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Cytokinin; a developing story

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Abstract

In the past decade, tremendous advance has been made in understanding the biosynthesis, perception and signaling pathways of the plant hormone cytokinin. It also became clear that interfering with any of these steps greatly impacts all stages of growth and development. This has spurted renewed effort recently to understand how cytokinin signaling affects developmental processes. As a result, new insights on the role of cytokinin signaling and the downstream targets during e.g. shoot apical meristem, flower, female gametophyte, stomata and vascular development are being unraveled. In this review, we aim to give a comprehensive overview of recent findings on how cytokinin influences growth and development in plants and highlight areas for future research.

Cytokinins: from signaling to development

Cytokinins, a class of phytohormones with diverse molecular structures [1], were first discovered in the late 1950's while looking for the molecule responsible for the growth-promoting activity of autoclaved herring sperm DNA [2]. Since then, the signaling pathway of cytokinins has been intensely studied. In our current understanding, cytokinins are synthesized by the ISOPENTENYL TRANSFERASE (IPT) and LONELY GUY (LOG) enzymes, whereas cytokinin conjugation act mainly through CYTOKININ OXIDASE (CKX) enzymes. Cytokinins are next perceived by the ARABIDOPSIS HISTIDINE KINASE2-4 (AHK2-4) receptors which initiates a phosphorylation signaling cascade. This phospho-relay starts with auto-phosphorylation of the receptors and will ultimately lead to phosphorylation and activation of B-type ARABIDOPSIS REPSONSE REGULATORS (ARRs) through the ARABIDOPSIS HISTIDINE PROTEINS (AHPs). The active ARRs will then induce cytokinin responsive genes, such as those encoding the cytokinin signaling repressors A-type ARRs or CYTOKININ RESPONSE FACTORS (CRFs). For detailed information on the biosynthesis, transport, perception and signaling cascades of cytokinins, we refer to several excellent recent reviews [1, 3-5]. With the now well studied aspect of cytokinin signaling, the next step is to understand how these processes impinge on plant growth and development. Over the past few years, an increasing number of studies highlight the importance of cytokinins during many developmental processes. In this review, we discuss how these insights aid in understanding how cytokinins controls development at a molecular level.

Shoot development

Already in the early years of cytokinin research, it was clear that these molecules have a big influence on plant growth, especially together with auxin [6, 7]. Plants grown on high levels of auxin and cytokinin massively proliferate and dedifferentiate leading to **callus** (see Glossary). Growing callus on high cytokinin levels induces shoot regeneration [6]. Based on these classical experiments, cytokinins are considered the main class of hormones involved in shoot development.

Cytokinin controls shoot cell fate

Cytokinin signaling was shown to be vital in the context of **shoot apical meristem** (SAM; see Glossary) development (Key Figure 1), being able to induce shoot formation in callus [6, 8]. Under specific conditions, exogenous cytokinins are even able to **trans-differentiate** (see

Glossary) lateral root primordia into a SAM [9, 10]. These observations suggest that cytokinins can specify shoot cell fate starting from different cell types. *In planta*, it has been shown that cytokinins are important for SAM formation as reduced cytokinin levels or signaling results in a smaller SAM [11-13]. Reduction of root derived cytokinin transport by ARABIDOPSIS ATP-BINDING CASSETTE G14 (ABCG14), was shown to be important for correct SAM development [14-16] while confinement of cytokinins within the SAM was suggested to be mediated by PURINE PERMEASE 14 (PUP14) transporters [17]. In the SAM, cytokinins have previously been shown to be positive regulators of *WUSCHEL* (*WUS*) [18], while more recent observations indicated that B-type ARR's directly bind the promoter region of *WUS* and subsequently promote its expression [19-23]. *WUS* is expressed in the organization center of the SAM and is necessary for proper stem cell niche maintenance in the SAM [20]. Both WUS and cytokinin signaling are required during shoot specification and regeneration, as *wus* and *arr1/10/12* mutant explants are unable to form new shoots, whereas other SAM-deficient mutants still have normal or reduced shoot regenerating capacities [19, 21]. Furthermore, WUS miss-expression leads to ectopic SAM formation in roots [24-26] and enables shoot regeneration of callus on hormone free medium [21]. Intriguingly, when treating lateral root primordia with cytokinins, *WUS* expression precedes the morphology change into a SAM [9], suggesting that WUS could be the direct mediator of cytokinin-induced shoot specification downstream of the cytokinin response pathway. This is further supported by the fact that normal cytokinin response seems to be necessary for proper *WUS* expression and that miss-expression of WUS is able to completely rescue the deficient shoot regenerative capacity of the *arr1/arr12* mutant [19].

Flower development

Cytokinins also plays a key role during several stages of flower development (Key Figure 1). For example, in loss-of-function lines of ARR1 and ARR10, **carpel** (see Glossary) regeneration from callus was impaired [27]. These B-type ARRs were shown to bind the *AGAMOUS* (*AG*) promoter region and induce expression of this carpel identity defining gene. Finally, carpel regeneration was impaired in *AG* amiRNA lines, confirming the requirement of this gene during carpel formation. Although these experiments were performed in a carpel-inducing system, they suggest that the cytokinin-dependent control of *AG* expression, through ARR1 and ARR10, might also be functional during normal carpel development [27].

Unlike the SAM, which continuously divides to generate new tissues, flower meristems generate a defined number of flowers and then terminate growth [28]. This determinacy is

impaired in *ag-10* mutants when treated with exogenous cytokinins, creating additional tissues within the carpels [28] with similar phenotypes observed in the *arf3-29* mutant. Under normal conditions, ETTIN/AUXIN RESPONSE FACTOR 3 (ARF3) reduces cytokinin signaling on multiple levels by repressing *IPT*, *LOG* and *AHK* genes [28]. The prolonged *WUS* expression within the *arf3-29* mutant further suggests that ETTIN/ARF3 repression of cytokinin signaling is necessary for flower determinacy, potentially by limiting *WUS* expression.

Later in flower development, during **gynoecium** (see Glossary) development, a maximum of TCS expression (Box 1) suggest the involvement of cytokinin signaling [29, 30] (Key Figure 1). Indeed, the *arr1/10/12* mutant carries fewer ovules, has defects in septum fusion and shows a reduction in transmitting tract tissues [30]. By contrast, plants with increased cytokinin levels show over-proliferation of the medial tissues [29-31]. The bHLH transcription factor *SPATULA* (*SPT*) is expressed in these tissues [30, 32] and loss-of-function phenocopies the *arr1/10/12* mutant. Moreover, SPT influences cytokinin signaling as TCS expression is lowered in the *spt* mutant and increased upon overexpression of *SPT*. This seems to be a direct regulation, as SPT is able to directly bind the *ARR1* promoter and as such induce cytokinin signaling in the medial tissues [30]. Intriguingly, confinement of cytokinin signaling within the medial tissues seems to be required for correct development of the outer tissues. This is achieved by inducing the cytokinin inhibitor AHP6 within these tissues [30, 31, 33], whereas AG represses cytokinin signaling by directly inducing A-type ARRs [34].

The role of cytokinins in gynoecium development has also been shown in other species, such as the dioecius plant *Actinidia*. Here, a male sex determining gene called *SHY GIRL* encodes for a C-type ARR, which acts as a negative regulator of cytokinin signaling. Presence of *SHY GIRL* results in female lethality without affecting male sterility in the flower, leading to dioecius flowers [35]. This example suggests that cytokinins plays important roles during gynoecium development across the plant kingdom.

Female gametophyte development

Within the carpels of the gynoecium, ovules with the **female gametophyte** (see Glossary) will eventually develop. During female gametophyte development, cytokinins plays important roles in cell fate specification (Key Figure 1). Many cytokinin associated genes like *cytokinin insensitive* (cki) single, *arr7/arr15* double and *ahp2-2/ahp3/ahp5-2* triple mutants are female gametophyte lethal [36-38]. *CKI* encodes for a histidine kinase that activates cytokinin response in absence of cytokinins [39]. In the CKI/*cki* loss-of-function mutant, a miss-specification of cell fates occurs with the antipodal and central cells obtaining egg cell fate. The role of cytokinin

signaling is emphasized as the TCS expression (Box 1) in these cells is reduced or absent in the CKI/*cki* and *cki1-9* mutant backgrounds respectively [37]. Given that AHP1, AHP2 and AHP5 act downstream of CKI, the standard cytokinin signaling pathway is used to control cell fate specification [36]. In contrast, overexpression of CKI leads to ectopic TCS expression and specification of the egg cell into a central cell. Upon fertilization, the sperm cell will fuse with this miss-specified egg cell and develop into a diploid endosperm instead of an embryo. Based on these results and the specific expression in the different cell types of the female gametophyte [37], it is clear that CKI is important to provide antipodal and central cell fate, whereas repression of CKI is required for synergid and egg cell specification. Despite these important insights, the actual mechanisms by which CKI provides these cell fates remains unknown.

Female gametophyte development as described above is however not conserved in all plants. For example, although gymnosperms encode a CKI ortholog, they do not contain central cells or endosperm. In *Ginkgo biloba* for example, the CKI ortholog does not fully rescue the arabidopsis (*Arabidopsis thaliana*) *cki* mutant as it was unable to confer central cell specification even though cytokinin signaling was induced. This could suggest that during angiosperm evolution, neofunctionalization of CKI helped the formation of central cells and endosperm establishment [40].

Cytokinin controls cell divisions in leaf epidermis

Similar to the cell division promoting function of cytokinins in many developmental processes [41], this role was recently extended to the leaf epidermis. Cytokinins and SPEECHLESS (SPCH), a transcriptional inducer for asymmetric divisions in the stomatal lineage [42], mutually regulate each other on multiple levels. For example, cytokinins induce expression of SPCH, whereas SPCH induces the A-type ARR16/17 and CLAVATA3/ESR9/10 (CLE9/10) peptides that will reduces cytokinin sensitivity [43] (Key Figure 1). As such, modifying cytokinin levels influences cell proliferation in the stomatal lineage and controls stomata numbers.

Root Development

Besides being the main determinant for shoot development, cytokinins have also been implicated in many aspects of root development. This is for example very clear when looking at the wide range of root-related phenotypes in biosynthesis, perception and signaling mutants

[7]. In this section, we will highlight recent findings exemplifying how cytokinins regulate root development.

Maintaining bilateral symmetry

The arabidopsis root shows a clear **bilateral symmetry** (see Glossary) within the vascular tissues with a central **xylem** axis (see Glossary) flanked by two **phloem** poles (see Glossary) and intervening procambium cells. In this diarch set-up, there is high auxin signaling in the xylem cells, whereas neighboring procambium and phloem cells are typed by high cytokinin signaling. This bilateral character of the vasculature is a consequence of a tight interplay between auxins and cytokinins, as the auxin signaling in xylem cells induces AHP6 which in turn represses cytokinin signaling [44]. Additionally, cytokinin signaling in procambial cells affects auxin efflux through PIN-FORMED (PIN) protein expression and localization [45]. Mathematical modeling suggests that this interplay is sufficient for achieving the bilateral symmetry within the vasculature [46-49] (Key Figure 1). The bilateral symmetry of the arabidopsis root is perhaps most clear in the pericycle, as lateral roots only develop from sets of xylem-pole pericycle cells with high auxin signaling. Here, AHP6 is thought to repress cytokinin signaling [50]. Additionally, cytokinins have a negative effect on lateral root initiation and organization by disturbing PIN protein localization and therefore perturbing local auxin accumulation [50, 51]. This repression of lateral root initiation by cytokinins is also important to achieve a regular spacing between lateral root primordia [52].

Perhaps more surprising is the increasing body of evidence suggesting that bilateral symmetry extends beyond the pericycle into the ground tissue [46, 53, 54]. Endodermal cells at the xylem pole contain **passage cells** (see Glossary), have increased division rates and are much shorter compared to those neighboring the phloem pole [53, 55]. Again in this case, bilateral symmetry is the result of the higher cytokinin levels in the protophloem endodermal cells, whereas AHP6 provides repression of cytokinin signaling in the protoxylem pole endodermis [54]. As such, cytokinins guide correct tissue patterning by generating a bilateral symmetry within the root in concert with auxins.

Vascular development

Cytokinins are tightly linked to vascular development as classical mutants in the signaling pathway such as *wooden leg (ahk4/cre1/wol)* and *ahp6* were identified because of their vascular defects. In the past few years, the heterodimer transcription factor complex formed by the bHLH transcription factors TARGET OF MONOPTEROS 5 (TMO5) and LONESOME HIGHWAY (LHW) emerged as an important regulator of vascular proliferation [46, 56-58]. Loss of

function of these factors results in plants with almost no vascular cell files, whereas ectopic misexpression increases vascular cell file numbers. This suggests that the TMO5/LHW complex is required and sufficient to control radial expansion in at least the embryo and the primary root meristem [46, 56-58]. In the xylem axis, this heterodimer complex directly binds the promoter regions of the cytokinin biosynthesis genes *LOG3* and *LOG4*. The locally produced cytokinins are thought to diffuse to the neighboring procambium cells where it induces cell divisions [46, 56] (Key Figure 1). Given that the vascular bundle size of *lhw* can be rescued by exogenous cytokinin application and radial proliferation is suppressed in the receptor mutant [46], cytokinins seem to be essential for radial expansion of vascular tissues. Indeed, perturbing cytokinin biosynthesis, transport or signaling results in reduced vascular cell file numbers [12, 13, 15, 16, 46, 59, 60]. Yet, the only direct link between cytokinins and cell division so far involves induction of *CYCLIND3;1* and *AINTEGUMENTA* (*ANT*) [61, 62]. In knock-out mutants of these genes, a decrease in vascular bundle size was found. However, cytokinins are still able to increase the vascular bundle size in these mutant backgrounds, suggesting that other factors next to *CYCD3;1* and *ANT* are involved in this process [62].

Besides the effect on radial proliferation, mutants in cytokinin biosynthesis or signaling also show strong patterning defects in which all vascular cells (excluding the pericycle) adopt protoxylem identity [12, 44, 46, 63, 64]. This is very different compared to higher order mutants of TMO5/LHW and in overexpression lines of their SUPPRESSOR OF ACAULIS5-LIKE (SACL) repressors, as these plant lines still contain all cell types, although in lower numbers [65, 66]. Additionally, increasing cytokinin levels by exogenous cytokinin treatments or removing the AHP6 repression leads to a complete or partial loss of protoxylem differentiation [44]. Taken together, cytokinins are tightly associated with vascular development as they influence radial proliferation, radial patterning and xylem differentiation processes. Yet, it remains unclear how cytokinins themselves control these different developmental processes in space and time at a molecular level.

Root meristem size determination

Besides a role in controlling radial patterning and proliferation, cytokinins also regulate root development in a longitudinal sense. In the root, cytokinins control root meristem size by limiting auxin activity in the transition zone. Here, cytokinins repress auxin activity through direct induction of *SHY2/IAA3* by ARR1 and ARR12. SHY2/IAA3 acts as a repressor of ARF activity and negatively regulate PIN proteins leading towards auxin redistribution [67-69]. Auxins themselves promote the degradation SHY2/IAA3, which activates auxin signaling [68].

This intricate interplay between auxins and cytokinins results in the creation of a distinct zone with low auxin signaling in the transition zone. The position of this auxin minimum was suggested to define the boundary between dividing and differentiating cells and as such controls the size of the root meristem [70].

Root nodule formation

Plant-microbe interactions between plants from the *Fabaceae* family and nitrogen fixating bacteria lead to the formation of specialized plant structures called root nodules and are strongly linked with cytokinin signaling. In *Medicago truncatula* and *Lotus japonica*, inoculation of nitrogen fixating bacteria leads to an increase in cytokinin biosynthesis and signaling within the affected roots [71-74]. In *Lotus japonica, lhk1-1 lhk1a-1 lhk3-1* triple receptor mutants show hyper infection after *Mesorhizobium loti* inoculation but the infection threads are not able to develop into nodule primordia [75]. This suggests that cytokinins might not be strongly involved in the infection event, but rather play important roles during root nodule development. However, some nodulation factors like MtNSP are shown to be induced downstream of cytokinins [76], implying the necessity of cytokinin signaling for nodulation. Moreover, overexpressing cytokinin biosynthesis genes also leads to root nodule formation in absence of nitrogen fixating bacteria, suggesting that cytokinin signaling is necessary and sufficient for normal development of root nodules [74].

Concluding remarks and future perspectives

In recent years, we gained new insights into how cytokinins influence key developmental processes. Although great advances have been made, the real challenge will be to understand how cytokinins regulate these developmental processes at a molecular level. For example, regulation of *WUS* and *AG* expression levels through specific B-type ARRs explains how cytokinins can promote shoot formation [19-23, 27]. However, examples of these direct links to key developmental regulators are still very scarce. Yet, understanding these connections will be vital to grasp how cytokinins control plant development. More specifically, similar to other plant hormones, a major hurdle is to understand how cytokinins are able to regulate very distinct processes throughout development. Indeed, besides promoting shoot formation, cytokinins are also involved in vascular proliferation, female gametophyte development, lateral root development and many other processes discussed above. To achieve this, cytokinins must be able to regulate different subsets of genes under different conditions. Part of this specificity might be brought about via the large diversity of the cytokinin signaling cascade components.

Currently, three receptors, five AHP's, eleven A-type ARRs, ten B-type ARRs and three C-type ARRs have been identified. Moreover, these ten different B-type ARRs can provide further selectivity due to their expression domain or binding specificity to certain promoter sequences. Indeed, some but not all B-type ARRs can rescue the *arr1;arr12* double mutant [77], hinting towards some specificity among the B-type ARRs. Although, major leaps forward were made to chart the direct downstream targets of mainly ARR1, ARR10 and ARR12 [22, 23, 78], recently, the targets of other B-type ARRs have not been studied as extensively. These might be a good starting point to unravel the complex downstream regulatory pathways and the observed specificity (see Outstanding Questions Box). The presence of other transcriptional regulators that co-regulate the cytokinin response could also partly explain how diverse cytokinin responses can be activated. For example, BARLEY B-RECOMBINANT/BASIC PENTACYSTEINE (BBR/BPC) or CYTOKININ RESPONSE FACTOR (CRF) gene families has been shown to induce a subset of cytokinin responsive genes [79, 80]. Additionally, the use of molecular tools including CKI, SRDX and EAR motifs in combination with tissue specific promoters could be useful to study the role cytokinin signaling in specific developmental processes. In summary, elucidating how cytokinins are able to induce specific sets of genes in different processes at certain moments in development will be key in understanding the ability of phytohormones to guide plant development in general.

As we slowly start to unravel how cytokinins guide developmental processes, it becomes clear that cytokinins by themselves are not sufficient for the formation of complex tissues. Indeed, as postulated decades ago, the specific interplay between cytokinins and other phytohormones emerges as an essential property for normal plant development. For example, the intimate interplay between auxins and cytokinins remains enigmatic for plant development. Indeed, ever since the classical experiments by Skoog and Miller [6], the important interaction between auxins and cytokinins have been extensively studied in many developmental systems [7, 33, 47, 70, 81]. Although the functional importance of interactions with other hormones is less established at the moment [82], it is very likely that also these interactions will be crucial for a holistic understanding of plant development (see Outstanding Questions Box).

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Glossary

Bilateral symmetry: One of many forms of symmetry found in plants in which two halves are the exact mirrors of each other.

Callus: Undifferentiated pluripotent cells induced by high auxin and cytokinin levels. Callus is able to differentiate into most plant tissues and transformation of most plant species is still based on an intermediate callus step.

Carpel: Flower organ at the outer part of the gynoecium, formed by fused leaf-like structures.

Female gametophyte: Part of the flower in which the egg cell resides and where the double fertilization takes place.

Gynoecium: The entire female reproductive organ.

Passage cells: Non-suberized cells in the otherwise impermeable suberized endodermis that are hypothesized to allow lateral transport through the Casparian strip**.**

Shoot apical meristem: Self-organizing population of cells that forms all shoot tissues. The shoot apical meristem comprises of a non-dividing organizing center and surrounding stem cells that actively divide to create shoot tissues.

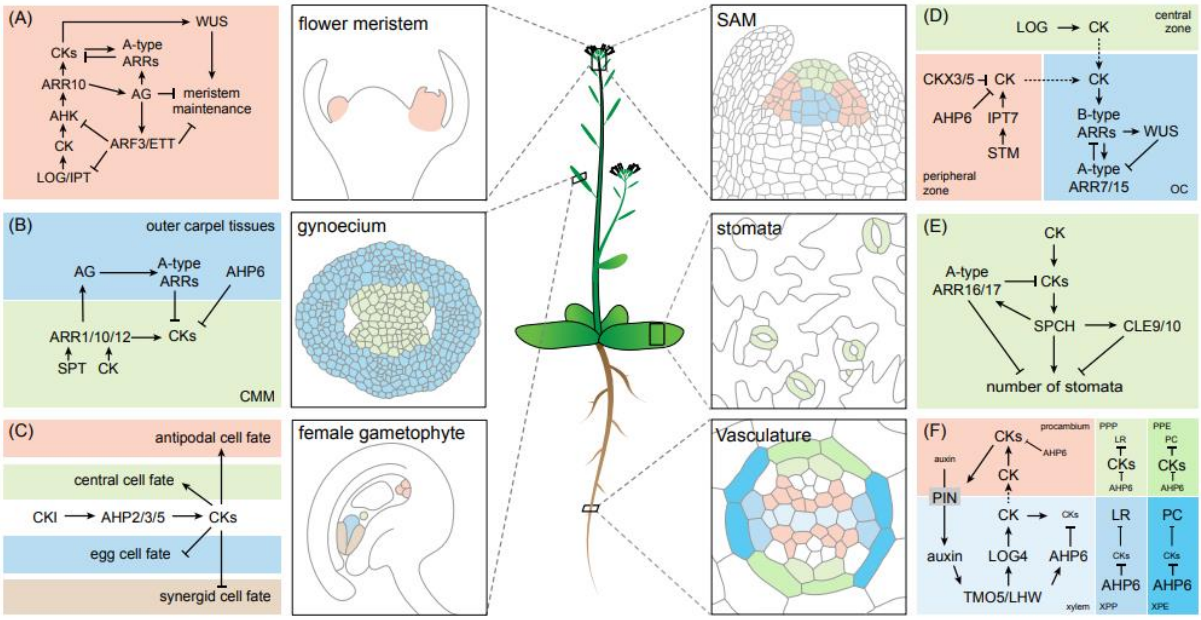
Trans-differentiation: the unique ability of differentiated plant cells to first dedifferentiate and next differentiate into another cell fate.

Xylem and Phloem: Vascular cell types that transport water, sugars and other nutrients and signaling molecules throughout the plant. Generally speaking, xylem and phloem respectively provide shootward and rootward transport.

Box 1. Seeing is believing: Visualizing cytokinins in plant development

Spatiotemporal signals direct plant cells towards specific developmental paths. Despite the very diverse nature of these signals, a solid *in vivo* visualization is in each case instrumental in understanding how and where these cues are regulating plant development. This has been clearly exemplified by the enormous impact of the heavily used auxin signaling reporters, including DR5, DR5v2 and R2D2 [83-85], on our understanding of the auxin signaling pathway. Also for visualizing cytokinin signaling, the TCSn reporter line has been a huge leap forward [9, 33, 37, 46, 52]. The TCSn promoter comprises 12 cytokinin responsive cis regulatory motifs, 5′-(A/G)GAT(C/T)-3′, present in the cytokinin responsive genes A-type

ARRs [86, 87]. This reporter gave researchers a tool to study cytokinin signaling during developmental processes in a spatial and temporal manner, many of which are mentioned in this review. The main limitation of the DR5 and TCS type of reporters is that they do not visualize actual cytokinin levels, but the responses. Cytokinin levels can be directly measured by using mass spectrometry with cellular resolution by using cell type specific markers combined with cell sorting [88]. However, these experiments are technically challenging and the specificity is limited by the availability of cell specific reporter lines. Alternatively, bioactive fluorescent labeled cytokinin species have been generated [89]. However, their use to study plant development is limited as they have to be implemented in the growth medium and do not take local production and degradation into account. In summary, although direct detection or visualization of cytokinins themselves is feasible (see Outstanding Questions Box), the TCSn reporter remains the most accessible and widely used tool to study the effect of cytokinin on plant development.



Key Figure 1. Schematic overview of key developmental processes influenced by cytokinin.

(A) Cytokinins induce expression of *AG* that together with ARF3/ETT will repress cytokinin signaling. This leads to decreased cytokinin levels in flower meristem and ultimately ensures meristem termination. **(B)** Interactions between the CMM and outer carpel tissues that helps with the restriction of cytokinin signaling to the CMM. **(C)** The effect of CKI and cytokinin signaling on cell fate specification in the female gametophyte. **(D)** Cytokinins made in the peripheral and central zone lead to activation of B-type ARRs in the organization center and induction of *WUS* expression which controls the shoot stem cell niche. **(E)** Induction of SPCH by cytokinins helps to promote stomatal lineage cell divisions, whereas SPCH reduces cytokinin sensitivity and dividing potential through ARR16/17 and CLE9/10. **(F)** Network generating zones of high auxin signaling in xylem cells and high cytokinin signaling in procambium cells. This also contributes to a bilateral symmetry that extends to the surrounding pericycle and endodermal cells by controlling lateral root and passage cells specification. **Abbreviations:** CK: cytokinins; CKs: cytokinin signaling; CMM: carpel marginal meristem; LR: lateral root; OC: organizing center; PC: passage cells; PPP: phloem pole pericycle; PPE: phloem pole endodermis; XPP: xylem pole pericycle; XPE: xylem pole endodermis.