissecting microscope
- Vacuum desiccator and pump
- Insulin needles
- Forceps (Dumont #3C)
- Disposable ophthalmic scalpel with a 15° angled point (FEATHER)
- Petri dishes
- Coverslips (24 X 50 mm; 0.13–0.17 mm thickness; LAUKA)
- CoverWell Perfusion chamber (PC8R-2.0; 8X9 mm DIA, 2.0 MM Depth; Grace Bio-Labs) (Optional)
- 5 mg/ml (7.4 mM) propidium iodide (PI) stock (Fluka; protect from light, store at 4°C)
- Glycerol (molecular grade)
- 0.8% agar 0.5×MS medium (results in less fluorescence signal) (Optional)
- Distilled water

Sample preparation

Longitudinal imaging

1. Put a drop of distilled water and a drop of 10 μM propidium iodide (PI) solution (counterstain) in a Petri dish.
2. Collect an inflorescence and place it in the drop of water to avoid desiccation.
3. Place the Petri dish under a dissecting microscope and dissect the gynoecium from a floral bud using small needles (we recommend insulin needles).
4. Place the dissected gynoecia in a drop of water until the desired amount is collected.
5. Place all gynoecia in the drop of 10 μM PI solution and incubate for 1–5 min.
6. Place a few drops of 20% glycerol (mounting medium) on a coverslip and place the dissected samples in it.
7. Place the coverslip in a vacuum desiccator and apply vacuum for 3–10 min (Optional: coverslip with samples in glycerol can be stored up to 20 min before starting CLSM for better visualization).
8. Add a coverslip onto the mounted samples and when necessary add more 20% glycerol.
9. Place the coverslip sandwich in a vacuum desiccator and apply vacuum for 10 min to remove possible air bubbles.
10. Observe samples using a CLSM (Optional: 10 μM PI may be added to the mounting medium to improve counterstaining).

Imaging of transverse sections

1. Put a drop of distilled water, a drop of 20% glycerol containing 50 μM propidium iodide (PI), and a drop of 25% glycerol in a Petri dish (Note: PI concentration may be lowered when observed counterstain is too intense).
2. Collect an inflorescence and place it in the drop of water to avoid desiccation.
3. Place the Petri dish under a dissecting microscope and dissect the gynoecium from a floral bud using small needles (preferably insulin needles).
4. Place dissected gynoecia in a drop of water till the desired amount is collected.
5. Take the gynoecium out of the water drop and then cut the gynoecia transversely using a scalpel (Note: Do not squeeze the gynoecium with the scalpel).
6. Transfer the cut gynoecia parts directly to the drop of 20% glycerol with 50 μM PI and incubate for 30–60 sec (Note: Time may be shortened when counterstain is too intense).
7. After counterstaining, transfer the samples directly to a drop of 25% glycerol (Note: Samples may stay in the glycerol up to 1 hr).
8. Place 1–2 μl of 50% glycerol (mounting medium) on a coverslip and, with the help of needles and forceps, place one dissected sample in it with its cut surface directed to the coverslip (Note: No coverslip is placed over the samples; optional: a silicone isolator may be used on the coverslip to divide/distinguish samples).
9. Mounting in agar (optional): after step 5, place the gynoecia in a drop of 50 μM PI solution and incubate for 1–5 min. After counterstaining, transfer the samples directly to a drop of water. Place a silicon isolator on a coverslip and fill a well with 0.8% agar 0.5× MS and, when still liquid, place the sample(s) in it and let the agar solidify (Note: Less fluorescence signal is observed compared to glycerol).
10. Observe samples using a CLSM (Note: Observe samples via the coverslip using an inverted CLSM).

Confocal laser scanning microscopy (CLSM)

Fluorescent images were captured using a confocal laser scanning inverted microscope LSM 510 META (Carl Zeiss, Germany). GFP was excited with a 488-nm laser line and VENUS and propidium iodide (PI) with a 514 laser line of an Argon laser. GFP emission was filtered with a BP 500–520-nm filter, VENUS emission was filtered with a BP 535–590-nm filter, and PI emission was filtered with a LP 575-nm filter. The following objectives were used: EC Plan-Neofluar 10x/0.3, EC Plan-Neofluar 20x/0.5, and EC Plan-Neofluar 40x/0.75. The pixel time was 12.8 μs and each image is the average of 4 scans.

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The role of cytokinin during Arabidopsis gynoecia and fruit morphogenesis and patterning

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SUMMARY

Cytokinins have many essential roles in embryonic and post-embryonic growth and development, but their role in fruit morphogenesis is currently not really known. Moreover, information about the spatio-temporal localization pattern of cytokinin signaling in gynoecia and fruits is lacking. Therefore, the synthetic reporter line *TCS::GFP* was used to visualize cytokinin signaling during gynoecium and fruit development. Fluorescence was detected at medial regions of developing gynoecia, and, unexpectedly, at the valve margin in developing fruits, and was severely altered in mutants that lack or ectopically acquire valve margin identity. Comparison to developing gynoecia and fruits in a *DR5rev::GFP* line showed that the transcriptional responses to cytokinin and auxin are frequently present in complementary patterns. Moreover, cytokinin treatments in early gynoecia produced conspicuous changes, and treatment of valve margin mutant fruits restored this tissue. The results suggest that the phytohormone cytokinin is important in gynoecium and fruit patterning and morphogenesis, playing at least two roles: an early proliferation-inducing role at the medial tissues of the developing gynoecia, and a late role in fruit patterning and morphogenesis at the valve margin of developing fruits.

Keywords: cytokinins, Arabidopsis gynoecium and fruit development, valve margin, cell proliferation, *TCS::GFP*, auxin.

INTRODUCTION

Flower development is a key process for all living angiosperms, and is essential for sexual reproduction. Fruits develop from the female reproductive part of the flower, which is also referred to as the gynoecium and consists of one or more ovule-bearing leaf-like structures, the carpels. Fruits are important for seed dispersal and have a high nutritional value.

In Arabidopsis, the gynoecium starts developing as a hollow tube from where the medial tissues initiate as two internal ridges that grow towards each other to form the septum (inside) and replum (outside). After fusion, ovules start to develop from the internal tissue (placenta). Meanwhile, cell proliferation at the apical region of medial tissues gives rise to the style, closing the hollow tube. The style is then crowned by stigmatic papillae, where pollen tubes germinate and grow through the transmitting tract to reach the ovules in a mature gynoecium (Bowman *et al.*, 1999; Alvarez and Smyth, 2002; Roeder and Yanofsky, 2006; Sundberg and Ferrándiz, 2009). After fertilization, the fruit elongates synchronically as the seed develops. Stigmatic papillae degenerate, and the valve margin, which is located between the valve and the replum, matures, involving lignification of special cells (including the valves), finally leading to dehiscense and seed release (pod shattering) (Ferrandiz, 2002). Some transcription factors involved in proper valve margin development are *INDEHISCENT* (*IND*) and *SHATTERPROOF1* and *2* (*SHP1/2*), and their absence results in indehiscent fruits (Liljegren *et al.*, 2000, 2004). Expression of the *IND* and *SHP1/2* genes is repressed in the valve by *FRUITFULL* (*FUL*). This transcription factor is required to prevent conversion of the valve into valve margin tissue, and valves acquire valve margin identity and fail to elongate in *ful* mutant fruits (Ferrandiz *et al.*, 2000; Liljegren *et al.*, 2000, 2004).

Hormones play key roles in diverse plant processes (Wolters and Jurgens, 2009), such as fruit development and patterning (Balanza *et al.*, 2006; Alabadi *et al.*, 2009; Sundberg and Ostergaard, 2009). Auxin has received special attention. Alterations in biosynthesis, signaling or transport components can severely affect gynoecium development and patterning (Ståldal and Sundberg, 2009; Sundberg and Ferrándiz, 2009; Sundberg and Ostergaard, 2009). Auxin gradients in the root specify the position of the root meristem (Sabatini *et al.*, 1999), and auxin has also been proposed to act as an apical–basal gradient in fruit development (Nemhauser *et al.*, 2000). Moreover, auxin biosynthesis genes are regulated by transcription factors guiding fruit patterning (Alvarez *et al.*, 2009; Trigueros *et al.*, 2009; Eklund *et al.*, 2010). In contrast, it was recently shown that lack of auxin but the presence of gibberellins is required for correct valve margin formation (Sorefan *et al.*, 2009; Arnaud *et al.*, 2010).

Cytokinins are signaling molecules derived from adenine, and they have many essential roles in embryonic and post-embryonic growth and development (Muller and Sheen, 2007, 2008; Werner and Schmulling, 2009; Argueso *et al.*, 2010). However, their role in gynoecium and fruit development is just starting to be explored. Recently, an important role for cytokinins for placental growth and ovule number has been uncovered by mutations in *CYTOKININ OXIDASE/DEHYDROGENASE* (*CKX*) genes. CKX enzymes are responsible for cytokinin breakdown, and mutations in some of them result in increased seed yield (Ashikari *et al.*, 2005; Bartrina *et al.*, 2011). Bartrina *et al.* (2011) have shown that cytokinins delay the differentiation of cells in the reproductive meristems, and regulate the activity of the ovule-forming placenta (Bartrina *et al.*, 2011). Furthermore, it has been reported that exogenous cytokinin application can cause parthenocarpic fruit growth (Vivian-Smith and Koltunow, 1999) and ectopic trichome formation on carpels in transgenic plants expressing the cytokinin biosynthetic gene *ISOPENTENYL TRANSFERASE* (*IPT*) under the control of a carpel-specific promoter (Greenboim-Wainberg *et al.*, 2005).

However, it is still not well known whether cytokinins are important for the process of fruit patterning and morphology. Moreover, detailed information about the spatio-temporal localization pattern in gynoecia is lacking, and could help to uncover further roles of cytokinins at later stages of gynoecia and fruit development or patterning.

Here we report on analyses of the cytokinin signaling pattern during various gynoecia and fruit developmental stages in wild-type plants and mutant backgrounds. Furthermore, we compared cytokinin signaling with auxin signaling, and investigated the effects of endogenous and exogenous cytokinin alterations during gynoecium and fruit development. The results strongly suggest that cytokinins play important roles in fruit patterning and morphogenesis, including a previously unexpected role in valve margin formation in fruits.

RESULTS

The *TCS::GFP* cytokinin reporter line reveals a dynamic fluorescence pattern in developing gynoecia and fruits

To uncover as yet unknown roles of cytokinin in fruit development, we sought to visualize cytokinin signaling during fruit development *in vivo*. We employed the synthetic reporter *TCS::GFP* (two-component output sensor), which contains six direct repeats of the cytokinin-induced B-type Arabidopsis response regulator binding motif (Muller and Sheen, 2008). Gynoecia and fruits of *TCS::GFP* transgenic plants at progressive floral developmental stages (according to Smyth *et al.*, 1990) were analyzed using confocal laser scanning microscopy, and the optical sections obtained are shown in Figure 1.

The gynoecium forms at the center of the floral meristem at stage 6. At stage 7, the gynoecium grows as a hollow tube, with two inner ridges, which will later give rise to the medial tissues of the gynoecium and fruit, growing towards each other. These incipient medial tissues show fluorescence, with the highest intensity from the bottom to the middle (Figure 1a). Although low, fluorescence is also found at the center of the top, as observed in the transverse picture from above (Figure 2a). At stage 8, the tube increases in size and the inner ridges keep on growing towards each other. Fluorescence can now be detected all along the developing gynoecium, from the bottom to the top, at the center of the medial region, and the contact zone of the internal ridges (Figures 1b and 2a). Furthermore, the flanks of the ridges along the whole gynoecia also show fluorescence, except at the top (Figures 1a and 2b). The medial ridges fuse at stage 9, at which the septum originates. The medial region shows fluorescence all along the gynoecium (longitudinal view, Figure 1c), and at the edges of the internal ridges that contact each other (top transverse view, Figure 2b). At stage 10, after fusing, the ridges grow to the sides and begin to form ovule primordia, arranged as interlocking projections. On top of the developing gynoecia, stigmatic papillae start forming. In the longitudinal axis, the abaxial or external face of the gynoecium shows fluorescence as two blurry lines at the sides of the incipient replum (the abaxial medial tissue), along the ovary (Figure 1d). A transverse section view reveals fluorescence at the center of the medial region (Figure 2c). At this stage, the transmitting tract, through which pollen tubes grow to reach the ovules, is differentiating in this region. The fluorescence signal continues during stage 11, when developing ovules initiate inner and outer integuments, and, at the top of the gynoecium, the style is covered by stigmatic papillae. An abaxial longitudinal view reveals fluorescence as two blurry lines at the valve–replum junction along the ovary (Figure 1e), and a transverse section shows strong fluorescence at the center of the medial region that coincides with the position of the transmitting tract (Figure 2d). It also shows fluorescence as

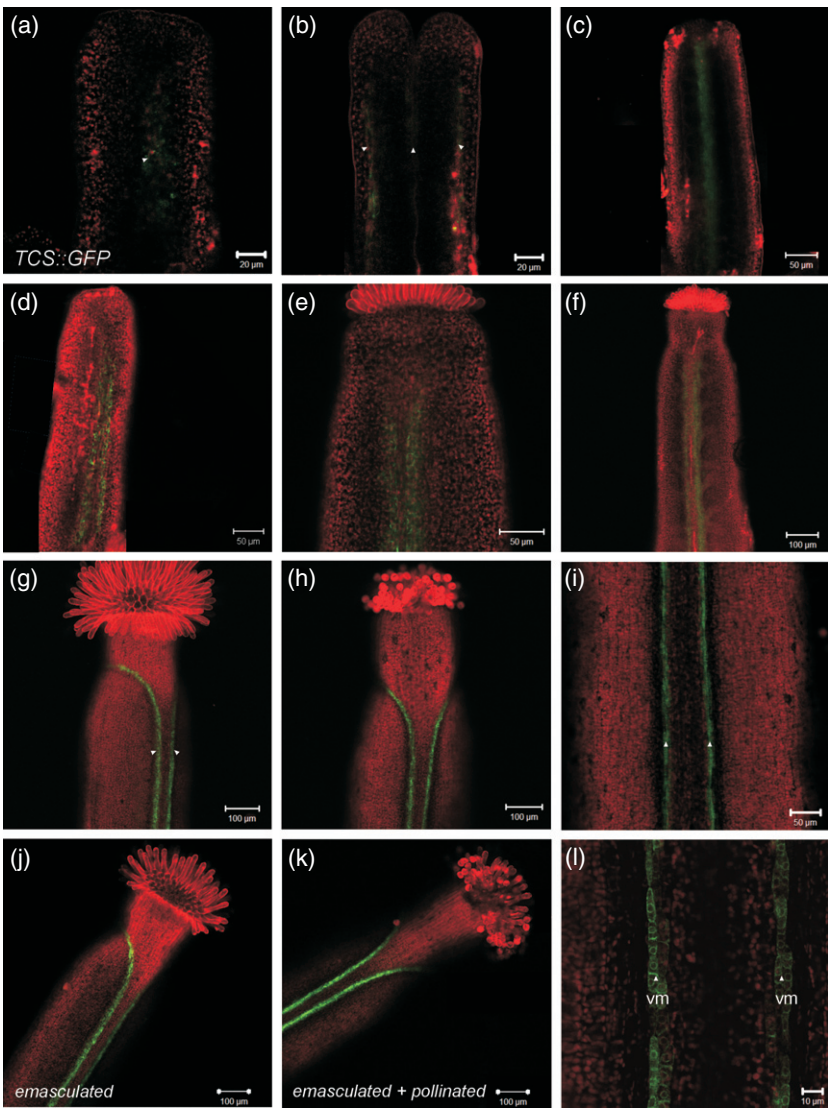

Figure 1. Fluorescence detection in single optical sections of gynoecia and fruits of the the cytokinin signaling marker line *TCS::GFP* at consecutive floral developmental stages.
(a–i) The fluorescence signal is observed in developing gynoecia and fruits at stages 7–8 (a), 8–9 (b), 9–10 (c), 10 (d), 11 (e), 12 (f), early 13 (g), 14 (h) and 17 (i). Stages are according to Smyth *et al.* (1990).
(j, k) *TCS::GFP* fluorescence detection in non-pollinated (j) and pollinated (k) gynoecia of emasculated flowers, 24 h after emasculation and manual pollination.
(l) Closer view of a stage 16–17 fruit showing localized cytokinin signaling fluorescence at the junction between replum and valves. Arrowheads in (a), (b), (g), (i) and (l) indicate the location of fluorescence in those optical sections. vm, valve margin.

two faint lines at the lateral edges of the inner medial region (Figure 2d). At stage 12, various tissues in the gynoecium (valves, valve margins, replum and style) start to adopt their specific morphological characteristics, and the gynoecium is mature. An abaxial longitudinal view shows two fluorescent lines along the valve–replum junction, and slight fluorescence in the funiculi (Figure 1f). A transverse section (Figure 2e) shows fluorescence at the center of the medial region and the funiculus, and two well-defined lines at the valve–replum junction (the position where the valve margin will be in the future fruit). At stage 13 of anthesis (opening of the flower), a longitudinal abaxial image shows localized fluorescence along the valve margin (Figure 1g). From this stage on, we only analyzed fluorescence in the abaxial face in the longitudinal axis of the gynoecium to follow this unexpected localization. At stages 14–15, the fertilized fruit starts to elongate. The fluorescence pattern is similar to that at stage 13, with two well-defined fluorescent lines at the valve–replum junction (the position of the valve margin) all along the valves (Figure 1h). To determine whether this precise localization of cytokinin signaling at the incipient valve margin at the abaxial side of the gynoecium was promoted by fertilization, we emasculated *TCS::GFP* floral buds and compared fluorescence of pollinated and unpollinated pistils 24 h after pollination. No difference in the abaxial pattern was observed (Figure 1j,k). Finally, at stages 16–17, when floral organs start to fall, leaving only the fruit attached to the pedicel, the abaxial localization of the fluorescence remains similar to the previous stages (Figure 1i). A close-up view revealed that fluorescence is observed at the valve margin (Figure 1l).

In summary, the cytokinin signaling pattern changed from early to later developmental stages, starting mainly at the incipient medial region of young gynoecia, formed by internal medial ridges, and later detected at the valve margins of maturing fruits.

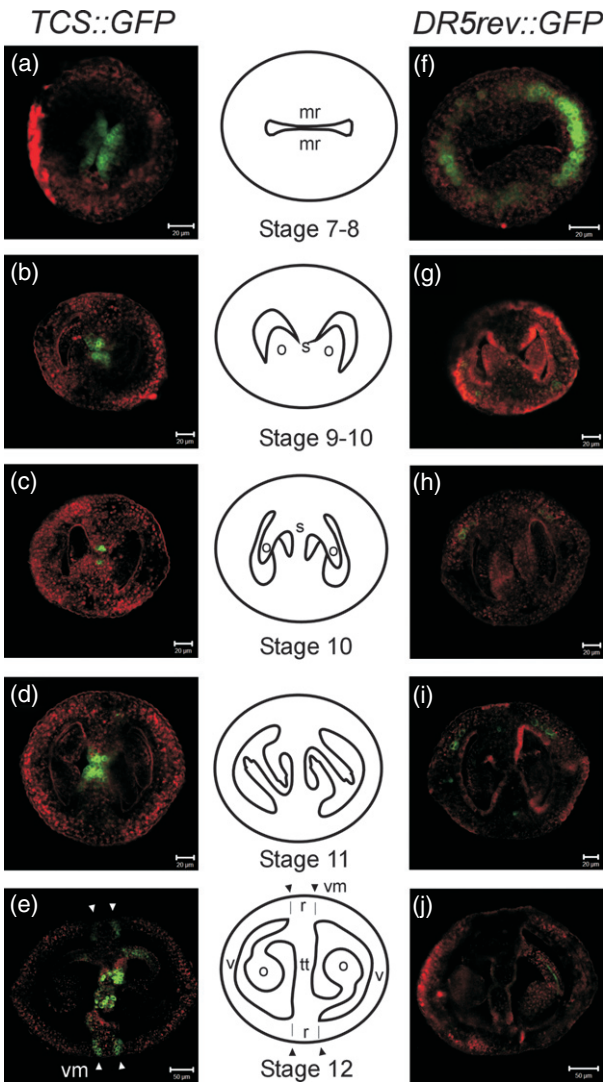

Figure 2. Fluorescence detection and comparison of fluorescence patterns in single optical transverse sections of gynoecia of the cytokinin signaling marker line *TCS::GFP* and the auxin signaling marker line *DR5rev::GFP* during development. (a–e) Cytokinin marker *TCS::GFP* gynoecia showing fluorescence in the medial tissues at various stages of development as indicated in the drawings. Stages are according to Smyth *et al.* (1990). (f–j) Auxin marker *DR5rev::GFP* fluorescence signal in developing gynoecia. The fluorescence signal is observed as a circle around the top of the gynoecium at stage 7 (f). Transverse sections showing the inner tissues of developing gynoecia at further stages reveal fluorescence mainly at the vasculature (g–j). The images shown in (a) and (f) were taken from the top of the gynoecium, and images (b–e) and (g–j) are transverse sections showing the inner tissues of the gynoecium. mr, medial region; o, ovule primordium/ovule; s, septum; r, replum; v, valve; vm, valve margin; tt, transmitting tract. Arrowheads indicate the valve margin, which is indicated by thin lines in the drawing.

Contrasting cytokinin and auxin localization patterns in gynoecia and fruits

Cytokinin and auxin together regulate many key plant processes, from growth and development to responses to the environment, and they also actively regulate each other through homeostatic feedback loops (Moubayidin *et al.*, 2009; Bishopp *et al.*, 2011a; Su *et al.*, 2011). Auxin plays a very important role in fruit development (Sundberg and Ferrándiz, 2009; Sundberg and Ostergaard, 2009). For instance, at the valve margin, an 'auxin minimum' is required for proper development of this region (Sorefan *et al.*, 2009).

In order to visualize the relationship between the hormones in the context of gynoecium and fruit development in more detail, the fluorescence pattern of the auxin reporter *DR5rev::GFP* (Benkova *et al.*, 2003) in developing gynoecia and fruits was observed using confocal laser scanning microscopy, and compared to the *TCS::GFP* pattern (Figure 2).

Opposite patterns were observed for these hormones in specific tissues and stages of development. Images taken from the top of young developing gynoecia showed cytokinin signaling inside the developing structure, especially at the growing ridges of the medial region (Figure 2a). In contrast, the reporter line for auxin signaling showed fluorescence outside this region, as a circle around the developing 'tube' (Figure 2f). Longitudinal imaging of these gynoecia revealed a circle of strong *DR5* fluorescence at the top (Figure 3a–e). Below the top, auxin reporter fluorescence was only observed at the incipient vasculature (Figure 3a–c), which probably is unrelated to gynoecia and fruit patterning and unrelated to cytokinin interaction. At stages 10–12, gynoecia of the auxin marker line still showed fluorescence at the vasculature and at the top (Figure 3d–f and Figure S1), which was full of developing stigmatic papillae, and where no fluorescence was observed for the cytokinin marker line (Figure 1).

Stage 13 *DR5* gynoecia kept showing fluorescence at the base of stigmatic papillae and the vasculature (Figure 3g). At stage 17, close observation of the auxin marker line (Figure 3i) showed fluorescence at replum and valve cells, and diminished fluorescence at the valve margins, as previously reported (Sorefan *et al.*, 2009). In contrast, the cytokinin marker only showed fluorescence at the valve margins (Figure 1i,l).

Taken together, these observations indicate that auxin and cytokinin transcriptional responses frequently occur in complementary patterns during gynoecia and fruit development.

Alterations in endogenous cytokinin levels affect medial region development in fruits

After observing the fluorescence patterns of the cytokinin reporter line, we tested the effects of altering endogenous cytokinin levels in fruit development. For this, the *lac*- and *Gal4*-based transactivation system (*pOp* × *LhG4*) was used to drive fruit expression of enzymes involved in cytokinin biosynthesis or degradation (Moore *et al.*, 1998; Shani *et al.*, 2010).

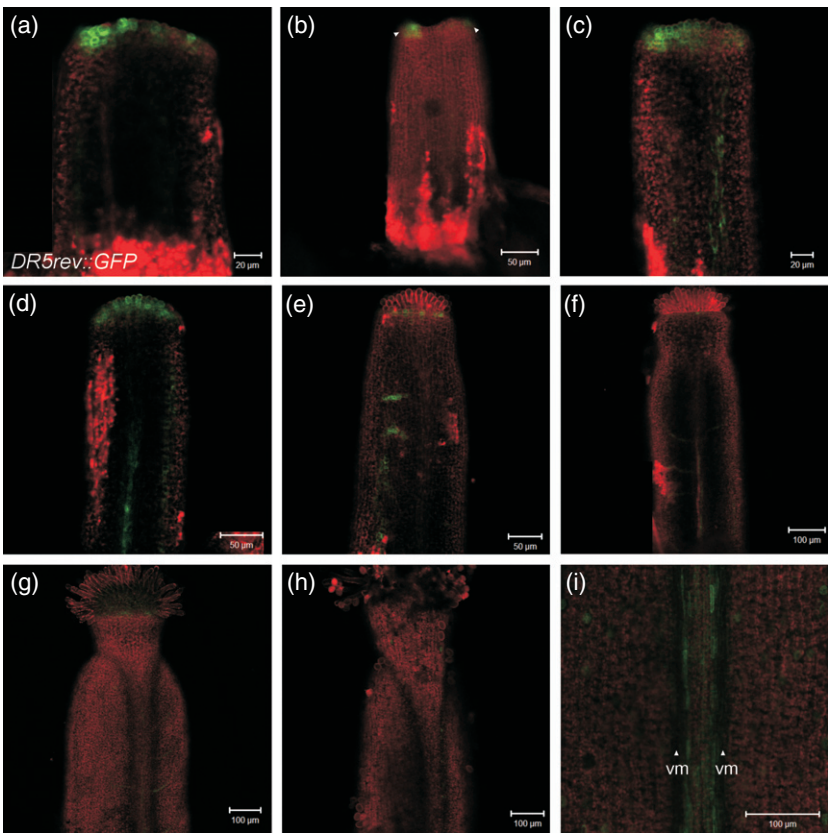

Figure 3. Fluorescence signal detection in auxin marker *DR5rev::GFP* gynoecia and fruits during development (longitudinal sections). Fluorescence signal was observed in developing gynoecia and fruits of stages 7–8 (a), 8–9 (b), 9–10 (c), 10 (d), 11 (e), 12 (f), early 13 (g) and 14 (h), and a close-up of a fruit at stage 17 (i). White arrowheads in (b) indicate localized *DR5rev::GFP* fluorescence at the top of the young gynoecium. The fluorescence signal that forms a circle at the top is observed as two dots in this optical section along the gynoecium. White arrowheads in (i) indicate the location where the cytokinin signaling marker *TCS::GFP* fruits show the most intense fluorescence, which coincides with the lowest fluorescence signal in the auxin marker *DR5rev::GFP* fruits (i). Stages are according to Smyth *et al.* (1990). vm, valve margin.

The driver lines used were *pFUL::LhG4* and *pSHP2::LhG4* (Y. Eshed, Department of Plant Sciences, Weizmann Institute, Israel, personal communication). These driver lines were crossed to the operator lines *Op::IPT7* (*ISOPENTENYLTRANSFERASE7*) and *Op::CKX3* (*CYTOKININ OXIDASE3*) (Werner and Schmulling, 2009; Shani *et al.*, 2010). To visualize the expression patterns directed by the promoters, crosses to an *Op::GUS* line were included (Shani *et al.*, 2010), which showed GUS expression in fruits (Figure 4g,h). The regulatory regions used lack introns that have been shown to be essential for a correct spatial expression pattern for other MADS box genes (Sieburth and Meyerowitz, 1997; Kooiker *et al.*, 2005; de Folter *et al.*, 2007); however, although variations in intensity and pattern were observed in different lines and fruits, in general, the *pSHP2::LhG4* line drove GUS expression in the valve margin, while *pFUL::LhG4* drove GUS expression at the valves (Figure 4g,h). Interestingly, both promoters (driver lines) produced very similar results. However, opposite effects in plant and replum development were observed for crosses with the cytokinin biosynthesis (*IPT7*) or degradation (*CKX3*) operator lines (Figure 4a–f). The transactivation lines, including the *Op::IPT7* genotype, also showed clear alterations in other tissues, resembling cytokinin-treated plants, as they showed wider stems and serrated cauline leaves (Figure 4i–n), while transactivation lines including *Op::CKX3* had narrow stems.

The *pSHP2 >>CKX3* and *pFUL>>CKX3* fruits showed a reduction in replum width in comparison to wild-type fruits (Figure 4a–c,f). In contrast, *pSHP2 >>IPT7* and *pFUL>>IPT7* fruits showed an increase in the size of their repla (Figure 4d–f). The *pFUL::LhG4* promoter showed variations in the intensity of GUS staining when crossed to the *Op::GUS* line, and moderate to large increases in replum width when crossed to the *Op::IPT7* line. Remarkably, the most altered repla had double the width of wild-type repla and contained stomata, a type of cells not observed in wild-type repla, but present in the style and valves of wild-type fruits, and in tissues such as leaves or stems (Figure 4e). In conclusion, alterations in the endogenous levels of cyto kinins were able to affect the growth of the replum, an external medial tissue.

Application of exogenous cytokinins severely alters gynoecium morphology

The expression of enzymes altering endogenous cytokinin levels in fruits affected the development of their repla. However, internal cytokinin levels can be modulated by feedback regulatory mechanisms that act upon other enzymes in cytokinin metabolism. To reduce the influence of these feedback loops, we tested the effects of continuous exogenous application of this hormone in developing gynoecia. Flowering wild-type Arabidopsis Col plants

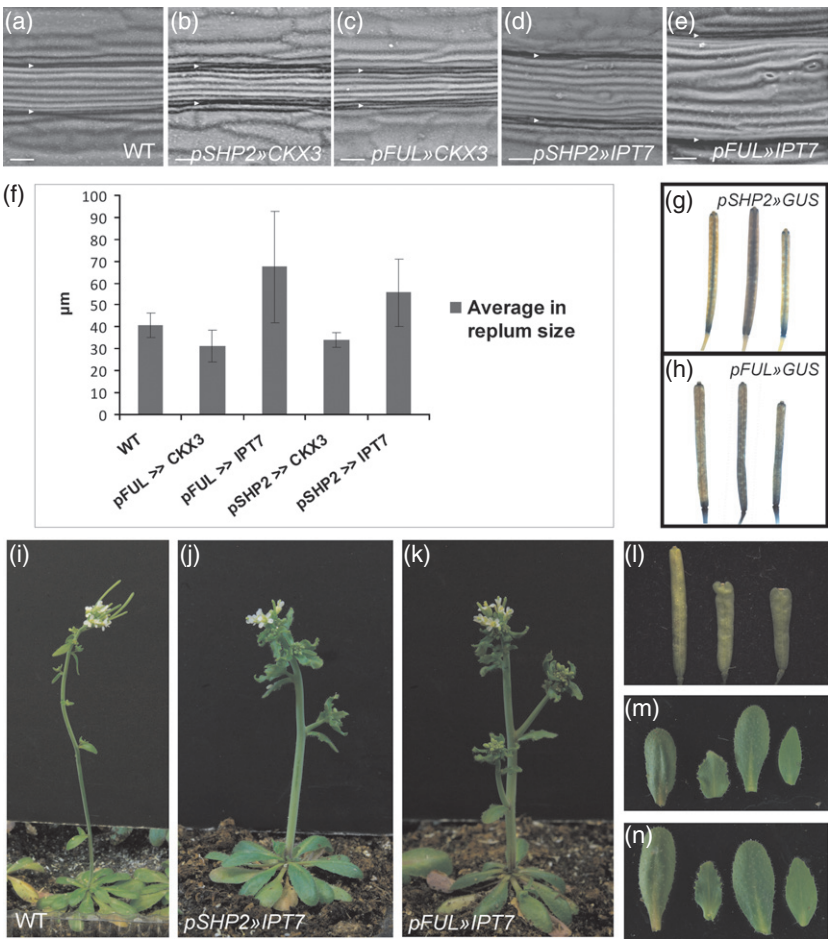

Figure 4. Alterations in endogenous cytokinin levels affect replum width.
(a–e) Representative scanning electron micrographs of a wild-type replum (a), compared with repla of reduced size observed in the transactivation lines *pSHP2 >>CKX3* (b) and *pFUL>>CKX3* (c), and repla of increased size in the transactivation lines *pSHP2 >>IPT7* (d) and *pFUL>>IPT7* (e).
(f) Comparison of mean replum size measurements in fruits of various transactivation lines compared to wild-type fruits (n = 19). Error bars indicate standard deviation.
(g, h) GUS staining of *pSHP2 >>GUS* (g) and *pFUL>>IPT7* (h) fruits. (i–n) The transactivation lines including the *Op::IPT7* genotype showed alterations in other tissues, resembling cytokinin-treated plants, i.e. wider stems, serrated cauline leaves and altered fruit shape when compared to wild-type plants.
(i–k) Whole-plant phenotype of wild-type (i), *pSHP2 > >IPT7* (j) and *pFUL>>IPT7* (k) transactivation lines.
(l) Siliques (from left to right) of wild-type, *pSHP2 >>IPT7* and *pFUL>>IPT7* plants.
(m, n) From left to right, rosette and cauline *pSHP2 >>IPT7* (m) and *pFUL>>IPT7* (n) leaves followed by rosette and cauline wild-type leaves.
Scale bars = 200 μm (a–i).

were sprayed 5 days a week with a 100 μM benzylaminopurine (BAP) solution or a mock solution. The treatment was initiated 1 week after bolting to avoid detrimental effects on development during the vegetative phase. After 3–4 weeks of treatment, the plants showed clear known effects of cytokinin application, such as short thick stems and serrated cauline leaves. Remarkably, treated gynoecia showed a conspicuous overgrowth of green tissue, crowned by colorless tissue (Figure 5b,c). Close observation revealed that the colorless tissue at the top resembled stigmatic papillae (Figure 5c), and transverse sections of these fruits showed that the growth arose from the replum (Figure 5e). Other aerial tissues did not show a comparable over-proliferation response and extreme morphological change. Fruit valves of treated plants showed short cells of variable sizes (Figure 5c), contrasting with the elongated, regularly sized cells of untreated valves. Petals and cauline leaves showed serrations at their edges, and anthers became shorter, but no other striking effect comparable to conspicuous growth of the replum was observed.

The developmental stage of the gynoecium at the start of the treatment determined the severity of the outgrowth effect. Treatment of the earliest floral buds (stages 6–8) produced the most severe outgrowth, which was observed in all gynoecia. The treatment of flowers at subsequent stages resulted in a reduction of severity (stages 8–10, intermediate; stages 10–12, slight). The treatment of fruits (stages ≥ 13) did not produce any detectable outgrowths after 4 weeks.

The effects suggest that cytokinins are also able to severely alter fruit morphology, in addition to altering organ number (Venglat and Sawhney, 1996; Lindsay *et al.*, 2006; Gordon *et al.*, 2009), fruit size and yield (Ashikari *et al.*, 2005; Bartrina *et al.*, 2011), inducing trichome formation in valves (Greenboim-Wainberg *et al.*, 2005) or triggering parthenocarpy (Vivian-Smith and Koltunow, 1999), as previously reported.

Cytokinin treatments of hormone signaling reporters and fruit patterning mutants show altered responses

To better understand the striking outgrowth phenotype in developing gynoecia sprayed with BAP (Figure 5), we treated the cytokinin and auxin marker lines, and mutants that lack various fruit tissues. First, *TCS::GFP* inflorescences were sprayed to obtain an indication of the cytokinin

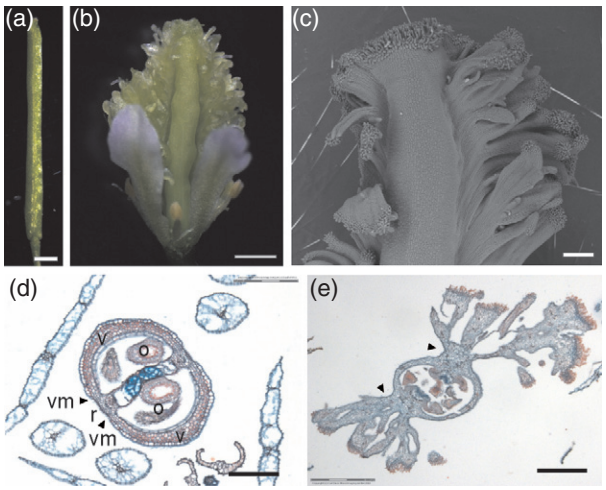

Figure 5. Exogenous cytokinin treatments induce extensive proliferation at the external medial region in developing gynoecia.
(a, b) Fruit of wild-type plants treated with a mock solution (a) or with 100 μM BAP (b).
(c) Scanning electron microscopy observations of a gynoecium of BAP-treated wild-type plants.
(d, e) Transverse sections show clear differences in the development of a gynoecium of a non-treated plant (d) compared to a gynoecium of a BAP-treated plant, in which extensive outer proliferation is observed (indicated by arrowheads) (e). Scale bars = 2 mm (a, b), 200 μm (c), 150 μm (d) and 300 μm (e). o, ovule, r, replum, v, valve, vm, valve margin.

transcriptional response after treatment. Intriguingly, an increased fluorescence signal was only observed at the internal medial tissues of the treated gynoecia, but no fluorescence was detected at the external proliferating tissue (Figure 6a,c,d). As the cytokinin and auxin pathways interact in various tissues, we investigated the auxin marker in cytokinin-induced ectopic tissues. *DR5rev::GFP* plants were subjected to the same cytokinin treatment, and, remarkably, a clear fluorescence signal was detected at the tip of the growing protuberances (Figure 6b).

Furthermore, various genotypes affected in the development of diverse fruit tissues were sprayed with BAP. The treated genotypes were *ful*, in which valves acquire valve margin identity, *shp1 shp2* and *ind* mutants, which lack the valve margin, and *35S::FUL*, in which valve margin and replum are absent (Ferrandiz *et al.*, 2000; Liljegren *et al.*, 2000, 2004). The *ful*, *shp1 shp2* and *ind* gynoecia and fruits were still able to moderately form ectopic proliferating tissues (Figure 6g–i). However, *35S::FUL* fruits that lack a replum and valve margin did not show any external proliferations, confirming that the external proliferation originated mainly from the replum (Figure 6f). Interestingly, *35S::FUL* and *shp1 shp2* fruits became wider, suggesting that proliferation was occurring internally (Figure 6f,h).

Together, the results of these experiments indicate that the internal tissues of developing gynoecia respond to external application of cytokinins by triggering proliferation, mainly observed at the external medial tissue (replum). External application of cytokinins also resulted in auxin presence, detected as a fluorescence signal of the *DR5rev::GFP* marker at the tips of the proliferating tissue.

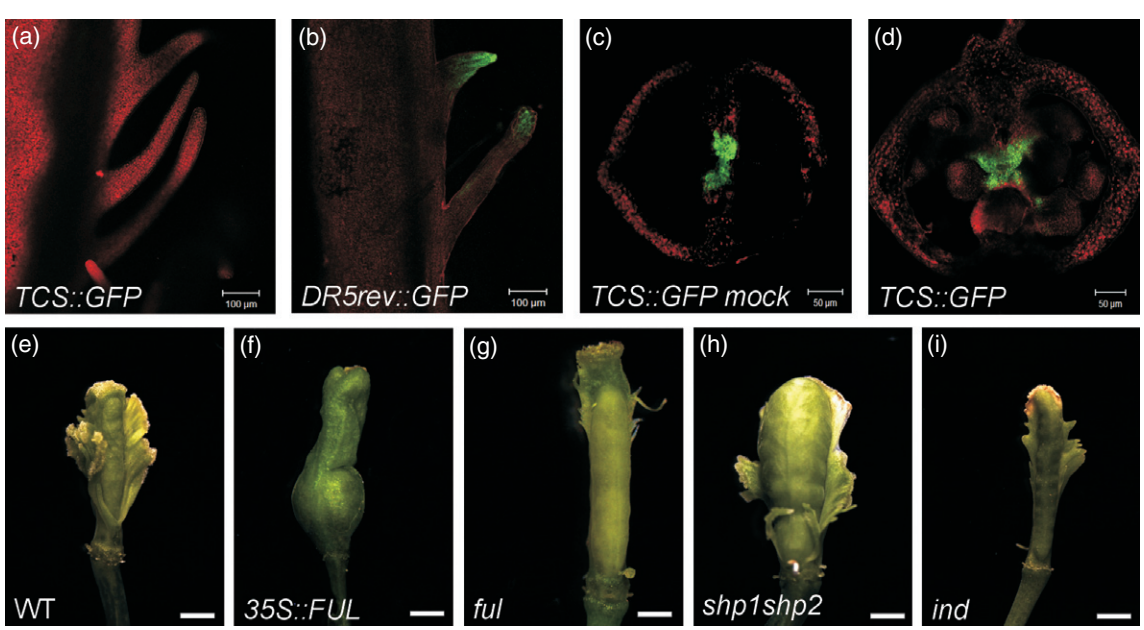

Figure 6. Cytokinin treatment of hormone signaling reporters and mutants affected in fruit patterning.
(a–d) Gynoecia of hormone signaling reporter lines.
(a, b) Treated (sprayed with BAP) *TCS::GFP* gynoecia (a) and *DR5rev::GFP* gynoecia (b).
(c, d) Untreated (c) and treated (sprayed with BAP) (d) *TCS::GFP* gynoecia.
(e–i) Gynoecia of various genotypes sprayed with BAP: Wild-type (e), 35S::*FUL* (f), *ful* (g), *shp1 shp2* (h) and *ind* (i). Scale bars = 1 mm (e–i).

Alterations in valve margin identity modify the cytokinin localization pattern

The cytokinin marker revealed fluorescence at the region between valves and repla in mature gynoecia and developing fruits, and, in order to explore the biological significance of this unexpected localization, we analyzed the cytokinin marker in fruits that lacked a functional valve margin. *TCS::GFP* fluorescence was analyzed in the *ind* mutant and in the *shp1 shp2* double mutant background. Interestingly, while *TCS::GFP* fruits showing wild-type phenotypes presented a very well-defined fluorescent line in the region between valves and replum, no fluorescence was detected in this region in homozygous *ind* or *shp1 shp2* mutants lacking a dehiscence zone (Figure 7b,c). This demonstrates that functional *IND* and *SHP1/2* are required for cytokinin accumulation at the valve–replum junction.

Conversely, in *TCS::GFP ful* mutant fruits, in which valves acquire valve margin identity (Ferrandiz *et al.*, 2000; Liljegren *et al.*, 2000, 2004), the whole valves showed very intense fluorescence (Figure 7d). This was not observed in wild-type valves, and further suggests that cytokinins and valve margin identity are indeed connected.

Cytokinin application restores valve margin formation and dehiscence in the *shp1 shp2* and *ind* mutants

After observing the changes in the pattern of cytokinin signaling in the three mutants affected in valve margin identity, and to test a possible functional role for cytokinins in this tissue, BAP was applied to valve margin mutants. In fruits of the *shp1 shp2* and *ind* mutants, the valve margin cannot be easily distinguished at the abaxial face, and the fruits fail to dehisce (Figure 8a,c). However, when BAP was locally applied to *shp1 shp2* and *ind* developing fruits (4 days after pollination), the characteristic abaxial morphology of the valve margin was recovered in maturing fruits (Figure 8b,d). Moreover, when dry, these fruits showed increased dehiscence when compared to mock-treated controls

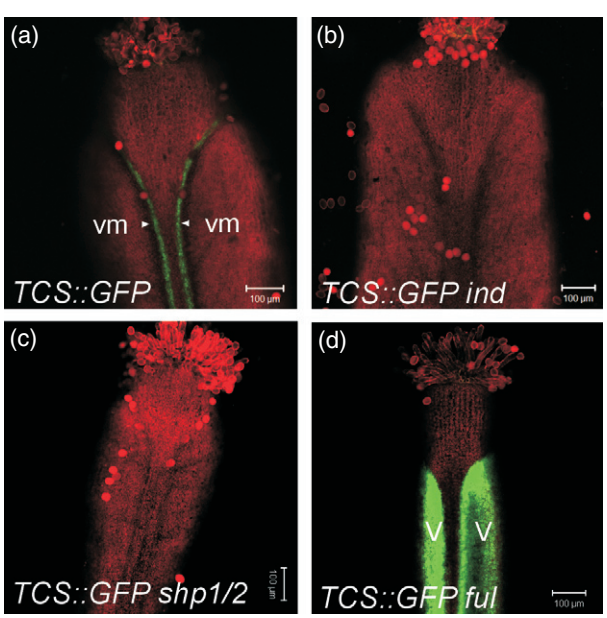

Figure 7. Cytokinin localization is severely altered in mutants that lack valve margins or that have ectopic valve margin identity in the valves. Cytokinin signaling marker *TCS::GFP* fluorescence signal is observed in the valve margins (indicated by arrowheads) of a segregating gynoecium with wild-type phenotype (a). However, no signal is detected in the *ind* (b) or *shp1 shp2* (c) mutants that lack valve margins. In the *ful* mutant, where valves acquire ectopic valve margin identity, intense fluorescence signal is detected in the whole valves (d). vm, valve margin, v, valve.

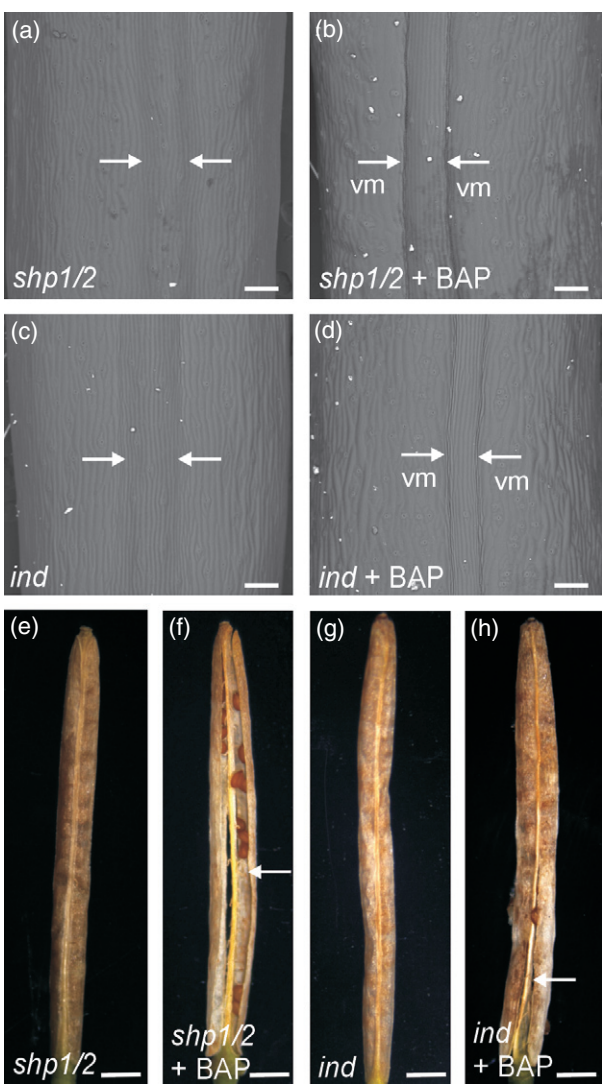

Figure 8. Local application of cytokinin restores dehiscense in valve margin mutants.
(a–d) Scanning electron micrographs of fruits of the valve margin mutants *shp1 shp2* (a, b) and *ind* (c, d) painted with a mock solution (a, c) or a solution containing BAP (b, d).
(e–h) Fruits of the valve margin mutants *shp1 shp2* (e, f) and *ind* (g, h) to which a mock treatment (e, g) or a mixture of lanolin with BAP (f, h) had been applied. The solution was painted on, and the lanolin was applied to the external medial region of developing mutant fruits. Scale bars = 100 μm (a–d) and 1 mm (e–h).

(Figure 8e–h). This suggests that cytokinins have a functional role in the process of valve margin formation that leads to proper dehiscence in fruits.

DISCUSSION

Cytokinins may play different roles depending on the context. For example, at the shoot apical meristem, they promote cell proliferation (Leibfried *et al.*, 2005; Lindsay *et al.*, 2006; Gordon *et al.*, 2009), but they play the opposite role at the root apical meristem, where they promote cell differentiation (Werner *et al.*, 2003; Dello Ioio *et al.*, 2008; Muller and Sheen, 2008). Ectopic cytokinin at the apical tissues activates shoot stem-cell genes such as *CLAVATA1* and *WUSCHEL*, while cytokinin at the basal cell in the early embryo results in failed root-stem cell determination (Werner *et al.*, 2003; Lindsay *et al.*, 2006; Dello Ioio *et al.*, 2008; Muller and Sheen, 2008; Gordon *et al.*, 2009). Other roles include delay of morphogenetic activity at the leaf edge of species that form compound leaves (Shani *et al.*, 2010), and, together with gibberellins, trichome formation (Gan *et al.*, 2007).

Here, we investigated the role of cytokinins in gynoecium and fruit development. For this, we first used the *TCS::GFP* synthetic reporter (Muller and Sheen, 2008) to visualize cytokinin output *in vivo*. As shown in Figure 9, the pattern of cytokinin output changed from early to late developmental stages, suggesting that cytokinins play at least two roles: an early proliferation-inducing role at the medial region of the developing gynoecia, and an unexpected, late role during formation of fruit valve margins.

The fluorescence signal was first observed at the internal tissues of developing gynoecia. Earlier observations in rice and Arabidopsis *ckx* mutants, presumably containing higher cytokinin levels and producing increased numbers of ovules and seeds, indicated that cytokinins play an important role in placental development (Ashikari *et al.*, 2005; Bartrina *et al.*, 2011). Medial tissues (which include the placenta, septum and replum, and from which the style and stigma develop) are considered to be quasi-meristems, as they possess characteristics of shoot apical meristems (Balanza *et al.*, 2006; Alonso-Cantabrana *et al.*, 2007; Girin *et al.*, 2009). Cytokinins promote cell proliferation at shoot apical meristems, and appear to perform this function also in the internal tissues of developing gynoecia (Leibfried *et al.*, 2005; Lindsay *et al.*, 2006; Gordon *et al.*, 2009; Bartrina *et al.*, 2011). In this work, we observed a clear effect of increased or decreased cytokinin levels on the size of the replum (the external medial tissue) by using a transactivation system to drive *IPT7* or *CKX3* expression from fruit promoters. Interestingly, the two promoters used produced similar results, but using a third promoter that did not show expression at the valves, valve margin or replum did not produce significant replum size changes. An explanation for the similar effect of the two promoters in the replum could be that, as cytokinins are able to regulate meristem size through a non-

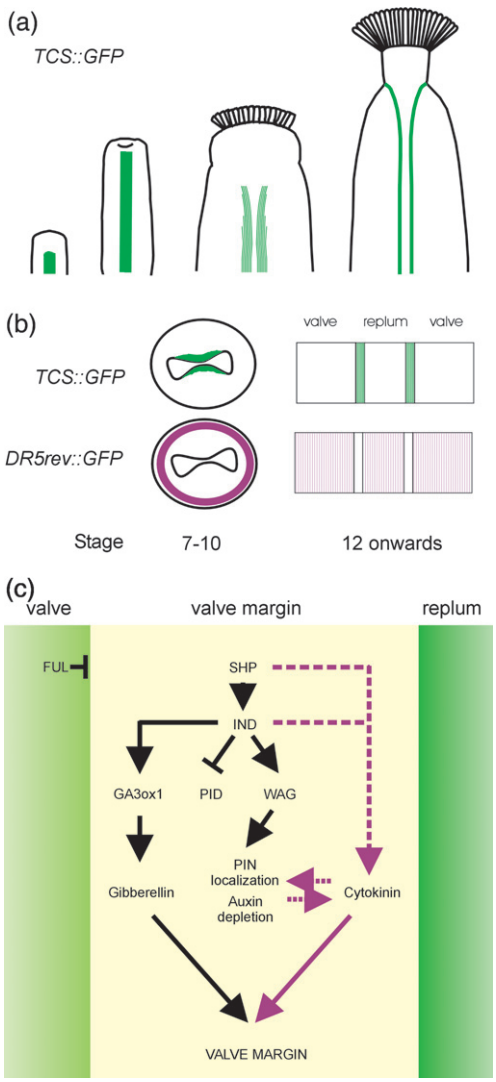

Figure 9. Schematic representations of cytokinin localization and working model of interactions with fruit patterning genes and hormones.
(a) Schematic representation of the fluorescence pattern observed in the cytokinin marker line *TCS::GFP* during progressive gynoecium and fruit developmental stages.
(b) Schematic representation of two contrasting fluorescence patterns observed in gynoecium and fruit internal and external tissues between cytokinin *TCS::GFP* and auxin *DR5rev::GFP* marker lines. Left, drawings representing the top apical view of stage 7–10 gynoecia. Cytokinin-induced fluorescence can be observed at the inner medial tissues, while auxin-induced fluorescence is observed as a circle at the top of the gynoecium. Right, drawings representing the abaxial view of a region at the medio-lateral axis of the ovary of a stage 12 (and onwards) gynoecium and fruit. Cytokinin-induced fluorescence is strongly detected at the valve margins, while auxin-induced fluorescence is undetectable in this tissue.
(c) Schematic model proposed for the interactions of cytokinins with the genes *IND*, *SHP1/2* and *FUL*, and the hormones auxin and gibberellin in the context of valve margin development. Cytokinin signaling is repressed by *FUL* at the valves, and promoted by *SHP1/2* and *IND* at the valve margins. Auxin signaling is absent from this tissue, while cytokinin signaling is strongly detected as well-defined *TCS::GFP* fluorescence. Components of the auxin and cytokinin pathways (including biosynthetic enzymes, transporters and/or signaling and response components) may interact with each other and reinforce this pattern. On the other hand, the combined presence of gibberellins and cytokinins may promote valve margin development in this region.

cell-autonomous mechanism, according to data reported by Bartrina *et al.* (2011), a similar mechanism could operate for quasi-meristems.

Furthermore, in the experiment in which cytokinins were exogenously applied to very young developing flowers, cytokinins dramatically enhanced the proliferative activity of the replum, and, although other tissues were affected by the treatment, none of them displayed such conspicuous overgrowth (Figure 5). Moreover, treated mutants lacking valves or valve margins were still able to form ectopic external tissue, while *35S::FUL* fruits that lack a replum did not. Intriguingly, when the *TCS::GFP* line was treated, increased fluorescence was observed in the inner medial tissues, but was not detected in the external ectopic tissues (Figure 6a,d), suggesting that externally applied cytokinins are able to trigger internal cytokinin signaling that induces external proliferation in a non-cell-autonomous manner.

Other studies have shown that application of BAP to developing inflorescences results in changes in floral organ identity and number, explained by extended meristematic activity (Venglat and Sawhney, 1996; Blahut-Beatty *et al.*, 1998; Lindsay *et al.*, 2006). Interestingly, BAP-treated mature flowers and fruits did not produce ectopic tissue (this study), suggesting that cytokinins have a proliferation-inducing activity in young gynoecia only, where the medial tissues show meristematic characteristics.

Remarkably, comparison of the cytokinin marker with the synthetic reporter for auxin output, *DR5rev::GFP* (Benkova *et al.*, 2003), revealed contrasting patterns in specific tissues. Cytokinins and auxins often play antagonistic roles in different tissues (Skoog and Miller, 1957). They show complex interactions through reciprocal regulation of the biosynthesis, signaling and transport components of each other's pathways in different cells. Auxin down-regulates cytokinin biosynthesis and induces cytokinin negative regulators (Arabidopsis response regulator type A) at various stages and tissues, such as early root development, the post-embryonic root and the shoot apical meristem (Muller and Sheen, 2008; Zhao *et al.*, 2010). On the other hand, cytokinin induces auxin negative regulators (Aux/IAA) and affects PIN auxin-efflux transporters (Laplaze *et al.*, 2007; Dello Ioio *et al.*, 2008; Pernisova *et al.*, 2009; Ruzicka *et al.*, 2009; Jones *et al.*, 2010; Bishopp *et al.*, 2011b). Due to these interactions, opposite localization patterns of auxin and cytokinins have been reported for various tissues (e.g. Muller and Sheen, 2008; Bishopp *et al.*, 2011b), and were also observed at different stages and regions of developing gynoecia and fruits (Figures 1–3 and 9). It would be interesting to investigate whether the same molecular mechanisms that connect these pathways in other tissues are also responsible of the contrasting patterns observed in gynoecia and fruits.

During development, auxin is localized at the apical part of gynoecia, where stigmatic cells develop (Aloni *et al.*, 2006; Benkova *et al.*, 2003; Figure 3). Cytokinins were not detected at this region (Figure 1), but external application of cytokinins to developing *DR5rev::GFP* flowers resulted in a fluorescence signal at the apex of the ectopic tissue that developed from the replum (Figure 6b), where stigmatic cells also developed, resembling the natural localization of auxin and stigma at the top of the fruit. Jones *et al.* (2010) have shown that cytokinin application in young, developing tissues leads to a rapid increase in auxin biosynthesis, and cytokinins are able to regulate PIN auxin efflux transporters in various tissues (Laplaze *et al.*, 2007; Dello Ioio *et al.*, 2008; Pernisova *et al.*, 2009; Ruzicka *et al.*, 2009; Jones *et al.*, 2010; Bishopp *et al.*, 2011b). Therefore, it is tempting to speculate that, in the context of gynoecium development, cytokinins in the medial tissue may stimulate auxin biosynthesis and/or transport, resulting in auxin accumulation at the top of the gynoecium, leading to stigma development.

The unexpected visualization of older *TCS::GFP* gynoecia and fruits at the valve margin suggested that cytokinins may not only play a role in early gynoecium medial tissue proliferation, but may also participate later in development (Figures 1–3 and 9). Auxin depletion is required for proper valve margin development (Sorefan *et al.*, 2009). The bHLH transcription factor IND promotes localization of PIN3 in the plasma membrane of valve margin cells such that auxin is 'pumped out' (Sorefan *et al.*, 2009). *IND* itself is activated by SHP MADS box transcription factors at the valve margin (Liljegren *et al.*, 2000, 2004). We detected a sharp fluorescence signal at the replum and valve junction in the cytokinin reporter line, which disappeared in *ind* and *shp1 shp2* mutant backgrounds, indicating that cytokinin signaling is working downstream of these valve margin regulators.

As auxin has been shown to down-regulate cytokinin biosynthesis (Nordstrom *et al.*, 2004), one possible scenario could be that auxin depletion by IND (Sorefan *et al.*, 2009) is required for cytokinin appearance. On the other hand, it cannot be ruled out that the valve margin regulators directly activate the cytokinin pathway. As cytokinin signaling regulates the radial localization pattern of the PIN auxin transporters in the root vasculature (Bishopp *et al.*, 2011b), if a similar phenomenon occurs at the valve margin, cytokinins may also contribute to auxin depletion in this tissue. The model presented in Figure 9 shows both scenarios.

IND also activates the gibberellin biosynthesis gene *GA3ox1* at the valve margin of fruits (Arnaud *et al.*, 2010). This may appear to contradict the cytokinin observations, as previous earlier work has shown antagonistic effects of cytokinin and gibberellin (Ezura and Harberd, 1995; Brenner *et al.*, 2005; Greenboim-Wainberg *et al.*, 2005; Jasinski *et al.*, 2005; Yanai *et al.*, 2005). However, these hormones can also act simultaneously upon transcription factors that stimulate trichome initiation (Gan *et al.*, 2007). As IND is also necessary for cytokinin presence (this work), valve margin formation may also require the cooperative action of both hormones.

On the other hand, proper valve development requires repression of valve margin identity in the valve tissue. FUL is a MADS box transcription factor that represses *SHP1/2*, *IND* and other genes involved in valve margin identity in the valve (Ferrandiz *et al.*, 2000; Liljegren *et al.*, 2000, 2004). Absence of *FUL* results in conversion of valve cells into valve margin cells, and *TCS::GFP ful* mutants showed intense fluorescence signal at the valves (Figure 7d). These results strongly indicate a relationship between cytokinins and valve margin identity. Furthermore, local application of cytokinin in developing fruits restored valve margin formation and increased dehiscence in *shp1 shp2* and *ind* mutants, suggesting that cytokinins play a functional role in valve margin formation.

However, if this is the case, it is not clear how this late role of cytokinins in valve margin formation is related to the early role of cytokinins in proliferation of cells in (quasi)meristematic tissues. Further work is required to unravel the molecular mechanisms by which cytokinins act in this tissue.

In conclusion, the results suggest that cytokinins play at least two different roles in gynoecium and fruit patterning and morphogenesis: an early role stimulating proliferation of the medial tissues, and a late role in valve margin formation, opening new paths for detailed studies about cytokinins in these processes. As observed for other parts of the plant, different tissues respond differently to cytokinins, which may be able to work in a non-cell-autonomous manner. Further studies will help to unravel the role of the specific cytokinin species synthesized, modified and degraded through various enzymatic routes, and the role of transport, signaling and response components in the distinct effects of cytokinins during fruit development. Moreover, cytokinins may act in concert with auxin, as contrasting patterns were observed for developing gynoecia and fruits of the cytokinin and the auxin marker lines, and, on the other hand, auxin signaling was observed in proliferating tissue growing in cytokinin-treated gynoecia. In the future, new attempts to understand the molecular interactions that relate cytokinins to gynoecium and fruit tissue identity, key transcription factors and other hormones will shed light on the processes that shape fruits.

EXPERIMENTAL PROCEDURES

Plant growth and plant materials

Plants were germinated in soil in a growth chamber at 22°C under long-day conditions (16 hrs light, 8 hrs dark), and further grown in soil under standard greenhouse conditions (natural light conditions, around 22–25°C). The *TCS::GFP* line (Muller and Sheen, 2008) and the *DR5rev::GFP* line (Benkova *et al.*, 2003) are in the Col background. Transactivation lines *pFUL::LhG4* and *pSHP2::LhG4* (Yuval Eshed, Department of Plant Sciences, Weizmann Institute, Israel) and *Op::GUS*, *Op::IPT7* and *Op::CKX3* (Werner and Schmulling, 2009; Shani *et al.*, 2010) are in the L*er* background. The *ind-2*, *ful-1* and *shp1 shp2* mutants (Ferrandiz *et al.*, 2000; Liljegren *et al.*, 2000, 2004) are in the L*er* background. *35S::FUL* (Ferrandiz *et al.*, 2000) is in the Col background.

Hormone treatments

Seeds were germinated in a growth chamber (long days at 22°C), and plants were grown in soil under standard greenhouse conditions. One week after bolting, wild-type and mutant plants were sprayed 5 days a week with 100 μM benzylaminopurine (BAP; Duchefa Biochemie, http://www.duchefa.com), 0.01% Silwet L-77 (Lehle Seeds, http://www.arabidopsis.com) or a mock solution (100 μM BAP and 0.01% Silwet together). All aerial tissues were sprayed, and effects were evaluated after 2 weeks (Figure 6a,b,d) or 3–4 weeks (Figures 5 and 6e–i). Alternatively, for the valve margin mutants, local application of cytokinins to the external medial region of the ovary of developing fruits was performed either by using a paintbrush to apply a solution of 0.01% Silwet L-77/100 μM BAP 5 days a week (Figure 8f,h), or by spreading once with a 250 μM BAP lanolin paste (Figure 8b,d). The paintbrush treatment started 1 day after pollination, and photographs were taken when the fruits were brownish (3–4 weeks after initiation of the treatment). Treatment with lanolin paste started 4 days day after pollination, and photographs were taken when the fruits reached stage 17.

Histology

For GUS analysis, Arabidopsis tissues were incubated overnight at 37°C with an X-Gluc solution (Gold Biotechnology, https://www.goldbio.com) (Jefferson *et al.*, 1987). Fruits of plants treated with cytokinins and control fruits were fixed in formaldehyde/acetic acid/alcohol solution, then dehydrated, embedded in Paraplast (Sigma-Aldrich, http://www.sigmaaldrich.com), and 10 μm sections were cut as previously described (Zúñiga-Mayo *et al.*, 2012). Tissue sections were stained to analyze the transmitting tract using a solution of alcian blue and counterstained with a solution of neutral red (Sigma-Aldrich) (Zúñiga-Mayo *et al.*, 2012). Tissue sections were observed by optical microscopy (Zeiss Axio Observer, http://www.zeiss.com).

Microscopy

Fluorescent images were captured using an LSM 510 META confocal scanning laser inverted microscope (Zeiss). GFP was excited using a 488 nm line of an argon laser, and propidium iodide was excited using a 514 laser line. GFP emission was filtered using a BP 500–550 nm filter, and propidium iodide emission (including autofluorescence) was filtered using an LP 575 nm filter. We noted variations in the fluorescence intensity when *TCS::GFP* plants were grown in different seasons (summer and winter). For scanning electron microscopy, plant tissue was collected from plants and directly observed in a Zeiss EVO40 scanning electron microscope with a 20 kV beam, using the SE detector (Figure 4) or the BSD detector (Figures 5 and 8). For measuring the replum, photographs taken at 215× magnification were used. Photographs of treated silique sections were taken using a Zeiss AxioCam MRc camera installed on a Zeiss Observer.Z1 inverted microscope. Images of treated siliques, transactivation GUS-stained tissues and phenotypes were obtained using a Leica EZ4 D stereomicroscope (Leica, http://www.leica-microsystems.com).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Confocal micrograph of a *DR5rev::GFP* gynoecium at stages 9–10.

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Hormones talking

Does hormonal cross-talk shape the Arabidopsis gynoecium?

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Keywords: gynoecium, fruit, patterning and development, auxin, cytokinin

Abbreviations: TCS, Two Component System; GFP, GREEN FLUORESCENT PROTEIN; CK, cytokinin; Aux, auxin; BAP, benzylaminopurine

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The proper development of fruits is important for the sexual reproduction and propagation of many plant species. The fruit of Arabidopsis derives from the fertilized gynoecium, which initiates at the center of the flower and obtains its final shape, size, and functional tissues through progressive stages of development. Hormones, specially auxins, play important roles in gynoecium and fruit patterning. Cytokinins, which act as counterparts to auxins in other plant tissues, have been studied more in the context of ovule formation and parthenocarpy. We recently studied the role of cytokinins in gynoecium and fruit patterning and found that they have more than one role during gynoecium and fruit patterning. We also compared the cytokinin response localization to the auxin response localization in these organs, and studied the effects of spraying cytokinins in young flowers of an auxin response line. In this addendum, we discuss further the implications of the observed results in the knowledge about the relationship between cytokinins and auxins at the gynoecium.

Fruits are plant organs that nurture, protect, and facilitate seed dispersal and are therefore very important for successful plant propagation through sexual reproduction in many species. Most fruits originate from the female reproductive organ, the gynoecium, after ovules inside become fertilized by the pollen. From top to bottom, the Arabidopsis gynoecium is formed by a stigma, style, ovary, and gynophore (**Fig. 1A**). Internally, the ovary contains the ovules that become seed when fertilized and is divided in two by an internal tissue: the septum (**Fig. 1B**). Externally, the ovary consists of two valves separated by the replum, the external continuation of the septum.[1] After fertilization, the Arabidopsis gynoecium produces a long, dry, dehiscent fruit named silique. In siliques, a specialized tissue, the "valve margin" develops between the valves and replum, and is the site where a mature silique opens to release the seed.[2] The gynoecium is the last organ to be formed from the floral meristem, and, in Arabidopsis, it starts as a short tube at the center of the developing flower. This "hollow tube" elongates while two internal ridges (medial ridges) grow toward each other until they fuse and give rise to septum and placenta.[3-7] Different internal and external tissues of the gynoecium and later the fruit, such as the ovules, placenta, transmitting tract, septum and replum, style, and stigma also derive from the medial tissues.[4,5] Different factors guide the processes that exquisitely shape the gynoecium and fruit, and hormones are an important part of these factors. In particular, auxin is known to be relevant for fruit patterning. The current model involves an apical-basal auxin gradient that specifies the different parts of the gynoecium and later fruit.[8] On the other hand, both the lack of auxin and the presence of gibberellin have been reported to be required for proper valve margin formation.[9,10] Recently, brassinosteroids have also been implicated in the development

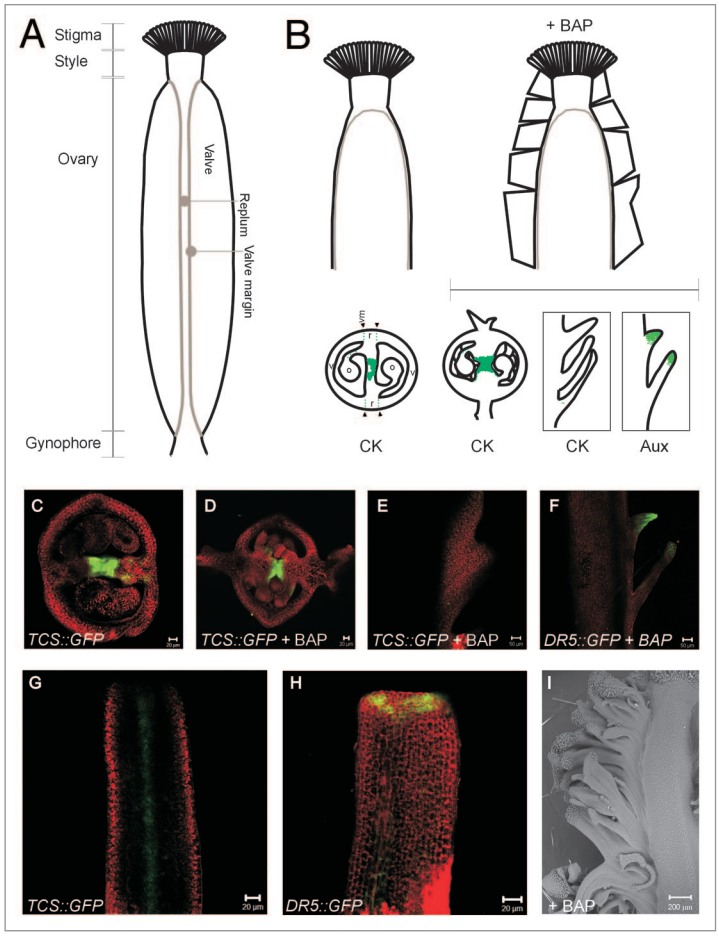

Figure 1. Cytokinin and auxin response in gynoecia and fruits. A) Parts of the Arabidopsis gynoecium. B) Drawings of the side view of a control (left) and BAP sprayed (right) gynoecium. The schemes below represent the localization of the cytokinin (CK) or auxin (Aux) response as indicated. Single plane confocal photographs showing the response to each hormone are also shown: C) transverse section of control *TCS::GFP*, D) and E) transverse (D) and longitudinal optical (E) sections of BAP sprayed *TCS::GFP*, F) longitudinal optical section of sprayed *DR5rev::GFP*. G) and H) Longitudinal confocal photographs (single plane) of young *TCS::GFP* (G) or *DR5rev::GFP* (H) gynoecia. I) Scanning electron micrograph of a BAP-sprayed gynoecium, where the ectopic tissue can be observed at the right side. v, valve; o, ovule; r, replum; vm, valve margin.

of the reproductive tract through which pollen tubes grow to reach the ovules.[11,12]

In other plant tissues, auxins and cytokinins are closely related and together guide the development of different organs.[13-17] In the gynoecium, cytokinins promote the growth of the placenta and ovules.[18,19] In different species, exogenous application or altered levels of cytokinins in developing flowers and gynoecia can promote the formation of extra floral organs, trichomes in valves, or trigger parthenocarpy.[20-23] However, there was little experimental evidence about the natural localization of cytokinin signaling in fruits, its role in fruit patterning, and the positional relationship to auxin signaling. We recently explored these questions in Arabidopsis, where cytokinin appears to fulfill at least two roles during gynoecium and fruit development: Initially, a role in promoting proliferation at the medial region during gynoecium development, and later, an unexpected role at the valve margin during fruit development.[25] Here, we speculate further about the relationship and implications of the localization of cytokinin and auxin during gynoecium development.

We compared the patterns of both hormones using the reporter lines *TCS::GFP* for cytokinin[15] and *DR5rev::GFP* for auxin signaling.[24] Complementary patterns were observed, particularly at specific locations and developmental stages such as: a) cytokinin response at the center of the gynoecium, and auxin response around it at early stages of development, and b) presence of cytokinin response and absence of auxin response at the valve margins in mature gynoecia and developing fruits.[25] However, while auxin has been proposed to act as a gradient to define the stigma-style, ovary and gynophore regions in the developing gynoecia,[8] a clear apical-basal gradient as revealed by the reporter line was not directly evident at the stages and tissues we analyzed: *DR5rev::GFP* gynoecia showed high fluorescence at the top and low fluorescence below (**Fig. 1H**). This localization of auxin at the top, as revealed by the synthetic reporter, has been reported before.[24,26,27] Based on this, Østergaard (2009) noticed that the auxin localization indicated a two-zone division of the gynoecium.[9] Therefore, he complemented the auxin gradient model by proposing that another morphogen, in combination with auxin, could provide positional information to define the stigma-style, ovary and gynophore regions, and suggested that cytokinin, in an inverted gradient, could be this second morphogen.[28] In our study, when gynoecia of the *TCS::GFP* cytokinin signaling marker were visualized longitudinally, an apical-basal gradient of fluorescence was not directly evident at the stages and tissues analyzed (an example of cytokinin localization in a young gynoecium can be observed in **Fig. 1G**). There appears to be a complementary pattern of both hormones at least in some of the specific tissues and developmental stages visualized, but, as far as we could determine, the marker lines did not reveal evident apical-basal gradients that converged at the same tissue at the same stage. It may still be that the current marker lines used do not allow the proper detection of the gradients in these tissues; that the gradients can only be observed at the earliest stages of development, for only a brief time; or that the gradients are so steep or subtle, that more detailed analysis are required to detect them. In any case, the lack of visualization of gradients keeps open the question of whether the patterning of the gynoecium and fruit is guided through these gradients or whether the hormones analyzed,

very relevant for gynoecium development and related to each other, are not necessarily organized as gradients to perform their functions in gynoecium and fruit patterning.

We also reported that cytokinin treatments induce overproliferation in the replum of developing gynoecia (**Fig. 1I**), and analyzed the pattern of the *TCS::GFP* and *DR5rev::GFP* markers in treated gynoecia. Interestingly, the cytokinin signaling pattern as revealed by the *TCS::GFP* marker showed a change in intensity at the center of the gynoecium, and was less evident at the new, external tissue (**Fig. 1B to E**). In contrast, *DR5rev::GFP* gynoecia showed intense fluorescence at the tips of the new ectopic proliferating tissue induced by cytokinin (**Fig. 1B and F**). Recently, Yoshida and collaborators showed that the light environment controls lateral organ initiation in the shoot apical meristem through the regulation of cytokinin and auxin, and proposed that "cytokinin is required for meristem propagation, while auxin redirects cytokinin-inducible meristem growth toward organ formation."[29] The medial tissues, the region where cytokinin signaling was observed in developing gynoecia, are considered to have meristematic properties.[4-6,30] Are we observing in the cytokinin-treated young gynoecia, a similar process to what Yoshida et al. propose to naturally occur in the shoot apical meristem? The external growth of the medial tissue could be explained by the cytokinin-inducible promotion of the meristematic activity of the medial tissue, which is then re-directed by auxin to produce a new ectopic "lateral organ." We can then speculate whether a similar cross-talk is naturally relevant for the development of the gynoecium.

Conclusions

In conclusion, the analysis of auxin and cytokinin response localization patterns and the effects that cytokinin treatments have on the auxin response marker suggest that, as has been observed in other tissues, these pathways are related, and possibly, we could speculate that their cross-talk might be shaping the gynoecium. More detailed experiments can help to better understand the mechanisms by which both pathways communicate, and the relevance of this communication in the patterning of the gynoecium and fruit.

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Review

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Inside the gynoecium: at the carpel margin

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The gynoecium, which is produced at the center of most flowers, is the female reproductive organ and consists of one or more carpels. The *Arabidopsis* gynoecium consists of two fused carpels. Its inner tissues possess meristematic characteristics and are called the carpel margin meristem (CMM), because they are located at the margins of the carpels and generate the 'marginal' tissues of the gynoecium (placenta, ovules, septum, transmitting tract, style, and stigma). A key question is which factors are guiding the correct development of all these tissues, many of which are essential for reproduction. Besides regulatory genes, hormones play an important part in the development of the marginal tissues, and recent reports have highlighted the role of cytokinins, as discussed in this review.

Importance of the carpel marginal region

Angiosperms produce flowers. Many of them give rise to the pistil or so-called gynoecium (Box 1) in their center. This female reproductive floral structure consists of one or several carpels, which protect the ovules that develop inside and create a selective barrier for pollen. Fertilized ovules go through a series of developmental events to transform into mature seeds that harbor the embryo inside, while in parallel the gynoecium mostly differentiates into a fruit [1–4].

The initiation of the gynoecium lays the foundation for correct fruit development very early on. Transcription factors that promote meristematic activity have important roles in these early events. In *Arabidopsis* (*Arabidopsis thaliana*), the gynoecium is composed out of two congenitally fused carpels that arise as a compound fused structure from a single primordium [5]. The two carpels are fused vertically at their margins, and these fused margins correspond to the medial domain of the gynoecium (see Figure I in Box 1). At the adaxial side (inside) of the growing cylinder, along each medial domain, a meristematic medial ridge develops [2,5,6] that is also called the carpel margin meristem (CMM) [7,8]. As development continues, the CMM gives rise to the carpel marginal tissues, which include the placenta, ovules, septum, transmitting tract, style, and stigma [5] (Box 1). All of these structures located in the carpel marginal region are critical for the reproductive competence of flowering species. At the abaxial side (outside) of the medial domain of the gynoecium, cells differentiate into the replum, to which the valve margins and valves are attached [4,5] (see Figure I in Box 1).

In this review, we focus on the CMM and its derived carpel marginal tissues. Flower development in *Arabidopsis* has been studied for over 20 years; many patterning and identity genes have been identified, and regulatory networks have been proposed for proper floral patterning (reviewed in [1–4,9–11]). The function of the flower is sexual reproduction. However, few studies have focused specifically on the development of the CMM and carpel marginal region, which produce the most important female reproductive tissues. Questions that come to mind are: how many genes are involved in the development of the CMM and the carpel marginal region? What are the unique features of CMM versus other types of meristems? Do tissue-specific genes exist? What are the consequences of alterations in the development of these tissues? And how did the carpel marginal region evolve? In this review we present an overview of what is known today about regulatory genes and take into account recent reports about the role of hormones in these tissues, especially cytokinins.

Genes involved in carpel marginal region development

After a literature survey looking for genes that are involved in processes related to CMM and carpel marginal tissue development in *Arabidopsis*, we found at least 86 genes, which are presented in Table S1 in the supplementary material online with their corresponding mutant phenotypes and expression patterns. Owing to space concerns, genes associated with microRNA regulatory pathways were left out, although microRNAs such as *miR164* [12,13] and various ARGONAUTE (AGO1 and AGO4) proteins [14,15] clearly affect carpel marginal tissue development. Furthermore, genes involved specifically in

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ovule development are also not considered in this review. Fifty-seven (65%) of the identified genes are transcription factors, and the rest of the genes in Table S1 correspond to transcriptional co-regulators, hormonal pathway components [auxin, cytokinin, and brassinosteroid (BR)], and other diverse functions.

Mutations in these genes cause phenotypes at different (early or late) stages of development of the medial tissues. Early alterations frequently affect many structures in the mature gynoecium, whereas late alterations are observed in specific tissues.

Mutations that produce alterations at early stages of the development of the carpel medial region

An example of an essential gene with a very early function in the formation of the whole gynoecium is the MADS-box gene *AGAMOUS* (*AG*), which encodes a protein that acts together with the MADS domain protein SEPALLATA3 (SEP3) in a multimeric complex [16]. *AG* expression initiates at floral stage 3, and the loss of AG activity causes a complete lack of gynoecium development owing to a loss of carpel identity and floral meristem determinacy [17–19]. AG is required to repress the transcription of the homeodomain transcription factor *WUSCHEL* (*WUS*) [20,21], either directly [22] or indirectly via the C2H2 zinc-finger transcription factor *KNUCKLES* (*KNU*) [23]. WUS establishes and maintains the floral meristem stem cell population, and the WUS–CLAVATA (CLV) signaling pathway maintains a dynamic balance controlling floral meristem size, as in the shoot apical meristem (SAM). At floral stage 6, *WUS* expression ceases and the floral meristem becomes consumed by the development of the two carpels [24–26]. No *WUS* expression is present in the CMM. This tight regulation is important because a larger floral meristem causes the production of more floral organs, and vice versa [27,28]. Furthermore, prolonged maintenance of the meristem causes indeterminate growth, as occurs in *ag* mutants as well as mutants for various other meristem-regulating genes, such as *ULTRAPETALA1* (*ULT1*), *REBELOTE* (*RBL*), and *SQUINT* (*SQN*) [29], *PERIANTHIA* (*PAN*) [30,31], *SUPERMAN* (*SUP*) [32], *SPATULA* (*SPT*) and *CRABS CLAW* (*CRC*) [33,34], and *JAIBA* (*JAB*) [35,36]. Moreover, recent observations of floral meristem determination and medial tissue phenotypes in *jab/crc-1* double mutants suggest that proper medial tissue development in gynoecia may require adequate floral meristem termination [35,36]. This conclusion is based on the observations that in *jab/crc-1* mutants, the development of the medial tissue decreases when the indeterminacy phenotype increases, and in gynoecia with a severe indeterminacy phenotype the septum virtually disappears and only a few ovules develop. However, it was recently reported that after correct floral meristem termination, extra carpel formation is observed in *clv* receptor mutants [37]. Initial floral meristem size was not altered, but increased cell proliferation was observed during early gynoecium development, which was linked to an expanded expression of the cell proliferation-promoting transcription factor *SHOOT MERISTEMLESS* (*STM*) [37].

STM is an important gene for meristem maintenance [38], and inducible *stm* mutants sometimes completely lack a gynoecium owing to an early consumption of the floral meristem, or gynoecia are produced but with reduced CMM development, resulting in a small placenta, a lack of septum formation, and (partially) unfused carpels [39]. Other genes with a meristem-promoting function are also expressed during early gynoecium development in the CMM, but their mutant phenotypes show alterations at later stages of marginal tissue development (Figure 1 and Table S1 in the supplementary material online).

Interestingly, until now no single mutation has been found that results in a gynoecium lacking all marginal tissues. However, some mutant combinations do have strongly reduced carpel marginal tissue development (reviewed in [4,10]). It has been proposed that the APETALA2 (AP2) transcription factor *AINTEGUMENTA* [*ANT* is involved {redundantly with the related gene *AINTEGUMENTA-LIKE6* (*AIL6*) (also known as *PLETHORA3*

Box 1. Flower and fruit development

When *Arabidopsis* commits to flowering, the shoot apical meristem (SAM) converts into an inflorescence meristem, which produces floral meristems on its flanks in an organized pattern [3]. Each floral meristem produces one flower that consists of four concentric whorls, which are, from outside to inside, four sepals, four petals, six stamens, and one gynoecium at the center. Flower and fruit development is divided into precise developmental stages numbered 1 to 20 [132]. At stage 5, the gynoecium primordium starts to develop and becomes visible. At stage 6, the two congenitally fused carpels begin to grow and start to form a hollow tube [5,34] (Figure I). At stage 7, the carpel margin meristem (CMM) is clearly visible in a transverse view of the gynoecium (Figure ID). As mentioned in the main text, this meristematic tissue gives rise to the carpel marginal tissues: placenta, ovules, septum, transmitting tract, style, and stigma [5,34]. As development proceeds, at stage 9 the two CMMs meet and form the septum through postgenital fusion. At the same time, on the flanks of the CMMs, placental tissue is formed. Notably, marginal placentation is not observed on all species. The placenta then produces ovule primordia that are visible at stage 10, and on the top of the gynoecium the first stigmatic papillae are visible. Finally, the gynoecium becomes fully closed at stage 11, and stigmatic papillae completely cover the stigma. During stage 12, the style and transmitting tract differentiate leading to the mature gynoecium. Also the valves, valve margins, and repla become morphologically distinct. At stage 13, anthesis occurs, the flower opens, and the gynoecium becomes self-fertilized, meaning pollen from the same flower lands on the stigma, germinates and pollen tubes grow through the internal part of the style and via the transmitting tract to reach the ovules. When fertilization occurs, the gynoecium will differentiate into a fruit, harboring the seeds. *Arabidopsis* produces a dry dehiscent fruit (or silique) and, along the mediolateral axis, the valves, valve margins (both lateral tissues), and the repla can be distinguished (Figure IB,J). During fruit ripening, lignification of the lateral tissues takes place, allowing fruit shattering and seed dispersal on maturity at stage 20 (reviewed in [1–4,9–11]).

In the late 1980s and early 1990s, various groups used molecular genetics to study floral homeotic mutants in *Arabidopsis* and snapdragon (*Antirrhinum majus*) (e.g., [18,133,134]), and very soon after the well-known 'ABC model' was postulated [135–137], which explains the formation of the floral organs by a combinatorial action of floral organ identity genes [138,139]. Most of these genes are members of the MADS-box family of transcription factors, which are active as higher-order protein complexes [16,140,141] and thereby orchestrate the regulation of their downstream target genes [142–145].

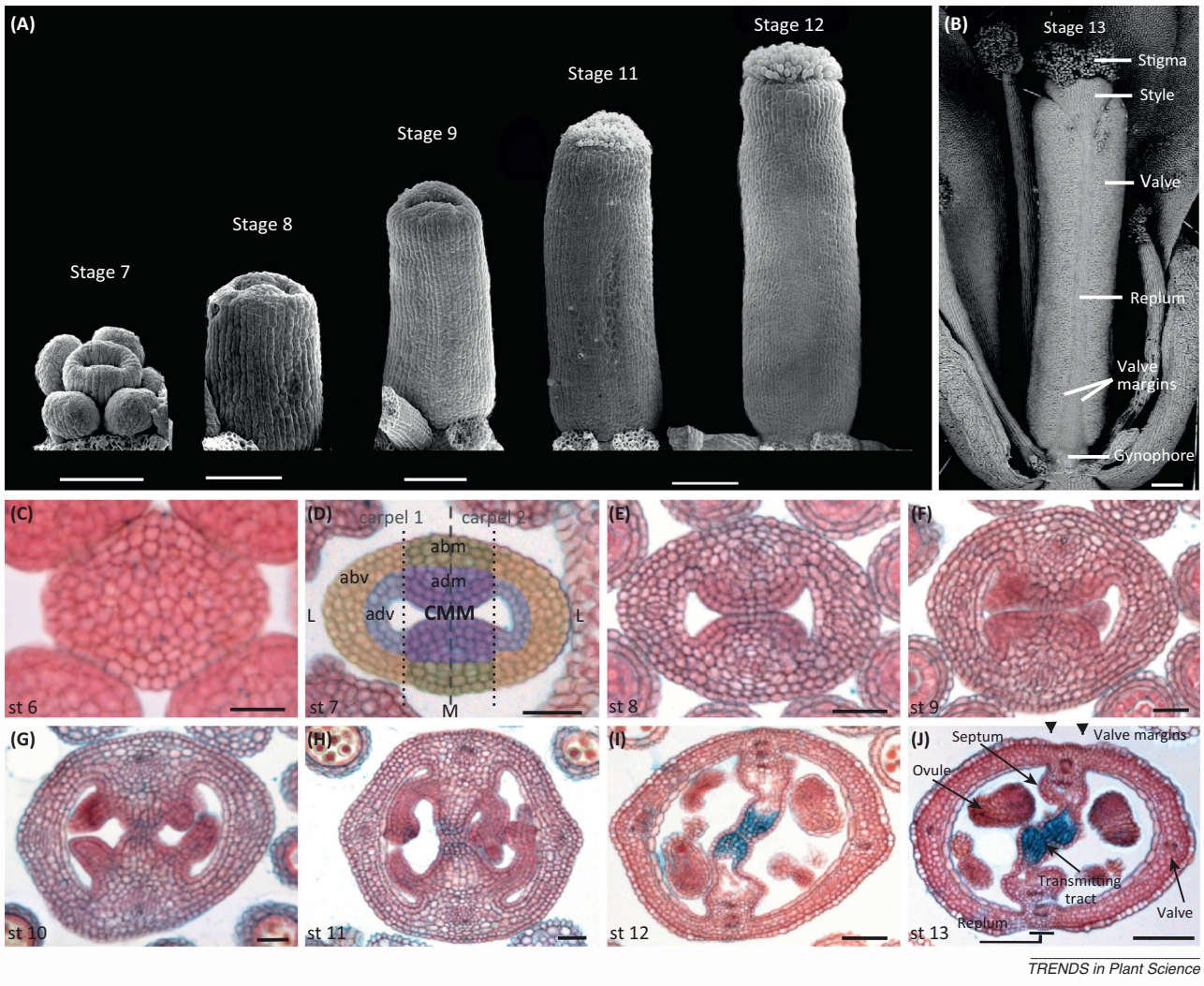

Figure I. *Arabidopsis thaliana* (ecotype Landsberg *erecta*) gynoecium development. **(A,B)** Scanning electron microscopy images of wild type gynoecia. (a) The gynoecium is shown at developmental stages 7, 8, 9, 11, and 12. At stage 13 (b), or anthesis, the flower opens and self-pollinates. The different external tissues that form the gynoecium are indicated. **(C–J)** Transverse sections of *Arabidopsis* gynoecia stained with neutral red (to visualize cell walls) and alcian blue (to visualize acidic polysaccharides, which are a major component of the extracellular matrix of the transmitting tract) at stages 6 (c), 7 (d), 8 (e), 9 (f), 10 (g), 11 (h), 12 (i), and 13 (j). Annotations in (d) explain the following: the broken line in the center separates the two congenitally fused carpels; and the dotted lines separate the lateral domains (L) from the medial domain (M). Gynoecial domains have been colored with approximation: yellow, abaxial valve (abv); blue, adaxial valve (adv); green, abaxial margin (abm)/replum; purple, adaxial margin (adm)/carpel margin meristem (CMM). Tissues derived from the CMM are indicated in (j). Abbreviation: st, floral stage. Scale bars represent: 50 μm for stages 7–9, and 100 μm for stages 11 and 12 in (a); 200 μm in (b); 10 μm in (c); 25 μm in (d–h); 50 μm in (i); and 100 μm in (j). (a) is adapted from [75], reprinted with permission from the American Society of Plant Physiologists.

(*PLT3*))}] in floral meristem initiation, growth, and patterning. Interestingly, the *ant9* mutant presents up to 30% of partially unfused carpels, a phenotype greatly enhanced in the *ant*/*ail6* double mutant [40–43]. However, in mutant combinations, such as *ant*/*seuss* (*seu*) [6], *ant*/*ap2* [40], *ant*/*seu*/*seuss-like 1* (*slk1*)/*slk2* [44], *ant*/*crc*, *ant*/*shp1*/*shp2*, and *ant*/*crc*/*shatterproof1* (*shp1*)/*shp2* [45], *ant*/*filamentous flower* (*fil*) and *ant*/*fil*/*yabby3* (*yab3*) [46], *ant*/*lug* (*leunig*) [47], and *ant*/*revoluta* (*rev*) [7], carpel fusion and marginal tissue development are severely affected or almost completely abolished. Some other mutants with similar phenotypes are *seu*/*cyp85a2* [48], *seu*/*crc* [6], *seu*/*lug* [49], and *crc*/*shp1*/*shp2* [45]. The SEU and LUG proteins physically interact and form a transcriptional co-regulator complex [49,50], and it has been suggested that ANT, SEU, LUG, and FIL may form a protein complex that is important for carpel medial tissue development [6,46].

The *CUP-SHAPED COTYLEDON* genes *CUC1* and *CUC2*, which encode a paralogous pair of NAC transcription factors, are also expressed in the CMM. They are called boundary genes because they affect organ fusion [51]. The double mutant presents several defects in carpel marginal tissue development, and in particular strong defects in both septum growth and fusion [52,53]. Furthermore, overexpression of *CUC2* prevents congenital carpel fusion [12,13,53]. Two other genes whose mutations cause partially unfused septa and a mild split carpel phenotype are *CRC* and *SPATULA* (*SPT*), with the double mutant presenting a dramatically enhanced phenotype of almost completely unfused carpels and dramatically reduced marginal tissue formation [33,34]. Apart from some larger cells observed in the septum in the *crc* mutant, carpel marginal tissues develop normally [34]. Notably, *CRC*, like other YAB family members, is also involved in the

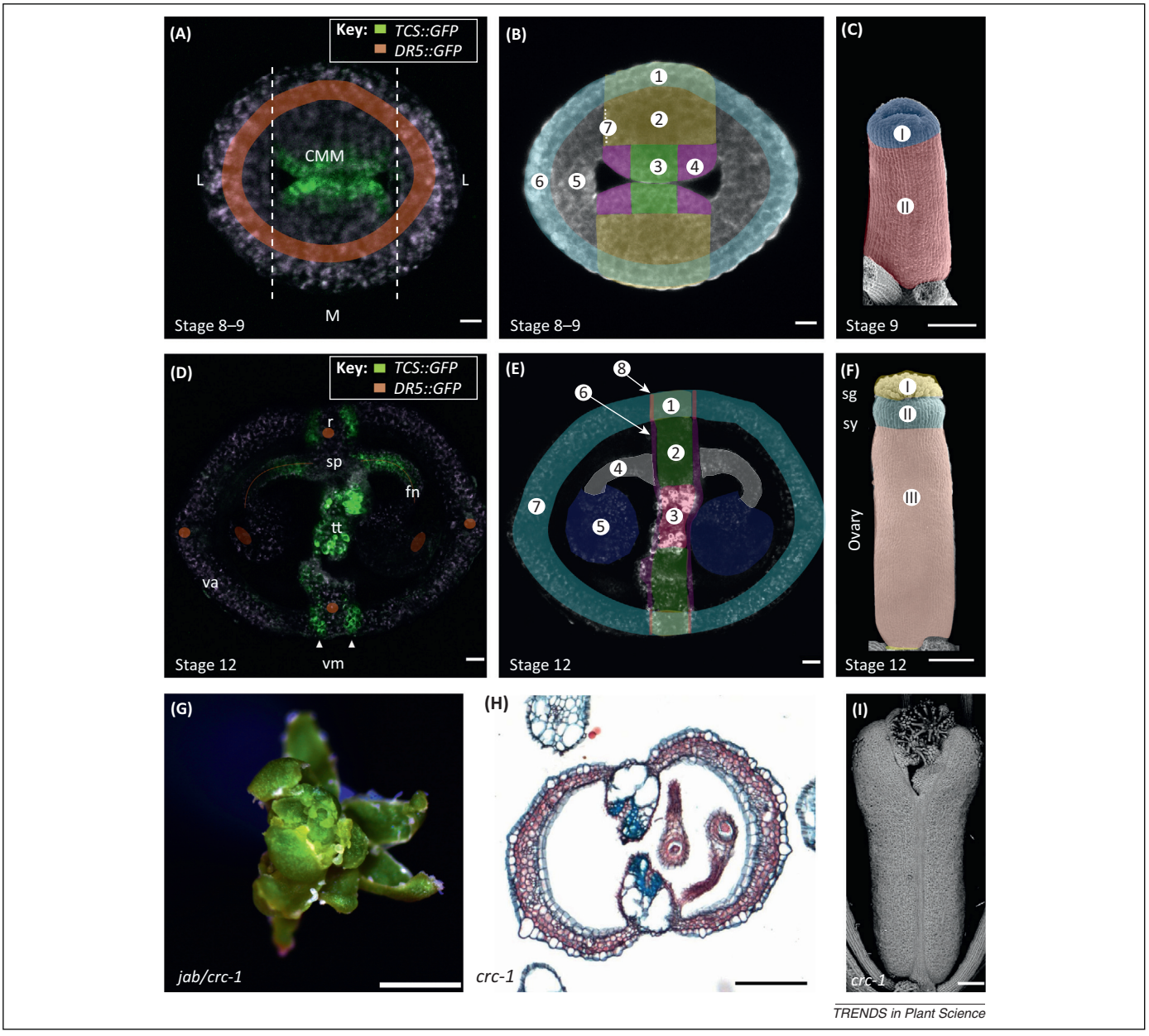

Figure 1. Expression domains of carpel marginal region genes, *DR5* (transcriptional response to auxin) and *TCS* (transcriptional response to cytokinin) patterns,, and examples of mutants with carpel marginal region defects. **(A,D)** Transverse sections of gynoecia at stage 8–9 (a) and 12 (d). *DR5* (brown) and *TCS* (green) expression patterns are indicated. Abbreviations: M, medial; L, lateral; va, valves; r, replum; sp, septum; tt, transmitting tract; fn, funiculus; vm, valve margins; CMM, carpel margin meristem; GFP, green fluorescent protein. Broken lines in (a) separate the lateral domains (L) from the medial domain (M). **(B,C,E,F)** Gynoecia at stages 8–9 (b,c) and 12 (e,f) were divided into the different regions indicated in Table S1 in the supplementary material online. Abbreviations: sg, stigma; sy, style. **(G–I)** Mutants with carpel marginal region defects. (g) *jaiba/crabs claw* (*jab/crc-1*), an indeterminate mutant. (h,i) The *crc-1* mutant. Septum (h) and style–stigma (i) fusion defects are visible. Scale bars represent: 10 μm in (a,b); 50 μm in (c); 20 μm in (d,e); 100 μm in (f); 2 mm in (g); 150 μm in (h); and 200 μm in (i). (c) and (f) were adapted from [75], reprinted with permission from the American Society of Plant Physiologists.

abaxial–adaxial polarity establishment of the carpel, presenting sometimes an ovule or ovule-like primordium produced at the abaxial part of the replum [34,54,55]. Lack of SPT function results in no transmitting tract formation, as well as reduced growth of the CMM and of the CMM-derived marginal tissues [33,34]. Recently, *spt* double mutant combinations showed that *ALCATRAZ* (*ALC*), *FUL* [56], and *INDEHISCENT* (*IND*) [57] are also involved in carpel fusion and septum, style, and/or transmitting tract development. Moreover, genetic interaction studies demonstrated that the split carpel phenotype seen in the *spt* mutant, which has overexpression of *CUC1* and *CUC2*, can be rescued in a *cuc1* or *cuc2* mutant background [53]. By contrast, the septum fusion defect in the *spt* mutant was enhanced in a *cuc1* or *cuc2* mutant background. Furthermore, *SPT* expression analyses in the *cuc1*/*cuc2* double mutant demonstrated that although *SPT* expression in the apical medial domain of the gynoecium is unaltered, it is missing in the basal part. Therefore, it is proposed that whereas negative regulation of *CUC1* and *CUC2* by *SPT* in the apical part of the gynoecium is necessary for correct congenital carpel fusion, in the more basal region all three genes would act together in promoting the formation of CMM-derived structures [53].

Mutations that produce phenotypes at later stages of carpel medial region development

Mutations in some genes with a meristem-promoting function that are expressed during early gynoecium development in the CMM produce altered phenotypes at later stages of marginal tissue development. Single mutants of *BREVIPEDICELLUS* (*BP*; also known as *KNAT1*), *REPLUMLESS* (*RPL*), and *WUSCHEL-LIKE HOMEOBOX13* (*WOX13*) present a reduction, whereas overexpression of *BP* and *WOX13* cause an increase in replum width [58–60]. Interestingly, repla phenotypes are also observed in mutants whose genes are not expressed in the medial domain but are expressed in the lateral domains, such as *JAGGED* (*JAG*), *FIL*, *YAB3*, *ASYMMETRIC LEAVES1* (*AS1*) and *AS2*. These genes act antagonistically with genes expressed in the medial domain [59,61,62], so it was recently suggested that an increase in the expression of medial factors, together with a decrease in lateral factor activities, leads to the overproduction of medial (or marginal) tissues, along with a large reduction in the size of the lateral domains [62]. Furthermore, a mutation in the *AP2* transcription factor also results in enlarged repla owing to enhanced expression of the medial factors *BP* and *RPL*, although AP2 is also expressed in the replum [63,64]. However, ectopic expression of *FRUITFULL* (*FUL*), which is normally expressed in the valves and represses valve margin identity genes, has no effect on the adaxial medial domain [65,66].

Genes expressed at later stages during gynoecium development include the three redundant *HECATE* genes (*HEC1*, *HEC2*, and *HEC3*) [67], *NO TRANSMITTING TRACT* (*NTT*) [68,69], *HALF FILLED* (*HAF*; also known as *CESTA*) [70,71], *SHORT INTERNODES/STYLISH* (*SHI/STY*) family members [72,73], and the redundant *NGATHA* genes (*NGA1*, *NGA2*, *NGA3*, and *NGA4*) [74,75]. The *ntt* and *haf* mutants, and the *hec1/hec2/hec3* triple mutant all have an altered transmitting tract development, resulting in reduced seed set. Recently, it has been shown that *IND* can also affect transmitting tract development [57]. In addition, the *hec1/hec2/hec3* triple mutant is affected in septum fusion and stigma formation, a phenotype not observed in the *ntt* or *haf* single mutants [67,68,70]. Interestingly, the three HEC proteins can interact with SPT in yeast two-hybrid experiments [67], and the SPT–IND [76], SPT–ALC [56], and ALC–IND [77] interactions have also been reported, suggesting that various functional complexes might exist guiding carpel fusion and septum, style, and/or transmitting tract development. *HAF* functions redundantly with two closely related basic helix–loop–helix (bHLH) transcription factors, *BRASSINOSTEROID ENHANCED EXPRESSION1* (*BEE1*) and *BEE3*. When *haf* is in the *ntt* or *bee1/bee3/ntt* mutant backgrounds, a reduction in seed set is observed [70]. Moreover, the authors show that programmed cell death in the septum is affected in these mutants, which in turn interferes with pollen tube growth [68,70]. The *nga1/nga2/nga3/nga4* quadruple mutant and *sty1/sty2/shi/lateral root primordium1* (*Irp1*)/*shi-related sequence 5* (*srs5*) quintuple mutant have apical carpel fusion problems, and marginal tissue development is affected, with no style nor stigma formation [72,74]. Furthermore, a genetic interaction was observed when *nga3* and *sty1* were combined [74], showing strong phenotypes similar to those observed in the *nga1/nga2/nga3/nga4* mutant or higher-order mutants of *sty1* with related *SHI* family members.

Hormones involved in carpel medial region development

Different lines of experimental evidence indicate that hormones also play a very important part in the development of the carpel marginal tissues. The first line of evidence is based on the reported effects of mutations in biosynthesis, transport, signaling, and response components of hormonal pathways. Mutations in genes involved in the auxin pathway — such as the auxin biosynthesis *YUCCA* and *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS* (*TAA*) genes [78,79], the auxin efflux transporter *PIN-FORMED* (*PIN*) genes [80,81], the PINOID protein kinase that regulates intracellular localization of PINs [82,83], or the auxin response factor (ARF) transcription factors [70,84] — can lead to alterations in gynoecium development and patterning, including clear defects in marginal tissues. The phenotypes of these mutants range from moderate transmitting tract defects to very conspicuous gynoecium morphologies, such as hollow tubes, stem-like structures, and alterations in the normal patterning of marginal tissues. In addition, mutations in components of other pathways, such as BRs and cytokinins, indicate that these hormones also participate in marginal tissue development, although in some cases combined mutations are required to produce altered phenotypes, suggesting a high degree of redundancy in the functions of the genes involved in these pathways. The mutation of *CYP85A2*, a cytochrome P450 required for the biosynthesis of BRs, produces, in a *seu* background, a gynoecium with split apex and horn-like protuberances [48]. Conversely, mutations in cytokinin oxidase/dehydrogenases (CKXs), which are responsible for cytokinin catabolism, cause increased activity of the placenta, leading to more ovules and therefore increased seed set [27]. Finally, the quintuple mutant of the *Arabidopsis* DELLA transcriptional repressors of gibberellin signaling shows a moderate increase in style length and stigma width, although this could be related to cell elongation and not to carpel marginal tissue development [85].

A second, related line of evidence is the effect of mutations in transcription factors that are involved in the regulation of hormonal pathways (such as in biosynthesis and transport). Some of these examples are: (i) the regulators of style development *STY* and *NGA*, which regulate the expression of auxin biosynthesis enzymes [74,86,87]; (ii) the CUC1 and CUC2 transcription factors [52], which, in other tissues, are required for proper PIN1 polarization, therefore affecting auxin transport [88]; (iii) the *spt* mutant, which in the root context shows a broader expression of PIN4 and an increased auxin response [89]; (iv) the positive regulator of BR biosynthesis, *HAF*, which works together with the closely related *BEE1* and *BEE3* in the promotion of the reproductive tract [70,71]; (v) the *STM* gene, which is essential for gynoecium and proper CMM development and is known to control cytokinin biosynthesis at the SAM [90]; (vi) the *TEOSINTE*

BRANCHED1/*CYCLOIDEA*/*PROLIFERATING CELL FACTOR* (*TCP*)*14* and TCP15 transcription factors, which mediate cytokinin responses [91,92] and cause excessive proliferation along the boundaries of the replum when fused to the EAR repression domain [91]; (vii) the ETTIN (ARF3) transcription factor, which when mutated affects gynoecium development [84] and, interestingly, binds to the promoter of an isopentenyltransferase involved in cytokinin biosynthesis, *ISOPENTENYLTRANSFERASE 5* (*IPT5*) [93].

A third line of evidence has been obtained from exogenous treatments of hormones, inhibitors, or endogenous hormonal alterations, alone or in combination with mutants. For example: NPA (an auxin transport inhibitor) enhances the carpel marginal region defects of the *ant*/*rev* double mutant, indicating that proper auxin transport is required for the development of the CMM [7]. Interestingly, although this treatment disrupts early development of the medial domain, it causes an enlargement of the style and stigma regions [94], and can rescue some marginal region phenotypes of mutants such as *spt* [95]. Moreover, either polar auxin transport inhibition or increased auxin biosynthesis can also restore style and stigma proliferation in mutants such as *lug*, *seu*, *ant*, *sty1*, *crc*, and *jag* [96]. By contrast, cytokinin treatments can produce impressive overgrowth from the marginal region of developing gynoecia, but not from valves [97].

Interestingly, this proliferation resembles the phenotype observed for the constitutive repressor form of TCP14 expressed using the *TCP14* native promoter (*pTCP14::TCP14:SRDX*) [91]. The gynoecia of these plants were reported to produce striking tissue outgrowths at the boundaries of valves and gynophore and the replum, similar to those observed after cytokinin treatment. Cells in this ectopic tissue expressed the mitotic marker *CYCLIN B1;2*, and the phenotype was enhanced in a *tcp14*/*tcp15* double mutant. Moreover, these outgrowths formed stigmatic papillae at their tips and grew nearly perpendicular to the direction of the elongation of the gynoecium, also resembling those produced by cytokinins [97]. Remarkably, these two genes have been shown to facilitate cytokinin responses in leaves and flowers. They interact with the O-linked N-acetylglucosamine transferase *SPY* and mediate the promotion of cytokinin responses [92]. However, it has also been observed that *TCP14* and *TCP15* modulate proliferation in a context-dependent manner [91].

Finally, hormone signaling during gynoecium development has been visualized using synthetic hormone-responsive promoters fused to reporter genes, such as the *DR5* (highly active synthetic auxin response element) auxin-responsive [81] and the two-component system (*TCS*) cytokinin-responsive [98] lines [97,99]. At early stages, *DR5* was visualized as a circle at the top of the developing gynoecia [76,97,99] and around the CMM, but not on the CMM itself [97] (Figure 1). Interestingly, by contrast, the *TCS* cytokinin-responsive marker shows high activity in the CMM in young gynoecia, and later in the transmitting tract (Figure 1; [97]). Cytokinins control proliferation at the SAM [90], and its precise localization in the early gynoecium [97] highlights the meristematic nature of the CMM, where they have been reported to also promote proliferation [27].

The localization of the *TCS* reporter at the early stages of CMM formation suggests that cytokinins might be defining this important precursor of the later reproductive tissues. Investigating the regulation of cytokinin biosynthesis and signaling genes by transcription factors may also help us to understand some differences between the SAM and CMM. *STM*, which is present in both the CMM and SAM, induces cytokinin biosynthesis [90], and *WUS*, which is present in the SAM but absent in the CMM, is known to repress the negative regulators of cytokinin signaling — type A ARABIDOPSIS RESPONSE REGULATORS (ARRs) — therefore maintaining the sensitivity of the tissue to cytokinins [100,101]. In the CMM, the absence of *WUS* could lead to the expression of these negative regulators in response to cytokinin after some rounds of proliferation and explain the deterministic nature and shorter lifespan of this meristem.

At later stages, expression of *TCS* tagged with green fluorescent protein (*TCS::GFP*) is observed at the center of the gynoecium, the early transmitting tract, and is also observed in the external layer of the septum, but was barely detectable in the style. Interestingly, in older, mature *TCS::GFP* gynoecia, fluorescence is precisely localized at the valve margins [97]. Some genes required for valve margin formation are also expressed at the central regions of the very early gynoecium, suggesting a connection between both tissues that would be worth to investigate further.

By contrast, the *DR5::GFP* marker has been observed in the presumptive replum of stage 9 gynoecia [76]. In growing gynoecia, *DR5* was observed mainly in the vasculature and as a ring at the top, just below the region where the stigmatic papillae form [76,97,99]. A minimum of auxin has been reported to occur at the valve margins of mature gynoecia and fruits [102], contrasting with the precise expression of the cytokinin marker at this tissue, as also occurs with the early CMM in young gynoecia [97].

Interestingly, the activities of both auxin and cytokinin seem to be correlated in the gynoecium because cytokinin-induced ectopic medial region proliferations express *DR5::GFP* at their tips [97]. It would be interesting to investigate whether this cytokinin-triggered proliferation from the medial region reflects the promotion of meristematic activity in the medial tissue by cytokinins, which is then redirected by auxin to form a new pseudo lateral organ formed by the external growth, mimicking what has been proposed to occur during lateral organ formation at the SAM [103,104].

The observation of the complementary localization of the expression of the cytokinin and auxin reporter lines in very young gynoecia, together with the induction of the auxin marker by cytokinins and away from the natural localization of cytokinin response, suggests a possible cytokinin–auxin crosstalk in shaping and maintaining the different tissues (i.e., the CMM and surrounding tissue) of the early gynoecium [104], as has been described for other tissues (reviewed in [105]). If this were the case, it could explain why auxin, which is clearly a crucial hormone for proper medial region development, is not observed (at least as a *DR5*

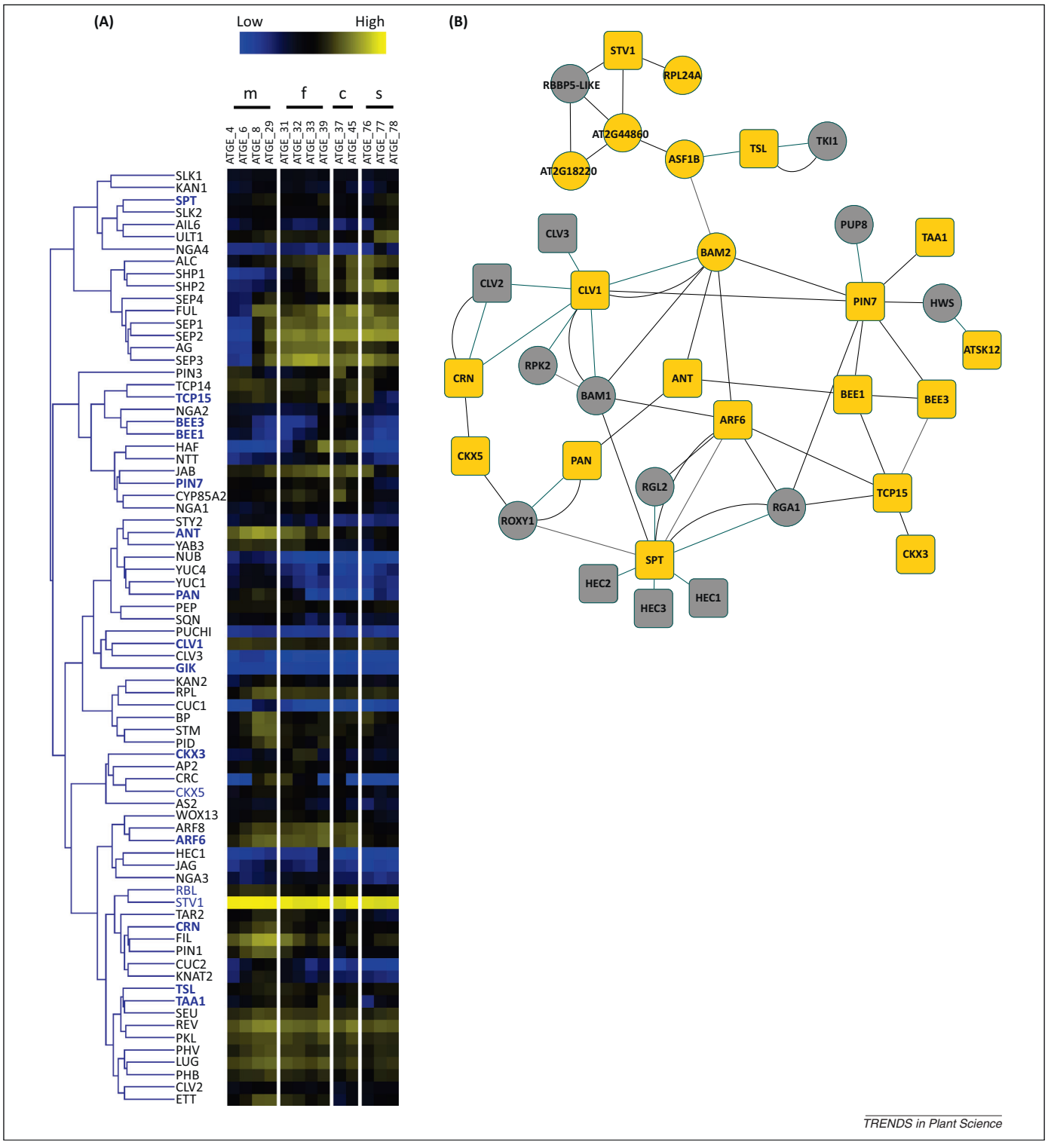

Figure 2. Hierarchical clustering of microarray expression values for genes involved in gynoecium development and a GeneMANIA-generated network for cytokinin-responsive carpel genes. **(A)** Hierarchical clustering. Microarray expression data correspond to the normalized data of AtGenExpress developmental series (http://www.weigelworld.org/resources/microarray/AtGenExpress/) [146] for wild type apical meristem (m), flower (f), carpel (c), and silique (s) samples. Expression data ranges from low (blue) to high (yellow) values. Cytokinin-responsive genes are highlighted in blue. **(B)** GeneMANIA-generated network for cytokinin-responsive carpel genes. The 18 cytokinin-responsive genes from Table S1 in the supplementary material online were used as input for the GeneMANIA prediction server [108]. Square nodes indicate genes present in Table S1 and yellow nodes indicate cytokinin-responsive genes. Edge color represents co-expression (black), colocalization (gray), or protein–protein interactions (blue). Genes represented as circular nodes are either paralogs of genes in Table S1 (*RPL24A*, *AT2G44860*, and *AT2G18220*), genes known to be involved in flower development (*TKI1*, *ASF1B*, *RGA1*, *RGL2*, *HWS*, *RPK2*, *ROXY1*, *BAM1*, and *BAM2*), flowering time (*RBBP5-LIKE*), or cytokinin transport (*PUP8*). The observed correlations indicate that these genes could also be involved in carpel development through cytokinin response (circular yellow nodes), or the regulation of cytokinin pathways (circular gray nodes).

response) at the CMM of young gynoecia. It would be interesting to test this auxin–cytokinin crosstalk hypothesis in the medial region, link it to the current knowledge about the genes involved in its development and, if correct, further investigate its mechanism of action.

Support for this auxin–cytokinin crosstalk hypothesis is also suggested by the analysis of global expression studies of plants treated with cytokinin. In Figure 2A, which shows a hierarchical clustering of important genes for marginal tissues, gene names in blue letters indicate those genes with altered expression after cytokinin treatments [106,107]. Interestingly, expression of not only typically cytokinin-related genes, such as *CKX3* and *CKX5*, but also of genes involved in auxin biosynthesis (*TAA1*), transport (*PIN7*), or response (*ARF6* and *ANT*) are altered by cytokinin treatment. This group of cytokinin-altered genes includes three genes that also exemplify another hormonal crosstalk: *ARF6*, and two of its downstream genes involved in transmitting tract development, the closely related BR-induced genes *BEE1* and *BEE3* [70].

Using cytokinin-responsive genes (genes highlighted blue in Figure 2A, as well as *Arabidopsis thaliana SHAGGY-like kinase 12* (*ATSK12*), which is not represented in the ATH1 microarray) as input, a network was generated using GeneMANIA (http://www.genemania.org/) [108] to visualize how they were related to each other (Figure 2B). It is interesting to see how all of them, except *GIANT KILLER* (*GIK*), form a single interconnected network, suggesting they are part of a larger regulatory program involving the cytokinin response pathway(s). GeneMANIA also retrieved genes not present in our input list, including genes important for medial region development (*CLV2*, *CLV3*, *HEC1*, *HEC2*, and *HEC3*), the gibberellin signaling repressors *REPRESSOR OF GA1-3 1* (*RGA1*) and *RGA-LIKE 2* (*RGL2*) [85], other cytokinin-responsive genes, and *ARABIDOPSIS THALIANA PURINE PERMEASE 8* (*PUP8*), which might be involved in the transport of purine and purine derivatives (such as cytokinins) across the plasma membrane [109,110]. It would be interesting to check whether these GeneMANIA-retrieved genes also participate in marginal tissue development via a cytokinin-dependent regulatory pathway.

In summary, experimental evidence strongly supports a key role of the hormones cytokinins, auxins, and BRs during carpel marginal tissue development, and suggests that the interactions among these hormonal pathways may shape and guide this process.

Do specific carpel marginal region genes exist?

Two interesting questions raised about gynoecium development [5] are also suited to the particular case of its marginal region: how many genes are required for its development? And are these genes specific: that is, not expressed in other parts of the plant? Regarding the first question, and as the supplementary material online Table S1 shows, more than 80 genes required for correct carpel marginal tissues development are currently known, and it is very likely that more are yet to be identified. Interestingly, from their published expression patterns it emerges that about one-third of these genes (Table S1 in the supplementary material online) are expressed very early on at the CMM, suggesting that many players participate in the gene regulatory network that guides the early steps of marginal tissue formation.

From the analysis of microarray-based expression assays, it is evident that the relevant genes for carpel marginal tissue development are also expressed in other tissues and developmental stages (Figure S1 in the supplementary material online). Because the CMM possess a meristematic nature, the genes required for its development might be specific, or general meristematic-promoting genes. *STM*, *BP*, and *RPL* are good examples of genes that function in all aerial meristematic tissues, and, from Figure 2A, it is likely that this may be also the case for other genes. *HAF* may be the closest to a carpel marginal tissue-specific gene (Figure 2A and Figure S1 in the supplementary material online). Interestingly, Figure 2A shows that some genes from the same family share similar expression patterns. For example: eight MADS-box genes and pairs of *TCP*, *BEE*, *YUC*, *CLV*, and *ARF* genes cluster together.

Both the low specificity and the redundancy of some known genes involved in carpel marginal tissue development could explain why, until now, no single loss-of-function mutation has been found that, without affecting any other tissues in the plant, totally abolishes development of marginal tissues.

Evolution of the differentiation of the carpel marginal tissues

The gynoecium (with the carpels as its structural units) is a highly complex organ with a great diversity of forms, and confers selective advantages on the angiosperm evolution [111–114]. The origin and evolution of the carpels are still unknown, although various hypotheses have been proposed, and the most widely accepted are their origin as a leaf-like structure, or as a composed structure of a leaf-like part and a reduced shoot [4,112,113,115–117].

Based on morphological studies in the living basal-most angiosperms, the ANITA (Amborellaceae, Nymphaeales, and Austrobaileyales) grade, the proposed ancestral state of the gynoecium was apocarpous (i.e., separated carpels) with ascidiate (tubular or bottle-shaped) carpels sealed by a secretion, and with the presence of some stigmatic tissue [112,118], although some Nymphaeales have partial syncarpy. However, syncarpous gynoecia (i.e., several carpels fused into a single structure) and plicate ('folded') carpels are most commonly seen (in some magnoliids and most monocots and eudicots).

Many studies on gynoecium development in ancestral species exist, although information on the differentiation of the marginal region is limited. In species of the ANITA grade, the carpel marginal region consists only of placenta, producing ovules (or sometimes only one ovule) in a linear or a diffuse fashion [119]. In many magnoliids, the placentas are placed in the plicate carpel zone and the inner surface forms a canal filled with secretion, which serves as a pollen tube transmitting tract [120]. In syncarpous gynoecia, septa are often formed by the congenitally fused carpel flanks, and the placentae are then in each of the inner angles formed by two septa. The origin and evolution of the septum is complex, because it has evolved many times in numerous plant groups [114].

Notably, *Arabidopsis* has a syncarpous gynoecium with a false septum [4].

Despite recent advances in molecular mechanisms of the development of the carpel marginal region, there is still a fundamental lack of understanding of the molecular events that led to evolutionary differentiation of the tissues of the marginal carpel region. Phylogenetic analysis and functional studies in basal species of orthologous genes that control the development of the marginal carpel region may help (Table S1 in the supplementary material online). The putative *CRC* and *TSL* orthologs have been identified in basal angiosperms and, remarkably, their expression patterns in carpels, and the function of *CRC*, appear to be conserved [121,122].

Moreover, it would be useful to explore hormone localization, effects, and crosstalk in basal species and in species with contrasting gynoecia morphologies to evaluate whether variations can explain the different kinds of gynoecia and help us to understand the evolution of this important tissue and the whole gynoecium.

Challenges inside the gynoecium

It is clear that the CMM and some of its derived tissues are essential for reproductive competence. Over the years, genetic studies in *Arabidopsis* have increased our knowledge of the genes, and more recently of the hormones, involved in CMM and carpel marginal tissue development. The important biological functions of these tissues and the high redundancy among their regulators suggest that a robust regulatory network controls these processes. However, the nature of this gene regulatory network is far from understood. Although relative small modules are beginning to be elucidated, data are still missing that can connect them to form the big picture and help us to understand what makes CMMs unique.

Various technological approaches are at hand to identify new regulators and connect the ones already known. Temporal cell type-specific RNA sequencing (RNA-seq) experiments using laser-capture microdissection or cell sorting will be useful for discovering the presence and dynamics of CMM and carpel marginal tissue developmental regulators. Another strategy involves the identification of target genes of transcriptional regulators through chromatin immunoprecipitation followed by sequencing (ChIP-seq; the identification of binding events of a protein of interest to regulatory DNA sequences), or microarray hybridization experiments with inducible, specific expression constructs, mutants, or stage or tissue-specific hormonal induction or degradation. ChIP-seq experiments have been performed for *SEP3* [123], *AP2* [124], and *REV* [125], and microarray hybridizations were obtained for *AG:glucocorticoid receptor* (*GR*) [126], *SPT:herpes simplex virus protein* (*VP16*):*GR* [127], *STY1:GR* [128], *STM:GR* and *STM:VP16* [129], and *FIL:GR* [130], as well as the *seu/ant* double mutant [8]. Although these experiments resulted mostly in lists of thousands of putative (direct) target genes, we still lack a comprehensive framework that can help us decipher the complex genetic and molecular interactions between them. One step in this direction is the identification of the protein–protein interactions among identified genes in order to connect known functional modules or predict new ones (e.g., [131]). Unfortunately, protein–protein interaction data are still limited to a small number of the genes in Table S1 in the supplementary material online. A challenge for the future will be to generate more data, identify new connections and interactions (e.g., gene–gene, protein–gene, protein–protein, gene–hormone, and hormone–hormone interactions), perform functional analysis for identified target genes, identify functional conservation modules in various plant species, and integrate all of this information into comprehensive regulatory networks that will allow us to fully understand the mechanisms that guide carpel medial region development.

Disclaimer statement

The authors declare no conflicts of interest.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tplants.2013.08.002.

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Cytokinin treatments affect the apical-basal patterning of the *Arabidopsis* gynoecium and resemble the effects of polar auxin transport inhibition

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The apical-basal axis of the *Arabidopsis* gynoecium is established early during development and is divided into four elements from the bottom to the top: the gynophore, the ovary, the style, and the stigma. Currently, it is proposed that the hormone auxin plays a critical role in the correct apical-basal patterning through a concentration gradient from the apical to the basal part of the gynoecium, as chemical inhibition of polar auxin transport through 1-*N*-naphtylphtalamic acid (NPA) application, severely affects the apical-basal patterning of the gynoecium. In this work, we show that the apical-basal patterning of gynoecia is also sensitive to exogenous cytokinin (benzyl amino purine, BAP) application in a similar way as to NPA. BAP and NPA treatments were performed in different mutant backgrounds where either cytokinin perception or auxin transport and perception were affected. We observed that cytokinin and auxin signaling mutants are hypersensitive to NPA treatment, and auxin transport and signaling mutants are hypersensitive to BAP treatment. BAP effects in apical-basal gynoecium patterning are very similar to the effects of NPA, therefore, it is possible that BAP affects auxin transport in the gynoecium. Indeed, not only the cytokinin-response *TCS::GFP* marker, but also the auxin efflux carrier PIN1 (*PIN1::PIN1:GFP*) were both affected in BAP-induced valveless gynoecia, suggesting that the BAP treatment producing the morphological changes has an impact on both in the response pattern to cytokinin and on auxin transport. In summary, we show that cytokinin affects proper apical-basal gynoecium patterning in *Arabidopsis* in a similar way to the inhibition of polar auxin transport, and that auxin and cytokinin mutants and markers suggest a relation between both hormones in this process.

Keywords: apical-basal patterning, gynoecium, *Arabidopsis*, plant developmental biology, auxin, cytokinin

INTRODUCTION

The gynoecium is the female reproductive organ of the flower. Different axes can be distinguished during the development of the *Arabidopsis thaliana* gynoecium and one of them is the apical-basal axis. This axis can be divided into four domains: the stigma at the apical part, consisting of a single layer of elongated cells called papillae, followed by a solid cylinder below, called the style, then there is the ovary which is the most complex part of the gynoecium and contains the ovules, and finally in the basal part the gynophore, which is a short stalk-like structure connecting the gynoecium with the rest of the plant (Balanza et al., 2006; Roeder and Yanofsky, 2006; Alvarez-Buylla et al., 2010).

Plants produce different hormones, which are involved in many developmental processes throughout their life cycle (Durbak et al., 2012; Lee et al., 2013). One of the most widely studied hormones is auxin (Tromas and Perrot-Rechenmann, 2010; Sauer et al., 2013). It has been reported that alterations in polar auxin transport, as occurs in the *pin1* mutant (Okada et al., 1991), or treatment with the polar auxin transport inhibitor 1-*N*-naphtylphtalamic acid (NPA; Nemhauser et al., 2000), or alterations in auxin signaling, occurring in the *ettin* mutant (Sessions and Zambryski, 1995), or deficiency in auxin biosynthesis, shown in the *yuc1 yuc4* (Cheng et al., 2006) and the *wei8 tar2* (Stepanova et al., 2008) mutants, have strong impact on gynoecium development, affecting the establishment of their apical-basal patterning. It has been proposed that auxins act through a gradient in the establishment of apical-basal patterning of the gynoecium, where the highest concentration of auxin is in the apical end and decreases towards the basal part of the gynoecium (Nemhauser et al., 2000), though modified views have evolved related to the presence of an auxin gradient (Ostergaard, 2009; Larsson et al., 2013). Alterations in the apical-basal patterning of the gynoecium are distinguished by an increase in the style and gynophore domain sizes at the expense of the ovary, which in severe cases even completely disappears.

Another well-studied plant hormone is cytokinin, which is involved in different developmental processes such as shoot meristem formation and maintenance, organ formation, and seed germination, among others (Mok and Mok, 2001; Hwang et al., 2012; El-Showk et al., 2013). Recently, it has been reported that cytokinins are involved in the regulation of floral organ size,

ovule number, and ovule development in the gynoecium (Bartrina et al., 2011; Bencivenga et al., 2012). Furthermore, cytokinins are involved in medial tissue proliferation at early stages of the developing gynoecium and at more mature stages in valve margin differentiation (Marsch-Martinez et al., 2012a,b; Reyes-Olalde et al., 2013).

In recent years special attention has been paid to the study of interactions between different hormones. Hormonal crosstalk provides an extra level of regulation in biological processes conferring robustness and stability, as well as flexibility (Moubayidin et al., 2009; Wolters and Jurgens, 2009; Depuydt and Hardtke, 2011; Vanstraelen and Benkova, 2012). The cytokinin–auxin crosstalk is important for the establishment and maintenance of the root apical meristem (RAM) and the shoot apical meristem (SAM). These two hormones act antagonistically in the RAM, cytokinin by promoting cell differentiation and auxin by promoting cell division (Dello Ioio et al., 2007; Ruzicka et al., 2009). Conversely, in the SAM, auxin increases cytokinin response through the repression of cytokinin signaling repressors (Zhao et al., 2010). Several studies have demonstrated that the cytokinin–auxin crosstalk can occur at different levels, cytokinin can affect auxin synthesis, transport or signaling, and *vice versa*, auxin can affect cytokinin synthesis, degradation, or signaling (Hwang et al., 2012; El-Showk et al., 2013).

Despite the large number of studies on the role of cytokinins in plant development, their functions in gynoecium development are just beginning to be explored (Marsch-Martinez et al., 2012a; Reyes-Olalde et al., 2013), while it's possible interactions with other hormones in this organ have not been studied yet. In this study we analyzed the possible role of cytokinin in apical-basal patterning of the gynoecium and its possible interaction with auxin through exogenous application of the cytokinin benzyl amino purine (BAP) and the auxin transport inhibitor NPA to different mutants and cytokinin and auxin signaling markers. The results suggest that cytokinins are also involved in apical-basal patterning of the gynoecium, which is more evident when the auxin transport or signaling is affected.

MATERIALS AND METHODS

PLANT GROWTH CONDITIONS

All wild type and mutant plants used in this study are *Arabidopsis thaliana* ecotype Columbia. Plants were germinated in soil under long-day conditions (16–8 h, light–dark) in a growth chamber at 22°C. One week after germination, the plants were transferred to the greenhouse with a temperature range from 22 to 28°C, long-day conditions (13–11 h, light–dark approximately) and natural light.

HORMONE TREATMENTS

One week after bolting, wild type, mutant and marker line inflorescences were dipped five consecutive days in BAP, NPA, or mock solutions. The BAP and NPA solutions contained 100 μM benzylaminopurine (BAP; Duchefa Biochemie, http://www.duchefa.com) or 100 μM NPA (Sigma–Aldrich, St. Louis, MO, USA) respectively, and 0.01% Silwet L-77 (Lehle Seeds, Round Rock, TX, USA). The mock solution contained only 0.01% Silwet L-77. All treated plants with their respective controls were grown simultaneously under the same conditions. For each mutant background five plants were treated, of which 10–15 main and secondary inflorescences were analyzed. The gynoecia were analyzed after anthesis. The treated plants were frequently monitored; the apical-basal patterning phenotypes began to be observed after 2 weeks.

The standard deviation was calculated considering the phenotype frequency percentages between each inflorescence analyzed. To determine whether there was a significant difference in the different phenotypes between wild type plants and the different treated mutants a Student's *t*-test was performed comparing the phenotype frequency percentages of each mutant background versus wild type plants. The treatments for each mutant were performed twice with similar results. The results presented here are from one experiment.

MICROSCOPY

For light pictures and phenotype analysis the plant material was dissected and observed using a Leica EZ4 D stereomicroscope (Leica, Wetzlar, Germany). Scanning electron microscopy images were captured using a Zeiss EVO40 environmental scanning electron microscope (Carl Zeiss, Oberkochen, Germany) with a 20 kV beam, and the signal was collected using the BSD detector, for which plant tissue was collected and directly observed in the microscope. For fluorescent microscopy, the images were captured using a LSM 510 META confocal scanning laser inverted microscope (Carl Zeiss, Oberkochen, Germany). Propidium iodide (PI) was excited using a 514-nm line and GFP was excited using a 488-nm line of an Argon laser. PI emission was filtered with a 575-nm long-pass (LP) filter and GFP emission was filtered with a 500–550-nm bandpass (BP) filter.

RESULTS

EXOGENOUS APPLICATION OF CYTOKININ AFFECTS THE APICAL-BASAL PATTERNING OF THE *Arabidopsis* GYNOECIUM

Recently, we reported that cytokinins are important for the proliferation at the medial tissues in the gynoecium and for proper valve margin differentiation in *Arabidopsis* fruits (Marsch-Martinez et al., 2012a). It has been shown that auxin plays an important role in establishing the correct apical-basal patterning of the gynoecium (Nemhauser et al., 2000). Furthermore, it is known that cytokinin and auxin cross-talk at different levels in several developmental processes (El-Showk et al., 2013). With this in mind, we decided to analyze the effect of exogenous cytokinin applications on the apical-basal patterning of the *Arabidopsis* gynoecium. Inflorescences of wild type plants were treated once a day for a period of 5 days with 100 μM BAP solution. In parallel, we carried out a treatment with 100 μM NPA under the same conditions; this compound blocks the polar auxin transport, causing apical-basal patterning defects in the gynoecium (Nemhauser et al., 2000). This treatment was performed in order to compare the effect of exogenous cytokinin application versus polar auxin transport blocking.

We previously reported that prolonged BAP application (3–4 weeks) produced gynoecia with conspicuous tissue proliferation (Marsch-Martinez et al., 2012a). However, when the wild type inflorescences were treated with BAP during a shorter time (5 days)

a gradient of phenotypes were observed. The first open flowers (flowers 1–5) after the treatment contained gynoecia with no obvious phenotype. The next floral buds to open (flowers 6–18) contained gynoecia that showed the proliferation that was reported previously. However, floral buds that opened later (flowers 19–31) contained gynoecia that showed apical-basal defects which are the focus of this study. In some cases we observed gynoecia with both phenotypes, the proliferation and the apical-basal defects; these gynoecia were developed in the transition zone of these two phenotypes. Finally normal gynoecia were developed.

Two weeks after each treatment, the gynoecia of treated floral buds were analyzed. In both cases for wild type plants twelve to fifteen gynoecia per inflorescence showed apical-basal defects with different severities. The observed phenotypes were classified according to previously reported by Sohlberg et al. (2006). The classification consists of three categories based on valve development: (1) If the length of the valves was more than 50% the length of the gynoecium, but less than the length of valves of mock-treated gynoecium, were named "reduced valves"; (2) This category includes gynoecia with one valve and gynoecia with two small valves that occupied less than half of its length; and (3) If the gynoecium did not develop any valves the phenotype was named "valveless" (**Figures 1** and **2**).

The BAP-treated wild type gynoecia presenting apical-basal defects were analyzed, and the majority of them (88%) showed reduced valves, 10% developed very reduced valves and almost 2% were classified as valveless (**Figures 2** and **3A**). In the case of NPA-treated wild type gynoecia, 59% of them showed reduced valves, 25% developed very reduced valves, and 16% showed the valveless phenotype (**Figure 3B**). The data obtained for the NPA treatment (**Figures 1** and **3**) are similar to those previously reported (Sohlberg et al., 2006). Comparing the frequencies of the phenotypes in both treatments, the defects observed due to BAP are less severe than the defects due to NPA, however, the occurrence of these phenotypes are constant between BAP treatments and significantly higher than the frequency in which they appear in untreated plants. These results indicate that, like NPA, exogenously applied cytokinin affects proper establishment of the apical-basal patterning in the *Arabidopsis* gynoecium.

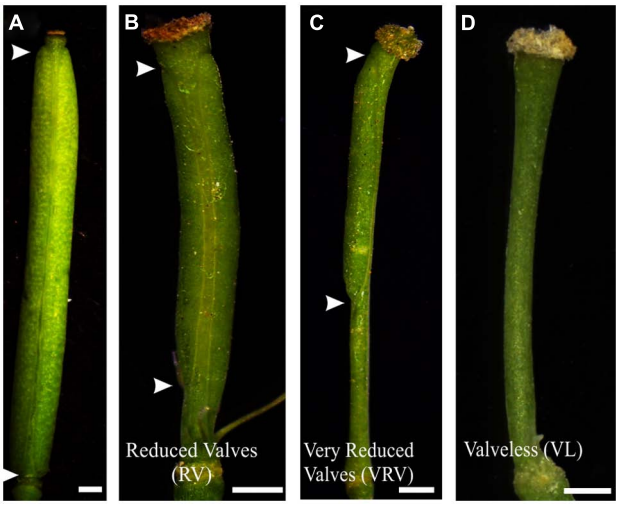

FIGURE 2 | Apical-basal phenotypes caused by exogenous BAP application. (A) Mock-treated wild type gynoecium. **(B)** A gynoecium with the "Reduced Valves" (RV) phenotype. **(C)** Gynoecium with a "Very Reduced Valves" (VRV) phenotype. **(D)** Gynoecium with the "Valveless" (VL) phenotype. The arrowheads indicate the beginning and the end of valves. Scale bars: **(A)** 1 mm; **(B,C)** 400 μm; **(D)** 200 μm.

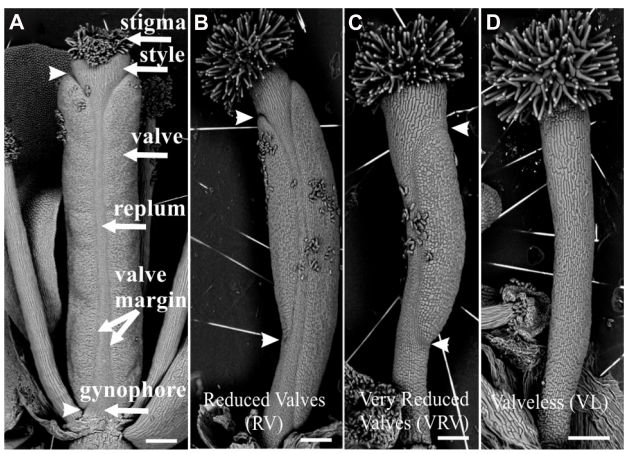

FIGURE 1 | Scanning electron micrographs of classification of apical-basal phenotypes in the *Arabidopsis* gynoecium. **(A)** Mock-treated wild type gynoecium. **(B)** Gynoecium presenting a "Reduced Valves" (RV) phenotype. **(C)** Gynoecium with the "Very Reduced Valves" (VRV) phenotype. **(D)** Gynoecium with the "Valveless" (VL) phenotype. These gynoecia were treated with NPA. The arrowheads indicate the beginning and the end of valves. Scale bars: **(A–D)** 200 μm.

BAP AND NPA APPLICATIONS HAVE SIMILAR EFFECTS IN AUXIN TRANSPORT AND SIGNALING MUTANTS

It has been reported that the apical-basal gynoecium patterning of auxin biosynthesis or signaling mutants gynoecia is hypersensitive to NPA treatment (Staldal et al., 2008). In order to know whether the the BAP effect on the apical-basal patterning was related with any auxin related processes, we performed BAP treatments in different auxin transport and signaling mutants.

In *Arabidopsis*, polar auxin transport requires the activity of polarly localized PIN-FORMED (PIN) auxin efflux transporters (Benkova et al., 2003; Friml, 2003). The *pin1* mutant produces hardly any flowers (Okada et al., 1991), so it was discarded for this study. On the other hand, the *pin3 pin7* double mutant gynoecia show alterations in apical-basal patterning, but its reproductive development is also severely affected (Benkova et al., 2003). However, the *pin3* and *pin7* single mutants do not exhibit visible apical-basal defects. Therefore, these two mutants represent an opportunity to explore the effect of BAP application in a background where polar auxin transport is affected but development is not severely altered. When the *pin7* mutant was treated with BAP, 39% of gynoecia developed reduced valves, 37% developed very reduced valves, and 24% showed the valveless phenotype

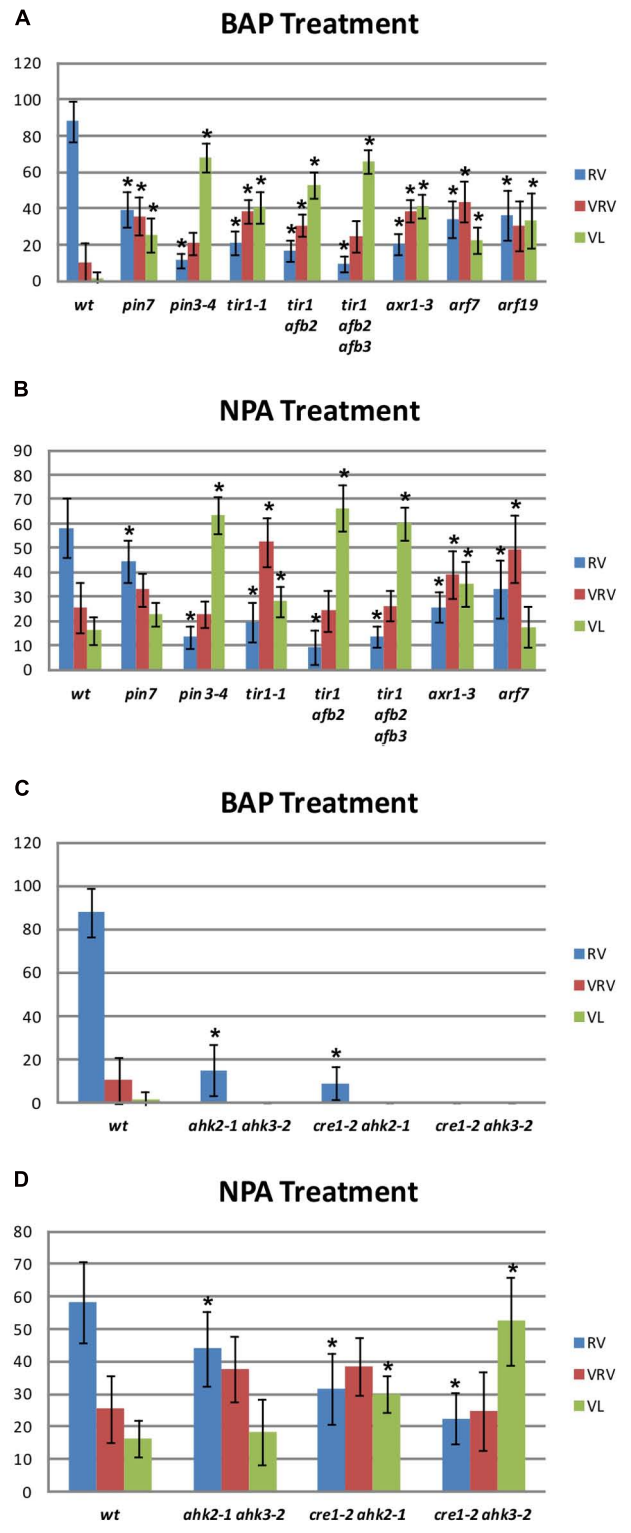

FIGURE 3 | Apical-basal gynoecium patterning phenotype frequency of NPA and BAP treatments in wild type and mutant backgrounds. (A,C) Distribution of the different categories of apical-basal phenotypes in BAP-treated gynoecia. **(A)** Auxin signaling mutants. Col, $n = 204$; *pin7*, $n = 111$; *pin3*, $n = 204$; *tir1*, $n = 145$; *tir1 afb2*, $n = 299$; *tir1 afb2 afb3*, $n = 383$; *axr1*, $n = 372$; *arf7*, $n = 122$. **(C)** Cytokinin signaling mutants. *ahk2 ahk3*, $n = 224$; *cre1 ahk2*, $n = 288$; *cre1 ahk3*, $n = 495$. **(B,D)** Distribution of the different categories of apical-basal phenotypes in NPA-treated gynoecia. **(B)** Auxin signaling mutants Col, $n = 231$; *pin7*, $n = 225$; *pin3*, $n = 258$; *tir1*, $n = 314$; *tir1 afb2*, $n = 557$; *tir1 afb2 afb3*, $n = 889$; *axr1*, $n = 406$; *arf7*, $n = 317$; *arf19*, $n = 434$. **(C)** Cytokinin signaling mutants *ahk2 ahk3*, $n = 163$; *cre1 ahk2*, $n = 148$; *cre1 ahk3*, $n = 177$. RV, Reduced Valves; VRV, Very Reduced Valves; VL, Valveless. Error bars represent standard deviation. The "*n*" indicates the total number of analyzed gynoecia for each background. Values on the *y*-axis are percentages. The asterisk (*) indicates significant difference.

(**Figure 3A**). In the *pin3* mutant 11% of gynoecia showed reduced valves, 21% developed very reduced valves, and 68% showed the valveless phenotype (**Figure 3A**). These same mutants were also treated with NPA (**Figure 3B**). In the *pin7* mutant 22% of gynoecia did not develop valves, whereas this alteration was observed in 64% of *pin3* mutant gynoecia. These results indicate that the apical-basal patterning of *pin3* and *pin7* gynoecia is hypersensitive to both treatments and the valveless phenotype frequencies are similar for both treatments in the same mutant. In addition, the *pin3* mutant appears to be more sensitive than the *pin7* mutant to both treatments, suggesting that PIN3 plays a more relevant role in the establishment of apical-basal gynoecium patterning than PIN7.Furthermore, auxin signaling mutants were treated with BAP or NPA. First, different auxin receptor mutants were treated: the single mutant *transport inhibitor response 1* (*tir1*; Ruegger et al., 1998), the double mutant *tir1 auxin signaling F-box protein 2* (*afb2*), and the triple mutant *tir1 afb2 afb3* (Dharmasiri et al., 2005). The untreated *tir1* and *tir1 afb2* gynoecia did not exhibit obvious apical-basal defects, while *tir1 afb2 afb3* gynoecia occasionally showed apical-basal defects under our growth conditions. However, all three genotypes were hypersensitive to BAP treatment, and the frequency of the more severe phenotype (valveless) increased when auxin perception decreased, such that in *tir1*, *tir1 afb2*, and *tir1 afb2 afb3* plants 40, 53, and 64% of gynoecia, respectively, showed the valveless phenotype (**Figure 3A**). When the mutants were treated with NPA, in *tir1, tir1 afb2*, and *tir1 afb2 afb3* plants 28, 66, and 61% of gynoecia, respectively, showed the valveless phenotype (**Figure 3B**), indicating that these mutants are also hypersensitive to the NPA treatment.

In addition, mutants affected in auxin signaling, downstream perception, were treated with BAP and NPA. These mutants were *auxin resistant 1* (*axr1*), where a protein related to the ubiquitin-activating enzyme E1 is affected, and *auxin response factor 7* (*arf7*) and *arf19* mutants, where transcription factors that mediate auxin response are affected (Leyser et al., 1993; Harper et al., 2000; Okushima et al., 2005). Untreated *axr1* gynoecia occasionally showed apical-basal defects under our growth conditions, but this was not observed for *arf7* and *arf19*. Regarding the BAP treatment, the *axr1* mutant developed 41%, the *arf7* mutant 24%, and the *arf19* mutant 35% of gynoecia without valves (**Figure 3A**). These results indicate that these three mutants are hypersensitive to the BAP treatment. In the case of the NPA treatment, the *axr1* mutant developed 34% and the *arf7* mutant 18% of valveless gynoecia (**Figure 3B**), indicating that *axr1* is hypersensitive to

NPA treatment. For the *arf19* mutant no data were obtained due to technical reasons.

In summary, the results indicate that the gynoecia of auxin transport and signaling mutants are hypersensitive to BAP application, resulting in apical-basal patterning defects. This phenomenon was already reported for NPA application (Staldal et al., 2008), therefore in this study NPA was used as reference, and produced similar results as seen for the BAP application.

THE ABSENCE OF CYTOKININ RECEPTORS ALTERS THE RESPONSE TO BAP AND NPA APPLICATIONS

The above results suggest that disruption of auxin transport or signaling has an impact on the effect caused by BAP treatments on the apical-basal patterning of the gynoecium, as had been reported and was also observed here for NPA treatments. The next step was to explore the possibility that disturbances in processes related to cytokinin perception might also have an impact on the effect of these treatments. For this purpose, the *cytokinin response 1* (*cre1*) *Arabidopsis histidine kinase 2* (*ahk2*), *cre1 ahk3*, and *ahk2 ahk3* cytokinin receptor double mutants (Higuchi et al., 2004; Nishimura et al., 2004) were treated. Untreated double mutant gynoecia never presented apical-basal defects under our growth conditions. After BAP treatment, two of the three cytokinin receptor double mutants showed slight apical-basal defects, but none of them developed gynoecia with severe apical-basal phenotypes. In *ahk2 ahk3* and *cre1 ahk2* mutants 15 and 9% of gynoecia developed reduced valves, respectively (**Figure 3C**). The *cre1 ahk3* mutant gynoecia did not show visible apical-basal phenotypes (**Figure 3C**). These results suggest that the cytokinin receptors CRE1, AHK2, and AHK3 are required for the full effect of exogenous BAP application on the establishment of apical-basal patterning of gynoecia observed in wild type plants. An opposite response was observed when the cytokinin receptor mutants were treated with NPA. In the *ahk2 ahk3*, *cre1 ahk2*, and *cre1 ahk3* mutants 19, 30, and 53% of the gynoecia, respectively, showed the severe valveless phenotype (**Figure 3D**), in comparison to only 16% in wild type plants. These results suggest that adequate cytokinin perception is necessary to attenuate the impact of the reduction in polar auxin transport on the establishment of apical-basal patterning of the gynoecium.

BAP AND NPA APPLICATIONS AFFECT THE EXPRESSION PATTERN OF CYTOKININ (*TCS::GFP*) AND AUXIN-RESPONSE MARKERS (*DR5::GFP*) AND THE AUXIN TRANSPORTER PIN1 (*PIN1::PIN1:GFP*) IN THE GYNOECIUM

It has been described that the cytokinin (*TCS::GFP*) and auxin-response (*DR5::GFP*) markers have well defined and mutually exclusive expression patterns in some regions of the gynoecium during development (Marsch-Martinez et al., 2012a). Besides, the auxin efflux carrier PIN1 is important for gynoecium development, because the *pin1* mutant produces almost no flowers and when flowers are produced their gynoecium show severe apical-basal patterning defects (Okada et al., 1991). We analyzed whether BAP or NPA application were able to cause changes in the expression pattern of PIN1 and the hormonal-response markers, and whether these changes could be related to the apical-basal gynoecium defects due to these treatments. For this purpose, each marker line was treated once a day for a period of 5 days, as done for the treatments described above, with the BAP or NPA solution for *TCS::GFP* and *DR5::GFP* and with BAP for *PIN1::PIN1:GFP*. The expression patterns of these marker lines were analyzed using confocal laser scanning microscopy when gynoecia with apical-basal defects were observed.

In wild type gynoecia between floral stages 8–10 (Smyth et al., 1990) the TCS::GFP signal was observed at the center, where the medial tissues are developing from the carpel marginal meristem (CMM), as we have observed before (Marsch-Martinez et al., 2012a; **Figures 4A,D**). After BAP or NPA treatment, the TCS::GFP signal was increased in the central zone of valveless gynoecia. However, these gynoecia had reduced development of the internal medial tissues (**Figures 4B,C,E**). For the DR5::GFP auxin-response marker in untreated gynoecia between stages 9–12 the signal was observed at the apical end of gynoecia and in the vasculature, as we have observed before (Marsch-Martinez et al., 2012a; **Figure 4H**). After BAP or NPA treatment, the DR5::GFP signal did not show obvious changes in these experiments (**Figures 4I,J**). However, in the wild type gynoecium at stage 10, the auxin efflux carrier PIN1 is expressed in the tissue that will give rise to the replum (**Figure 4F**), and after BAP treatment the *PIN1::PIN1:GFP* signal was observed in the whole valveless gynoecium (**Figure 4G**).

In summary, BAP and NPA application had comparable effects in the hormone reporter lines, this is, an increase in TCS::GFP activity in the central region of the gynoecium, but no detectable change in the DR5::GFP signal. Moreover, BAP application caused an increase in expression level and alteration of the localization of PIN1 in the gynoecium. These results correlate well with the observation that BAP and NPA treatments cause similar apical-basal patterning defects.

DISCUSSION

IMPACT OF CYTOKININ AND NPA APPLICATION ON APICAL-BASAL GYNOECIUM PATTERNING IN AUXIN TRANSPORT AND SIGNALING MUTANTS

Cytokinin is involved in different developmental processes throughout the *Arabidopsis* life cycle (Hwang et al., 2012; El-Showk et al., 2013), including proper gynoecium and fruit development (Marsch-Martinez et al., 2012a,b; Reyes-Olalde et al., 2013). Here, we evaluated the effect of exogenous cytokinin application on the establishment of apical-basal patterning of the *Arabidopsis* gynoecium.

BAP-treated gynoecia present the same apical-basal defects observed as when treated with NPA, but the frequencies in which altered phenotypes are observed are lower. Because the role of NPA is to block polar auxin transport and the phenotypes caused by both BAP and NPA treatments are similar, the results suggest that exogenously applied cytokinin might affect polar auxin transport and thereby cause the observed patterning phenotypes.

It has been reported that auxin biosynthesis or signaling mutant gynoecia are hypersensitive to NPA treatment in regard to apical-basal patterning (Staldal et al., 2008). In this study, we observed that the auxin transport mutants *pin3* and *pin7* were hypersensitive to both BAP and NPA treatments, and the sensitivity level was similar between treatments but different between mutants. In this case, the *pin3* mutant was more sensitive to either treatment

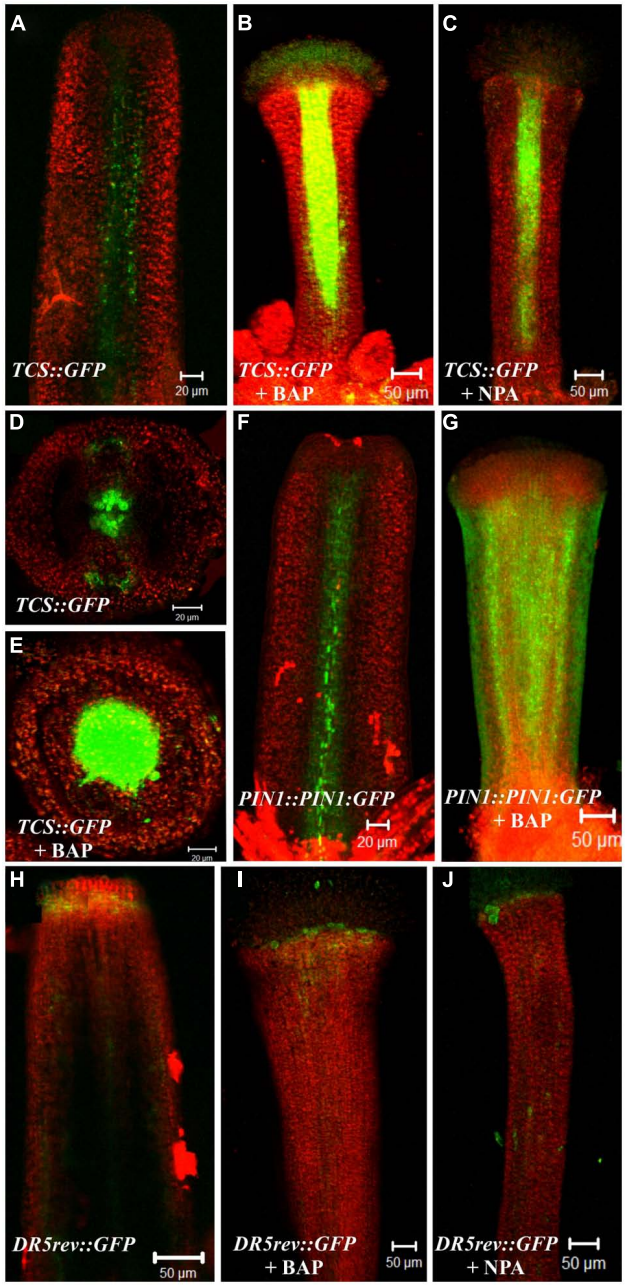

FIGURE 4 | Effect of cytokinin (BAP) and NPA application on the PIN1 (*PIN1::PIN1:GFP*), cytokinin (*TCS::GFP*) and the auxin-response markers (*DR5::GFP*). (A–E) The fluorescence signal of the cytokinin response marker *TCS::GFP* observed in the wild type gynoecium at floral stage 10 in a longitudinal view **(A)** and transverse view **(D)**. Valveless gynoecium at floral stage 11 caused by BAP treatment in a longitudinal view **(B)** and transverse view **(E)**. Valveless gynoecium at floral stage 11 caused by NPA treatment in a longitudinal view **(C)**. **(F,G)** The fluorescence signal detection of the PIN1 marker *PIN1::PIN1:GFP* observed in the wild type gynoecium at floral stage 10 **(F)**. Valveless gynoecium at floral stage 10 caused by BAP treatment **(G)**. **(H–J)** The fluorescence signal detection of the auxin response marker *DR5::GFP* observed in wild type gynoecium at stage 12 **(H)**. Valveless gynoecium at floral stage 12 caused by BAP treatment **(I)**. Valveless gynoecium at floral stage 12 caused by NPA treatment **(J)**. Scale bars: **(A,D–F)** 20 µm; **(B,C,G–J)** 50 µm.

compared to the *pin7* mutant, indicating that in the absence of the PIN3 function the imbalance caused by both BAP and NPA application has a greater impact on the establishment of apical-basal gynoecium patterning. This suggests that PIN3 and PIN7 contribute to different extent to proper gynoecium apical-basal patterning.

Furthermore, the different auxin signaling mutants analyzed in this study were also sensitive to both treatments. In the case of the auxin receptor mutants, only the mock-treated *tir1 afb2 afb3* gynoecia occasionally showed some apical-basal gynoecium patterning defects. However, the three different mutants were hypersensitive to BAP and NPA, suggesting that the proper establishment of the apical-basal gynoecium pattern is a robust process that even when auxin perception is severely affected can be carried out without major defects. However, when perturbations such as those caused by cytokinin application or by auxin transport inhibition occur, it becomes evident that a change in the level of auxin perception affects proper gynoecium development.

Auxin Response Factors (ARFs) are transcription factors that regulate transcription in an auxin-dependent manner. It is known that the *ARF7* and *ARF19* genes are involved in cell growth of leaves and in lateral root formation (Wilmoth et al., 2005; Okushima et al., 2007), and *ARF7* acts redundantly with *MONOPTEROS* (*MP/ARF5*) in the axial patterning of the embryo (Hardtke et al., 2004). We observed that the *arf7* and *arf19* mutants are hypersensitive to BAP application regarding apical-basal gynoecium patterning, suggesting a role of these genes in this process.

IMPACT OF CYTOKININ AND NPA APPLICATION ON APICAL-BASAL GYNOECIUM PATTERNING IN CYTOKININ SIGNALING MUTANTS

When the cytokinin receptor mutants were treated with BAP, less severe or no alterations were observed in apical-basal gynoecium patterning, suggesting that the exogenous cytokinin needs to be perceived by the plant to trigger these changes. Interestingly, the altered apical-basal patterning phenotypes caused by NPA treatments were increased in the cytokinin receptor mutants.

A comparison of the effects of both treatments in the different cytokinin receptor mutant backgrounds, suggested a negative correlation between the ability to respond to cytokinin and the severity of the phenotype caused by auxin transport inhibition. In the mutants where cytokinin perception was more affected, i.e., less alteration in patterning caused by BAP (least phenotypic effect observed in *cre1 ahk3*), the effect of NPA was increased, i.e., more visible alterations in patterning.

This may indicate that cytokinin (perception) buffers the effect of decreased auxin polar transport in apical-basal patterning.

IMPACT OF CYTOKININ AND NPA APPLICATION ON CYTOKININ (*TCS::GFP*) AND AUXIN-RESPONSE MARKERS (*DR5::GFP*) AND THE AUXIN TRANSPORTER PIN1 (*PIN1::PIN1:GFP*) IN THE GYNOECIUM

The cytokinin (*TCS::GFP*) and auxin-response (*DR5::GFP*) and PIN1 (*PIN1::PIN1:GFP*), markers were analyzed in gynoecia presenting apical-basal defects. The TCS::GFP signal was detected in the medial tissues during normal gynoecium development at

early stages. We followed the TCS::GFP signal in the BAP and NPA induced valveless gynoecia. In these gynoecia the medial tissue showed reduced development. However, the TCS::GFP signal was not only maintained, but interestingly, it was increased.

NPA treatments have been shown to inhibit the formation of lateral organs in shoot apical meristems (Reinhardt et al., 2000). The valves of gynoecia are considered lateral organs (Benkova et al., 2003), and NPA has a comparable effect, producing valveless gynoecia. In the shoot apical meristem context, NPA does not affect the meristematic activity as shown by the maintenance of the activity of various meristem markers (Reinhardt et al., 2000). At the gynoecium, the activity of the *TCS::GFP* marker suggests that a similar situation occurs in this tissue, i.e., that the valves are not formed, but the meristematic activity at the medial tissues continues. Interestingly, the cytokinin signaling was not only maintained after the NPA treatment, but seemed to increase, as revealed by the increased fluorescence observed at the medial tissues.

After BAP and NPA application, no evident changes were detected in the DR5::GFP signal in the abaxial (external) side of the valveless gynoecia, compared to the wild type. The model proposed by Sessions in 1997 suggests that the apical-basal patterning of the gynoecia is determined through the specification of two boundaries that are specified very early, during floral stage 6 when the gynoecial primordium is a radially symmetric dome of cells (Sessions, 1997; Larsson et al., 2013). Based on this, one possible explanation is that changes in auxin signaling (*DR5::GFP)* may occur in early stages (stage 5–7) during BAP or NPA-treated gynoecium development causing the apical-basal defects and such changes cannot be detected at later stages of gynoecium development. In order to test this hypothesis it would be necessary to analyze auxin signaling during earlier valveless gynoecia development, which is technically challenging, or by using a more sensitive auxin signaling marker like the DII-VENUS sensor (Brunoud et al., 2012).

On the other hand, cytokinin negatively affects PIN expression and localization in the root meristem (Laplaze et al., 2007; Dello Ioio et al., 2008; Ruzicka et al., 2009). In contrast, here we observed that the auxin efflux carrier PIN1 expression was increased and localized in whole valveless gynoecia due to cytokinin application. This suggests that cytokinin has an opposite effect on PIN1 expression in the gynoecium versus the root meristem, as similarly observed in the root vasculature (Bishopp et al., 2011).

The cytokinin–auxin interaction can occur at different levels, i.e., cytokinin can affect auxin synthesis, transport or signaling, and auxin can affect cytokinin synthesis, degradation or signaling (Hwang et al., 2012; El-Showk et al., 2013). With the generated data so far we cannot rule out any of these possibilities related to apical-basal gynoecium patterning. However, because the NPA role is to block polar auxin transport and the phenotypes caused by both treatments were very similar, the observations obtained from our experiments suggest that the exogenous BAP application may be able to affect polar auxin transport and therefore cause apical-basal gynoecium patterning defects. Supporting this hypothesis is the observation that cytokinin can affect PIN expression and localization in gynoecia. Further support comes from the fact that the different auxin transport or signaling mutants tested in this work showed a similar sensitivity level for both treatments and the TCS::GFP and DR5::GFP expression pattern, respectively, were also similar for both treatments. Another possibility is that exogenous BAP application affects auxin on more than one action level and that the induced apical-basal gynoecium patterning defects are due to the sum of these changes. Future work should give more insights into the molecular mechanisms.

AUTHOR CONTRIBUTIONS

Victor M. Zúñiga-Mayo and J. Irepan Reyes-Olalde performed experiments; all authors analyzed data; Victor M. Zúñiga-Mayo, Nayelli Marsch-Martinez, and Stefan de Folter drafted the manuscript. All authors provided intellectual content and contributed to manuscript revisions. All authors provided final approval of the manuscript. All authors agree to be accountable for all aspects of the work, including ensuring the accuracy and integrity of the work.

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