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REVIEW

The Yin-Yang of Hormones: Cytokinin and Auxin Interactions in Plant Development

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The phytohormones auxin and cytokinin interact to regulate many plant growth and developmental processes. Elements involved in the biosynthesis, inactivation, transport, perception, and signaling of these hormones have been elucidated, revealing the variety of mechanisms by which signal output from these pathways can be regulated. Recent studies shed light on how these hormones interact with each other to promote and maintain plant growth and development. In this review, we focus on the interaction of auxin and cytokinin in several developmental contexts, including its role in regulating apical meristems, the patterning of the root, the development of the gynoecium and female gametophyte, and organogenesis and phyllotaxy in the shoot.

INTRODUCTION

From their initial discovery as inducers of plant cell division in culture, cytokinin function has been linked to that of auxin (Miller et al., 1955, 1956). Subsequent studies revealed a plethora of processes regulated by their mutual control. In many cases, the regulatory interactions between these two hormones is characterized as antagonistic; however, the Chinese concept of yin-yang is probably more accurate, reminding us that they are complementary, rather than opposing forces. Indeed, the English translation of yin as "shady side" and yang as "sunny side" readily brings to mind plants and their integral relationship with light and shadow. As suggested by yin-yang, auxin and cytokinin act together dynamically, with roles that can be paradoxically antagonistic and supportive, to provide robustness to developmental processes and to confer distinct cell fates to precursor cells in close proximity, yielding a whole that is greater than the sum of its parts.

Recent studies have begun to shed light on the molecular mechanisms underlying auxin/cytokinin crosstalk at the levels of biosynthesis, degradation, transport, and signaling (Pernisová et al., 2011; Vanstraelen and Benková, 2012; El-Showk et al., 2013). Here, we review these recent studies together with some earlier work, focusing on the regulatory interactions that control growth and development. The literature on these two hormones is so extensive that an encompassing review is impossible, and we refer readers to excellent reviews on topics absent or only briefly touched on here (Argueso et al., 2009; Bishopp et al., 2011a; Domagalska and Leyser, 2011; Su et al., 2011; Hwang et al., 2012; Naseem and Dandekar, 2012; Del Bianco et al., 2013; El-Showk et al., 2013; O'Brien and Benková, 2013; Kieber and Schaller, 2014; Schaller et al., 2014; Zhao, 2014). We begin with an overview of cytokinin and auxin metabolism and signaling and then consider their interactions in controlling the development and growth of roots and shoots.

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METABOLISM, TRANSPORT, AND SIGNALING

Auxins

Recent studies suggest that the most abundant auxin, indole-3-acetic acid (IAA), is primarily synthesized in a two-step process from the amino acid tryptophan (Zhao, 2014) (Figure 1). In the first step, tryptophan is converted to indole-3-pyruvate by the TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) family of transaminases (Stepanova et al., 2008; Tao et al., 2008). Indole-3-pyruvate is then converted to IAA by the YUCCA family of flavin monooxygenases. Both the YUCCA and TAA enzymes are encoded by multigene families that are essential for plant development (Zhao, 2014). Intriguingly, the *YUCCA* and *TAA* genes display discrete, localized expression patterns, suggesting that in at least some cases, auxin biosynthesis is highly localized, which likely helps generate the precise spatial distributions of auxin involved in various developmental processes. There is evidence for other pathways of auxin biosynthesis, in particular a Trp-independent pathway, which may play a prominent role in some tissues (Tivendale et al., 2014). IBA is also a source of auxin as it can be converted to IAA via β-oxidation in the peroxisomes and likely serves as a source of auxin for specific processes (Strader and Bartel, 2011).

Auxin is transported throughout the plant in a polar manner by the PIN-FORMED (PIN) family of auxin efflux carriers (Zazímalová et al., 2010; Ljung, 2013; Adamowski and Friml, 2015). The pattern

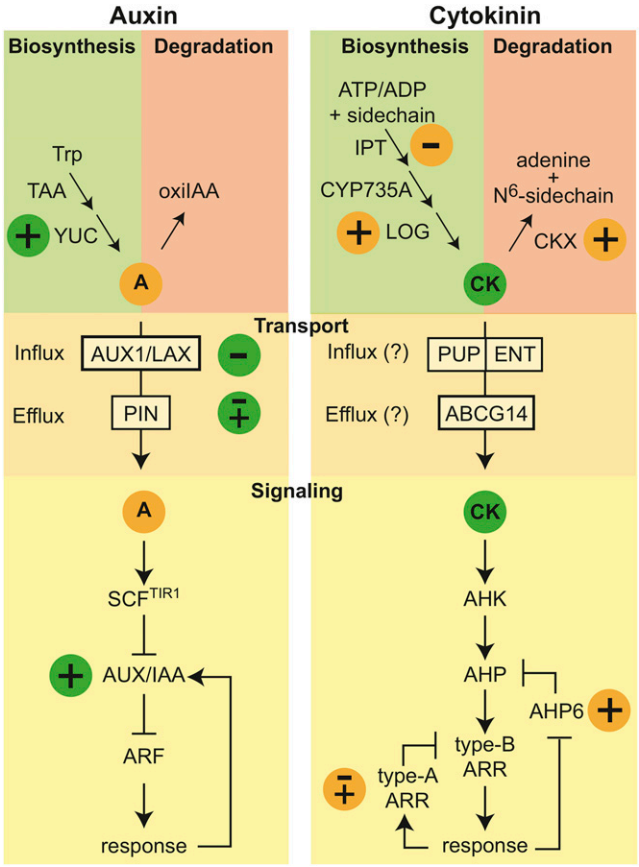

Figure 1. Key Elements of Auxin and Cytokinin Pathways.

Elements of the auxin and cytokinin pathways involved in biosynthesis, degradation, transport, and signaling are shown. Points of activating (+) or suppressing (−) crosstalk for the respective pathways are indicated by orange circles (auxin) or green circles (cytokinin). See text for additional details.

of expression of the various PIN genes and the localization of the PIN proteins on specific cell faces play the major role in determining the distribution of auxin in plant tissues. Auxin is transported into cells by the AUX/LAX family of proteins (Swarup and Péret, 2012). The MULTIDRUG RESISTANCE (MDR)-*p*-glycoprotein (PGP) family of proteins also plays a role in auxin transport and likely act in concert with the PINs to regulate the distribution of auxin (Geisler and Murphy, 2006). Unlike the polarly localized PINs, the MDR/PGP proteins are uniformly localized in the cell. The net effect of the distribution of these IAA influx and efflux carriers, coupled with localized biosynthesis, is to generate auxin asymmetries that regulate cell differentiation and division during plant growth and development. These cell-to-cell differences in auxin concentration regulate myriad developmental responses and have been the source of many experimental and theoretical studies. Initial modeling studies have confirmed that the spatial distribution of PIN transporters observed in the shoot and root meristems are sufficient to generate the auxin patterns observed there (de Reuille et al., 2006; Grieneisen et al., 2007); more recent studies have provided new insights into how auxin transport itself is regulated in these and other tissues (van Berkel et al., 2013; Goh et al., 2014).

Auxin levels can also be regulated through conjugation (Ludwig-Müller, 2011; Ljung, 2013). Auxin is inactivated by conjugation to sugars and to various amino acids, some of which can be hydrolyzed to yield active IAA by auxin amino acid conjugate hydrolases. The auxin-inducible GH3 family of acyl acid amido synthetases catalyzes the conjugation of amino acids to IAA (Staswick et al., 2005). Active IAA levels can also be decreased by degradation of IAA to 2-oxoindole-3-acetic acid (Kai et al., 2007), though the enzyme(s) catalyzing this reaction has not yet been identified.

The perception of auxin involves the Aux/IAA family of transcriptional repressors, the auxin response factor (ARF) transcription factors, and the TIR1/AFB1-AFB5 F-box components of the SCF complex (Peer, 2013; Salehin et al., 2015). In the presence of low levels of auxin, the Aux/IAA proteins, together with the TOPLESS transcriptional repressor, bind to the ARFs to block their function (Szemenyei et al., 2008). In the presence of elevated auxin, the Aux/IAA proteins form a complex with the TIR1/AFB1-AFB5 proteins, with IAA sandwiched between the proteins, acting as "molecular glue" to hold the components of the coreceptor together (Calderon-Villalobos et al., 2010). The formation of this complex results in the ubiquitination of the Aux/IAA proteins and their subsequent degradation by the 26S proteasome, thus relieving their repression of the ARFs (Ulmasov et al., 1999). The activated ARFs then modulate the expression of a large suite of auxin-regulated genes through their binding to auxin response elements (AuxREs) (Ulmasov et al., 1999). There are a large number of *Aux/IAA*, *ARF*, and *TIR* genes in *Arabidopsis thaliana* (29, 23, and 5, respectively), which provides an extremely large repertoire to modulate cellular responses to auxin. The AUXIN BINDING PROTEIN1, which is primarily localized to the endoplasmic reticulum (ER), has been implicated in an independent, non-transcriptional auxin signaling pathway, acting primarily in cell elongation and cell division (Sauer and Kleine-Vehn, 2011; Xu et al., 2014).

Cytokinins

The first step in the biosynthesis of cytokinin is the addition of a prenyl moiety from dimethylallyl diphosphate to ATP/ADP to yield N^6-isopentenyladenine (iP) ribotides, which is catalyzed by the enzyme isopentenyltransferase (IPT) (Sakakibara, 2006) (Figure 1). The *Arabidopsis* genome encodes nine *IPT* genes, designated as *IPT1* to *IPT9*, seven of which are involved in the biosynthesis of cytokinin, the other two acting to modify a subset of adenine bases on tRNA (Kakimoto, 2001; Takei et al., 2001). The iP ribotides made by IPT can be subsequently converted to *trans*-zeatin (*tZ*)-type cytokinins by hydroxylation of the isoprenoid side chain by the cytochrome P450 enzymes CYP735A1/CYP735A2 (Takei et al., 2004). The active forms of cytokinins are the free bases, which are made from cytokinin ribotides in a single enzymatic step catalyzed by the LONELY GUY (LOG) family of cytokinin nucleoside 5′ monophosphate phosphoribohydrolases (Kuroha et al., 2009).

The level of active cytokinin can be decreased through conjugation to glucose (Bajguz and Piotrowska, 2009). Glucosyl conjugates are inactive in bioassays, and these conjugated

cytokinins also fail to bind to the *Arabidopsis* cytokinin receptors (Spíchal et al., 2004). *N*-glycosylation of cytokinin occurs primarily at positions N^7 or N^9 of the purine ring and is thought to be irreversible (Hou et al., 2004). *O*-glycosylation, which is likely reversible, occurs at the oxygen on the side chains of zeatin or dihydrozeatin (Mok and Mok, 2001). Cytokinin levels are also regulated by cytokinin oxidases, copper-dependent amine oxidase enzymes that cleave the N^6-side chains from *tZ*- and iP-type cytokinins, thus irreversibly inactivating them (Schmülling et al., 2003; Werner et al., 2006). In most plant species, cytokinin oxidase is encoded by a multigene family whose members show distinct patterns of expression, intracellular locations, and enzymatic properties (Schmülling et al., 2003; Kowalska et al., 2010). Several genes encoding cytokinin oxidases are induced rapidly upon cytokinin and auxin treatment in rice (*Oryza sativa*) and *Arabidopsis* (Tsai et al., 2012; Bhargava et al., 2013; Gao et al., 2014).

The cytokinin signal transduction pathway is similar to bacterial two-component phosphorelays and involves three components: a "hybrid" receptor kinase that contains both histidine-kinase and receiver domains in one protein, a histidine-containing phosphotransfer (AHP) protein, and a separate RR (Stock et al., 2000; Müller, 2011; Schaller et al., 2011; Heyl et al., 2012; Hwang et al., 2012; Kieber and Schaller, 2014). In these multistep phosphorelays, the phosphate is transferred from amino acid to amino acid in sequence His to Asp to His to Asp.

The cytokinin signaling elements in *Arabidopsis*, rice, and other plants are encoded by small gene families whose members generally have overlapping function (Pareek et al., 2006; Du et al., 2007; Pils and Heyl, 2009). *Arabidopsis* has three cytokinin receptors (AHK2, AHK3, and CRE1/WOL/AHK4) that contain a conserved cytokinin binding CHASE domain, a histidine kinase domain, and a receiver domain (Inoue et al., 2001; Higuchi et al., 2004; Nishimura et al., 2004). The *Arabidopsis* AHK receptors play positive, partially redundant roles in cytokinin responses (Inoue et al., 2001; Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006). Cytokinin receptors are primarily located in the ER membrane in plants with the CHASE domain oriented into the ER, suggesting that the principal site of cytokinin binding in planta is in the lumen of the ER (Caesar et al., 2011; Lomin et al., 2011; Wulfetange et al., 2011).

The transporters that move cytokinin across the plasma membrane and into the ER have not as yet been compellingly demonstrated, though several purine permeases and equilibrative nucleoside transporter proteins have been implicated in transporting cytokinin (Bürkle et al., 2003; Sun et al., 2005; Hirose et al., 2008). Furthermore, an ATP binding cassette transporter (ABCG14) has been shown to be necessary for the movement of cytokinin, primarily *trans*-zeatin forms, from roots to shoots (Ko et al., 2014; Zhang et al., 2014).

The AHPs are the downstream targets of the AHK cytokinin receptors and act as intermediates in the transfer of the phosphate to the downstream response regulators (RRs) (Suzuki et al., 1998; Tanaka et al., 2004; Hutchison et al., 2006). There are five functional AHPs in *Arabidopsis* (AHP1-AHP5), with a sixth that is missing the His target of phosphorylation (AHP6). AHP1, AHP2, AHP3, and AHP5 are positive, partially redundant elements in cytokinin signaling (Hutchison et al., 2006; Hutchison and Kieber, 2007). In contrast, AHP6 negatively regulates cytokinin responsiveness, at least in part via an inhibition of the phosphotransfer reaction among the functional two-component elements (Mähönen et al., 2006). The AHPs are actively transported in and out of the nucleus, independent of their phosphorylation status or of cytokinin levels (Punwani et al., 2010; Punwani and Kieber, 2010), and could thus transfer phosphates either to or from the AHKs and the RRs.

The downstream targets of the AHPs, the RRs, fall into two main classes called type-A and type-B RRs. In both classes, an N-terminal receiver domain harbors an Asp residue that is the target of phosphotransfer. The type-B RRs are transcription factors that contain a DNA binding Myb domain. There are multiple type-B ARRs in *Arabidopsis* that act as partially redundant, positive elements that control the transcriptional response to cytokinin (Hwang and Sheen, 2001; Sakai et al., 2001; Mason et al., 2004). The protein levels of at least a subset of the type-B RRs (ARR1, ARR2, and ARR12) are regulated by the KMD family of F-box proteins (Kim et al., 2013). The type-A ARRs lack a DNA binding domain and negatively regulate cytokinin signaling (Hwang and Sheen, 2001; To et al., 2004). In contrast to the type-B ARRs, the type-A ARRs are rapidly induced by cytokinin treatment, both at the transcriptional (Brandstatter and Kieber, 1998; D'Agostino et al., 2000; Jain et al., 2006; Tsai et al., 2012) and in some cases the posttranscriptional levels (To et al., 2004; Ren et al., 2009). The type-A ARRs function as negative feedback regulators in cytokinin signaling.

These various elements of auxin and cytokinin biosynthesis, metabolism, and signaling represent potential points of interaction between these two phytohormones (Figure 1) (El-Showk et al., 2013). One major advance facilitating the identification of new interactions between these hormones is the development of synthetic sensors for both auxin and cytokinin. At their simplest, these involve concatemerized repeats of the known AuxRE binding sites to provide the synthetic DR5 reporter for auxin (Ulmasov et al., 1997) and a typical type-B ARR binding site to provide the synthetic TCS reporter for cytokinin (Müller and Sheen, 2008). Over the years these have been modified, in the case of DR5 by reversing this element to produce DR5rev (Benková et al., 2003) and by refining the sequences of TCS based on up-to-date binding information to give TCSnew (Zürcher et al., 2013). While these reporters have been used extensively, there are some limitations with their use. Extensive analysis in the embryo and elsewhere has shown that there is a strong cell-type dependence of auxin response and that this is controlled in part through different expression patterns of ARF transcription factors mediating cell-type-specific gene responses (Rademacher et al., 2012). Systematic comparison of the binding motifs of ARF1 and ARF5 using a protein binding microarrays identified a new response element that is strongly preferred by these proteins (TGTCGG) rather than the canonical sequence used in DR5. However, it was found that ARF1 and ARF5 show strong differences in their ability to bind sequences in which AuxREs pairs are spaced with differing sizes of intervening sequence between them (Boer et al., 2014). Collectively, these data suggest that reliance on the DR5 promoter may give a misguided impression of the complexity and specificity of auxin response. As an alternative system, an independent auxin sensor (DII-venus) has been developed based on the domain II of an AUX/IAA fused to yellow fluorescent protein (Brunoud et al., 2012). This is expressed under a fairly

constitutive promoter and is degraded in an auxin-dependent manner, providing a rapid, inverse auxin sensor that does not rely on transcription.

Recent studies using these sensors and the genes encoding the various signaling elements and metabolic enzymes have shed light on how cytokinin influences multiple aspects of auxin function and vice versa to achieve specific cellular responses.

AUXIN/CYTOKININ INTERACTIONS BELOW GROUND

Formation and Maintenance of the Root Apical Meristem

Root growth is driven by a group of undifferentiated cells in a region of the root known as the root apical meristem (RAM). Within this meristem, stem cells self-renew and produce daughter cells, which then differentiate with specific cell identities that pattern the root. In plants with closed meristems (such as *Arabidopsis*), a group of initial stem cells surround a small group of mitotically less active cells, known as the quiescent center (QC), which together make up the stem cell niche (Dolan et al., 1993). The QC produces non-autonomous signals that prevent differentiation of the surrounding initial cells (van den Berg et al., 1997). Auxin and cytokinin control almost every aspect of the activity of this meristem.

The root meristem first forms at the globular stage of embryogenesis when a cell at the base of the embryo called the hypophysis undergoes an asymmetric cell division (Figure 2). This cell division is a major event in patterning a stem cell niche in the root, as the upper lens-shaped cell goes on to become the QC and the larger basal cell gives rise to the columella (Laux et al., 2004). An elevated cytokinin response occurs in the hypophysis, as revealed by the expression of the cytokinin reporter TCS:GFP (green fluorescent protein). However, following the division of the hypophyseal cell, the cytokinin response was maintained in the apical, lens-shaped cell but repressed in this basal cell (Müller and Sheen, 2008) (Figure 2). When Müller and Sheen (2008) examined the expression of type-A ARRs (direct targets of the cytokinin response pathway that are often used as markers of cytokinin signaling), they made a surprising observation. Rather than observing high *ARR7* and *ARR15* expression in the apical, lens-shaped cell, where they noted a high cytokinin response as reported by TCS:GFP, they observed that *ARR7* and *ARR15* expression was substantially higher in the larger basal cell, suggesting that another factor may regulate *ARR7/15* expression.

This factor turned out to be auxin. Using the synthetic auxin reporter DR5:GFP, they observed high auxin output in the basal cell (Figure 2). A detailed analysis of the *ARR7/15* promoters and a series of genetic manipulations demonstrated that auxin signaling directly activated the transcription of *ARR7* and *ARR15*, their induction potentially serving to antagonize and reduce the cytokinin response in the basal cell. Additional analyses using constitutively active and dominant repressor versions of the type-B response regulator ARR10 confirmed a role for cytokinin signaling in embryogenesis. These results provided the first identification of a node of interaction between auxin and cytokinin signaling pathways. However, the degree to which ARR7/15 control root embryogenesis is not clear. Müller and Sheen observed altered root stem cells and aborted embryogenesis when an inducible RNA interference construct against *ARR7* was expressed in an *arr15* background, but subsequent analysis of an *arr7 arr15* double mutant revealed at most minimal effects on plant growth, including root development (Zhang et al., 2011). Thus, the effects of auxin on *ARR7* and *ARR15* expression alone are insufficient to mediate a regulatory role by auxin in formation of the root meristem; instead, rather like the proverbial canary in the coal mine, these effects on expression may signal more profound alterations in cytokinin regulation that have so far remained elusive. For example, auxin may also suppress expression of additional type-A *ARR*s or regulate the degradation of the type-A or type-B ARRs.

Postembryonic root growth is regulated by the activity of the root apical meristem. Three discrete spatial domains characterize the growing root. The proximal meristem lies closest to the QC, and within this domain, daughter cells undergo additional rounds of cell division. As cells exit the meristem they enter the transition zone (TZ) where they stop dividing before undergoing a period of rapid expansion and differentiating in the elongation/differentiation zone. The balance between the rate of cell division and differentiation is essential to allow continuous root growth and to maintain an appropriately sized meristem. Auxin and cytokinin lie at the very core of a mechanism controlling this balance.

Treatments with either auxin or cytokinin have significant effects on meristem size (Beemster and Baskin, 2000): Cytokinin application reduces the size of meristems and promotes cell differentiation in the TZ (Dello Ioio et al., 2007); treatment with auxin increases meristem size and promotes cell division in the proximal meristem (Blilou et al., 2005a). Analysis of type-B *ARR* mutants reveals that this process is under transcriptional control by cytokinin, with ARR1, ARR10, and ARR12 playing the largest role and ARR2 and ARR11 contributing to a lesser extent (Dello Ioio et al., 2008; Moubayidin et al., 2010; Hill et al., 2013). Not surprisingly, given the significance of the meristem to root growth, multiple points of interaction between cytokinin and auxin have been identified in the control of RAM size. The Aux/IAA protein SHY2 acts as a central switch in a mechanism controlling the balance of auxin and cytokinin signaling. Auxin switches off SHY2 by promoting its degradation via the SCF^{TIR1} complex (Tian et al., 2002; Dharmasiri et al., 2003); cytokinin induces SHY2 in the TZ via a direct activation of transcription by type-B ARRs (Dello Ioio et al., 2008; Moubayidin et al., 2010). In addition to modulating auxin responses, SHY2 also promotes cytokinin biosynthesis by elevating expression of *IPT5*. Together, these mechanisms provide a framework in which cytokinin reduces auxin output (via SHY2) and redistributes auxin, leading to cell differentiation in other tissues. Auxin in turn promotes SHY2 degradation to maintain an auxin response, PIN activity, and cell division.

The growth of a meristem over time is a dynamic process and the rates of cell division and differentiation change over time. Similarly, the expression levels of the type-B *ARR*s also vary over time (Dello Ioio et al., 2008; Moubayidin et al., 2010; Hill et al., 2013). The relative rate of cell division to differentiation is highest in the first 3 d after germination when cell division is needed to establish the final meristem size. While ARR1 is instrumental in maintaining the final meristem size, it has little effect during this early establishment of the meristem as its expression is suppressed by gibberellins during the early stages of growth (Moubayidin et al., 2010). During this period, ARR12, another type-B ARR, functions to promote a low level of

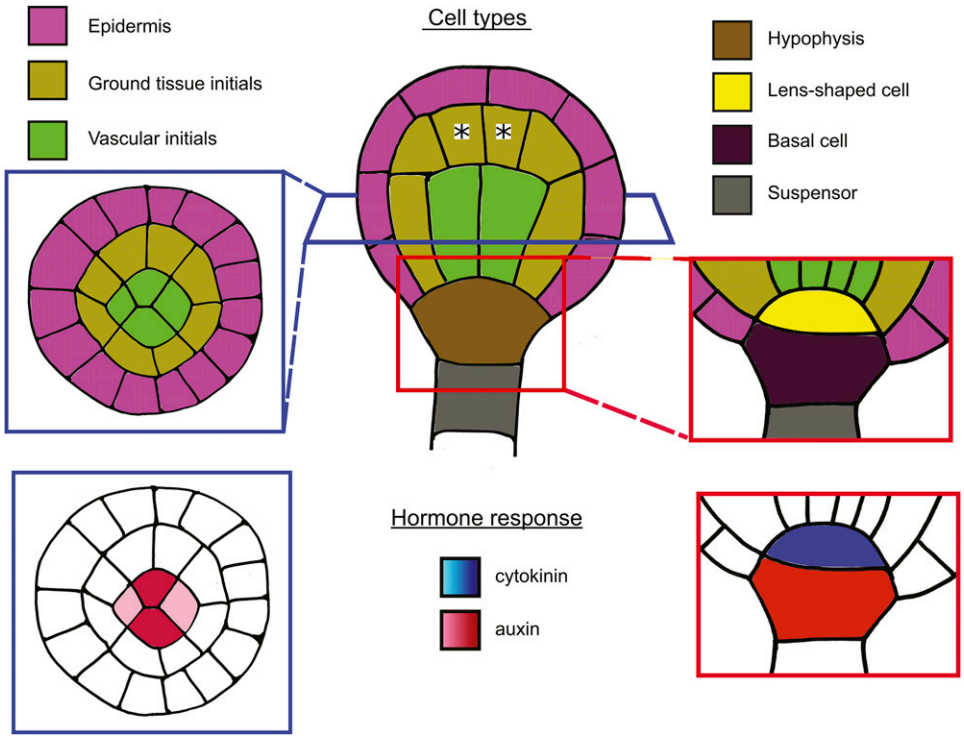

Figure 2. Cell Patterning in the Globular Stage Embryo.

The top images depict the cell types in early embryos with the colors corresponding to the indicated cell type. The upper image to the left shows a cross section through the embryo, and the four vascular initial cells represent a template similar to that used previously (De Rybel et al., 2014). The upper image to the right shows the asymmetric cell division of the hypophysis to generate the upper lens-shaped cell and the larger basal cell. This was shown to be controlled by both auxin and cytokinin (Müller and Sheen, 2008). The asterisks show the WUS-expressing domain in the embryo. The lower two images depict the relative cytokinin and auxin concentrations shaded in the indicated colors (Müller and Sheen, 2008; De Rybel et al., 2014). Note that in the cross section of the embryo, cytokinin activity is not depicted as this has not as yet been determined.

SHY2 (Moubayidin et al., 2010). SHY2 levels only reach sufficiently high levels to favor differentiation when gibberellin signaling is decreased to allow *ARR1* expression at a sufficient distance from the organizing center.

Cytokinins regulate auxin activity through other points of cross-talk in addition to the control of *SHY2* expression. For instance, cytokinins regulate auxin transport by inhibiting the endocytic recycling of the auxin efflux carrier PIN1, promoting its degradation in the vacuole (Marhavý et al., 2011). This regulation of PIN1 trafficking is independent of the type-B ARRs. Furthermore, ARR12 functions in conjunction with the RETINOBLASTOMA-RELATED protein to stimulate expression of *ARF19*, a transcriptional regulator of the auxin response (Perilli et al., 2013). *ARF19* usually functions as a positive regulator of auxin responses, but nevertheless negatively regulates root meristem size.

Similar to its role in the embryonic root, cytokinin also plays a key role in regulating the function of the QC in postembryonic roots, including the primary and adventitious roots (Della Rovere et al., 2013; Moubayidin et al., 2013; Zhang et al., 2013). Application of exogenous cytokinin or disruption of multiple type-A ARR genes, which negatively regulate cytokinin signaling, leads to loss of QC identity as measured by reactivation of cell division, reduced QC marker gene expression, and differentiation of the adjacent columella cells (Zhang et al., 2011, 2013). Consistent with this, expression of *WOX5*, a transcription factor required for the QC activity to maintain the undifferentiated state of adjacent cells in the stem cell niche (Sarkar et al., 2007), is repressed by elevated cytokinin (Zhang et al., 2013). Furthermore, the SCARECROW (SCR) transcription factor directly represses the expression of *ARR1*, a type-B ARR, in the QC cells (Moubayidin et al., 2013). Thus, the type-A ARRs and SCR act to repress cytokinin function in the QC, which is essential for its proper function. The role of cytokinin in the QC is mediated in part through multiple effects on auxin biosynthesis and transport. First, cytokinin downregulates the expression of the auxin influx carrier *LAX2*. The repression of *LAX2* requires ARR1 and ARR12 and occurs through direct binding of these type-B ARRs to the regulatory region of *LAX2* (Zhang et al., 2013). Disruption of *LAX2* phenocopies the effects of elevated cytokinin on the QC, indicating that reduced LAX2 is a key mediator of the effect of cytokinin on QC function (Zhang et al., 2013). Second, the suppression of *ARR1* by SCR in the QC regulates auxin biosynthesis (Moubayidin et al., 2013); in *scr* mutants, auxin levels are abnormally high, and this is suppressed by disruption of *ARR1*, indicating that ARR1 increases auxin biosynthesis in the root tip, but this effect is counterbalanced in the QC by repression of ARR1 by SCR. Finally, elevated cytokinin

function, through either disruption of the type-A ARRs or application of exogenous cytokinin, alters the level of the PIN auxin efflux carriers, primarily via a posttranscriptional mechanism, resulting in a change in the distribution of auxin in the stem cell niche of the root (Ruzicka et al., 2009; Zhang et al., 2013). Together, these results suggest that the mitotic inactivity and function of the QC appears to require a high auxin/low cytokinin environment, with cytokinin regulating auxin transport in the RAM to ensure an auxin maximum in the QC cells. However, as was first observed during organogenesis in tissue culture (Skoog and Miller, 1957), the response to these hormones is complex, as too high a level of auxin also leads to dedifferentiation of the QC.

Anatomical Patterning of the Root

The vascular tissues in the *Arabidopsis* root are arranged in a bisymmetric pattern, with a single xylem axis bisecting the vascular cylinder, two phloem poles at 90° to this axis, and intervening procambial cells separating these two domains (Figure 3). This pattern is a direct readout of bisymmetric domains of hormonal response. Cells within the xylem axis show high auxin response (Bishopp et al., 2011b; Dubrovsky et al., 2011), while cells within the phloem/procambial domains show the highest cytokinin response (Mähönen et al., 2006). A pair of mutually inhibitory interactions maintains these exclusive domains of auxin and cytokinin signaling output (Bishopp et al., 2011b).

The first of these involves the pseudo histidine phosphotransfer protein, AHP6, which is a negative regulator of cytokinin signaling (see above). The promoter of *AHP6* contains a series of AuxREs, is rapidly auxin inducible, and is an ARF5 target in in vitro assays (Bishopp et al., 2011b; Besnard et al., 2014). The second involves the cytokinin-mediated regulation of the PINs. Although multiple PINs are expressed in the stele (Blilou et al., 2005a), it is likely that PIN1, PIN3, and PIN7 play key roles in directing the flux of auxin toward the xylem axis, as all three of these PINs show asymmetries in the radial expression/localization within the stele (Bishopp et al., 2011b). Cytokinin regulates PIN expression both at the transcriptional and posttranscriptional levels (Laplaze et al., 2007; Dello Ioio et al., 2008; Pernisová et al., 2009; Ruzicka et al., 2009; Zhang et al., 2011, 2013). However, close examination of the PIN proteins in the vascular tissues revealed that cytokinin regulates several of these PINs in a distinct manner. For example, cytokinin promotes *PIN7* transcription; consequently, *PIN7* expression is restricted to the procambial cells flanking the xylem axis (Bishopp et al., 2011b). In contrast, cytokinin affects the polarity of the PIN1 protein. In cells with low cytokinin signaling, such as the xylem, PIN1 is polarized on the basal membranes, while in cells with high cytokinin signaling, such as in the procambium, PIN1 is additionally localized to the lateral membranes (Bishopp et al., 2011b). In both cases, the molecular mechanism for cytokinin regulation of PINs is poorly understood, though recent studies have provided some insight into how cytokinin modulates PIN1 polarity (Marhavý et al., 2011, 2014).

Taken together, these results suggest a mechanism for generating mutually inhibitory domains of auxin and cytokinin activity; however, when considered alone, they do not provide sufficient information as to why these domains should favor any particular pattern. Mathematical models have provided a sound framework for understanding how molecular components interact in space and time to regulate spatial patterning and this approach has recently been exploited in hormone biology (Voß et al., 2014). The interactions between auxin and cytokinin described above were incorporated into a mathematical model to test whether they were sufficient to maintain a stable pattern of hormonal response in the root meristem (Figure 4) (Muraro et al., 2014). Briefly, these simulations revolved around a realistic multicellular template composed of a root cross section incorporating a single representative PIN that was localized based on experimental data. Auxin and cytokinin were set to be synthesized equally in all cells and regulate each other's activity, with auxin directly promoting *AHP6* and cytokinin modulating PIN transport. These interactions were insufficient to produce the stable patterns of hormonal output that are observed experimentally. However, introduction of an additional set of components that have previously been demonstrated to regulate AHP6 (PHB, SHR, and microRNA 165/6) (Carlsbecker et al., 2010), modulation of the way in which these interact with each other, and introduction of an additional as yet unidentified inhibitor of cytokinin into this model provided the network with a framework for stable domains of hormonal signaling that were resistant to small fluctuations in either auxin or cytokinin levels. However, all these simulations were based around an initial asymmetry in PIN distribution, and while this model was able to maintain stable expression domains around this initial asymmetry, it wasn't clear how an initial asymmetry could be generated.

The obvious candidate for establishing an initial asymmetry was auxin; indeed, it had been noted that during embryogenesis, markers for auxin response seemed to migrate from the cotyledons to the root pole prior to the establishment of bisymmetry (Bishopp et al., 2011b). Further observations supported the notion that cotyledon number is important in defining the number of vascular poles as mutants with three or four cotyledons typically showed extra xylem files in embryonic tissues (Help et al., 2011). A definitive link between auxin, cytokinin, and the specification of vascular pattern was uncovered recently and involved the discovery of a new level of interaction between these components (De Rybel et al., 2014).

De Rybel et al. (2013) showed that the basic helix-loop-helix transcription factor TMO5 is a direct target of ARF5. TMO5, which heterodimerizes with another basic helix-loop-helix transcription factor called LONESOME HIGHWAY (LHW), is localized within the xylem axis and functions as a key regulator of vascular cell fate (De Rybel et al., 2013). TMO5/LHW act as part of a greater transcriptional complex to promote periclinal divisions within the stele. Surprisingly, TMO5 was found to directly promote the expression of the cytokinin biosynthesis gene *LOG4* within the xylem axis (De Rybel et al., 2014). While this seems counterintuitive, this observation was followed up with a suite of genetic experiments to conclusively demonstrate that auxin directly promotes cytokinin biosynthesis within the xylem axis (through the interaction of ARF5 and TMO5) and that this cytokinin production is necessary and sufficient to regulate TMO5-mediated periclinal cell divisions.

To resolve this conundrum (i.e., why cytokinin would be synthesized in the cells in which it does not show a response), De Rybel et al. (2014) turned to mathematical modeling (Figure 4). Their model contained many of the interactions described for the previous vascular patterning model; however, as cytokinin regulates

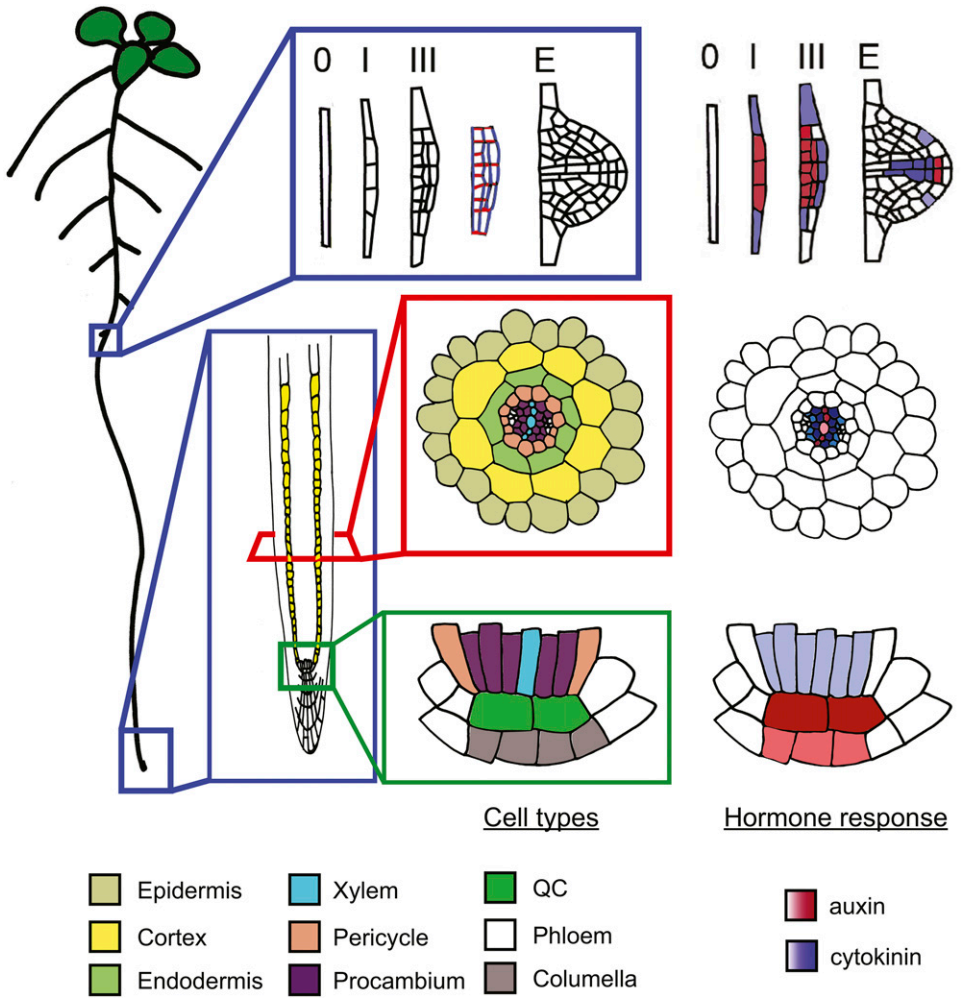

Figure 3. Cell Patterning in the Growing Root.

The central panels depict the cell types in various root tissues, with the cell types depicted in the indicated colors. A close-up of the root tip stem cell niche is boxed in green. The root cross section (boxed in red) shows the bisymmetric cellular pattern. The top panel boxed in blue depicts the some of the stages of lateral root organogenesis with E representing an emerged root. In the image of a stage III LRP, the anticlinal faces are marked in red and periclinal faces are marked in blue. The three panels on the right depict the relative levels of auxin or cytokinin signaling, depicted by the indicated colors, as measured by various reporters (based on results of Blilou et al., 2005b; Bishopp et al., 2011b; Della Rovere et al., 2013; Zürcher et al., 2013; Tian et al., 2014). See text for additional details. The lateral root image (top panel) is reproduced from Bishopp and Bennett (2014) (Figure 2).

cell division as well as cell differentiation, they opted for a template that evolved over time to incorporate the effects of cell division and growth and started with a cross section through an embryonic root prior to vascular morphogenesis. Based on anatomical analyses, they designated two “source cells” in the vascular initials with an uneven input of auxin as a consequence of auxin input from the cotyledons (Figure 2). Importantly, in order to generate stable, realistic patterns the initial geometry needed to include a small “bridge” connecting these source cells (Figure 2), which the authors demonstrated was present as a consequence of division planes at the two- to four-cell transition in the embryo. With these factors incorporated into their model, they tested the effects of allowing cytokinin production in all cells and compared this against restricting it to the xylem axis. By incorporating cytokinin-mediated cell division into their model, they observed that when cytokinin was produced everywhere, it created a large disorganized vascular cylinder with additional irregular cell divisions. However, restricting cytokinin to a nonresponding source in the xylem axis and allowing it to diffuse into the procambium allowed the restriction of maximal cytokinin response and cell division to the procambial cells closest to the xylem axis, which would correctly regulate both growth and patterning. Taken together, these studies show the complexity of interactions between auxin and cytokinin required to generate and maintain tissue patterning, where auxin regulates cytokinin biosynthesis and signaling and cytokinin regulates auxin transport. It also demonstrates the power of mathematical simulations in formulizing and testing complex interactions between multiple components in multicellular tissues.

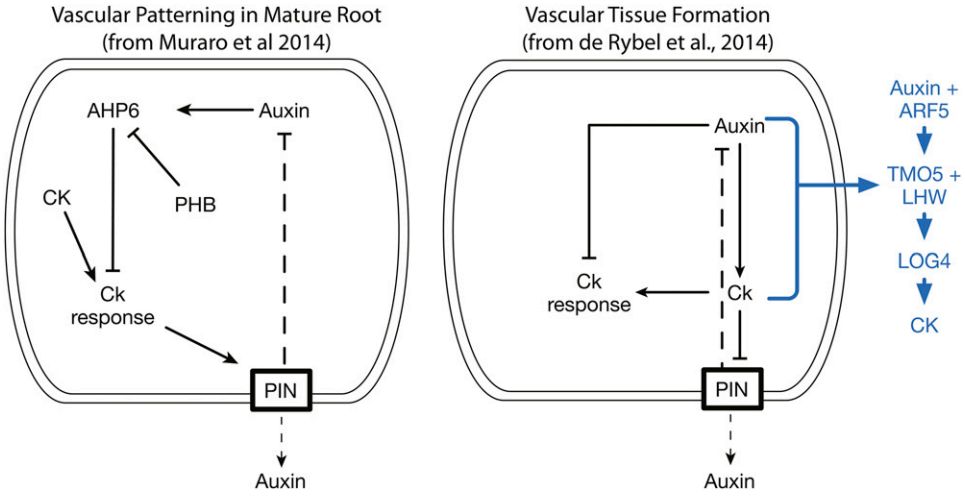

Figure 4. Regulatory Networks Controlling Vascular Development.

Models of the regulatory networks incorporated within two mathematical models of vascular development. Solid lines show activation or repression of targets. One key difference between these two models is the way in which cytokinin modulates PIN activity. In the model of vascular patterning by Muraro et al. (2014), PIN7 transcription is promoted by cytokinin response. In the model of vascular tissue formation by De Rybel et al. (2014), cytokinin inhibits the localization of PIN on the membrane independently of cytokinin response. Furthermore, in this model, there is an additional regulation on PIN polarity whereby auxin in neighboring cells polarizes auxin toward the membrane. In both models, the PINs transport auxin out of the cell and therefore reduce auxin concentration within the cell (dashed lines) and auxin inhibits cytokinin response (either through AHP6 or other as yet unidentified components). The regulation of cytokinin synthesis by auxin (via ARF5, TMO5, LHW, and LOG4) is a linear pathway, and as such De Rybel et al. didn't model the components individually. However, the complete pathway is shown alongside in blue, so that the reader can see how these components are included within the regulatory network.

Architectural Patterning of the Root

In most dicot species, the branching of the primary root plays an instrumental role in determining the overall size of the root network and maximizing the opportunities for resource acquisition. Auxin is instrumental in promoting the formation of new lateral root primordia (LRP) (Himanen et al., 2002; Marchant et al., 2002; Casimiro et al., 2003) (Figure 3). In addition, the developing LRP requires a redirecting of the auxin flux to define a new axis of growth at the apex of the primordia, and this can be seen by a gradual repolarization of PIN1 (Benková et al., 2003).

Cytokinin has an inhibitory role in lateral root organogenesis, and plants with reduced cytokinin levels or signaling display a greater number of lateral roots (Werner et al., 2003; Mason et al., 2005; Hutchison et al., 2006; Riefler et al., 2006), while cytokinin-hypersensitive mutants show fewer lateral roots (To et al., 2004). Furthermore, the cytokinin-insensitive mutant *ahk2 ahk3* exhibits increased sensitivity to auxin in the formation of lateral roots, emphasizing the significance of auxin-cytokinin crosstalk in this process (Chang et al., 2013). This crosstalk is mediated at least partly through cytokinin modulation of polar auxin transport, as perturbation of cytokinin in the protoxylem-associated pericycle cells led to alteration in PIN expression and auxin response pattern in LRP (Laplaze et al., 2007). Genes associated with cytokinin signaling, such as *CRE1/AHK4*, *AHK3*, and *AHP6*, are required for correct PIN1 localization (Marhavý et al., 2011; Moreira et al., 2013). The molecular basis for this interaction has been elucidated more recently when it was shown that cytokinin promotes the endocytic recycling of PIN1 by redirecting it for lytic degradation in vacuoles (Marhavý et al., 2011). This endocytic recycling of PIN1 occurs from the early stages (stage 1) of LRP development and correlates with the time at which cytokinin represses LR initiation.

Cytokinin also plays a key role in polarizing PIN1 during the later stages of lateral root organogenesis from stage III onwards, when the LRP forms a dome shape and PIN1 is clearly polarized on either anticlinal or periclinal membranes (Marhavý et al., 2014). Cytokinin signaling response affects this overall polarization, and cells with elevated cytokinin tend to have a greater amount of PIN1 localized on periclinal membranes. In lateral roots, this has the effect of channeling the auxin flux toward the apex of the primordia, and this increased auxin in this position is sufficient to promote lateral root development. This process of cytokinin-mediated fine-tuning of auxin flux is unlikely to be restricted to just the developing primordia, and experiments involving the mislocalization of specific PIN variants suggest that it also plays a role in gravity response.

AUXIN/CYTOKININ INTERACTIONS ABOVE GROUND

Molecular Mechanisms Underlying Shoot Apical Meristem Organization and Maintenance

The earlier introductory sentences describing the RAM can almost be recycled in the description of the shoot apical meristem (SAM). Shoot growth is driven by a group of undifferentiated cells within the SAM, a region where cells self-renew and produce daughter cells, which then differentiate with specific cell identities that pattern the

shoot (Kerstetter and Hake, 1997) (Figure 5A). In the SAM, a group of initial stem cells surround a small group of mitotically less active cells, known as the organizing center (OC), which is analogous to the QC in the RAM. Furthermore, a key homeodomain transcription factor, WUSCHEL (WUS), which is closely related to the QC-expressed WOX5, is expressed in the OC. The OC and the cells in direct contact comprise the stem cell niche and produce nonautonomous signals that prevent differentiation of these cells. The cells of the OC are positioned at the base of the central zone (CZ), a region with a low rate of cell division. Cells moving from the CZ into the peripheral zone (PZ), a region with a higher rate of cell division, give rise to the lateral organs. The region below the OC and CZ is called the rib zone and gives rise to the cells that form the stem.

However, there is a key difference when comparing the function of auxin and cytokinin in the SAM and the RAM as their roles are apparently reversed: The OC of the SAM is the site of maximal cytokinin activity, rather than of auxin activity as found in the QC of the RAM (Figure 5B). The roles of cytokinin and auxin in maintaining the SAM mirror their roles in the RAM, with the hormone maxima being reminiscent of the two contrasting black and white spots found on opposite sides of the yin-yang symbol. The general picture of auxin/cytokinin activity in the SAM, based in part on localization of their hormonal activity maxima, is that cytokinin promotes the proliferation of undifferentiated cells in the SAM, while auxin acts in the PZ to induce cellular differentiation and organ outgrowth. While this is generally correct, it is becoming increasingly clear that auxin and cytokinin interact with each other to various extents and by a variety of mechanisms throughout the SAM.

The SAM forms during embryogenesis, the first indication of its incipient formation in *Arabidopsis* occurring at the 16-cell globular stage when the transcription factor WUS is expressed (Mayer et al., 1998). WUS is required for formation of the SAM and is initially expressed in the four subepidermal cells of the apical half of the proembryo (Figure 2). The cues that lead to WUS expression are unclear but may involve auxin, which exhibits relatively high activity throughout the proembryo (Peris et al., 2010). Expression and polar localization of PIN transporters subsequently result in localized auxin maxima, coinciding with the increased patterning of the developing embryo, such that by the globular stage auxin maxima are found at the flanking cells of the apex.

The characteristic structure of the SAM becomes clearly established at the heart stage, at which point the cells expressing *WUS* define the OC. This is also the point when cytokinin activity becomes clearly associated with the SAM, which as we discuss below is a key element in maintaining *WUS* expression (Zürcher et al., 2013). The association of cytokinin activity with the embryonic SAM also occurs in monocots, even though monocots differ from dicots in the cell division patterns that occur during embryogenesis (Chen et al., 2014). Maintenance of a stem cell population in the SAM thereafter relies on a non-cell-autonomous feedback loop involving WUS and the CLAVATA (CLV) signaling pathway (Figure 5C). WUS functions in part by protein migration; the WUS protein produced in the OC migrates into adjacent cells of the CZ, thereby establishing a WUS gradient around the OC. WUS activity in the CZ activates expression of CLV3, which in turn negatively regulates WUS expression via the CLV1 receptor, which therefore serves to restrict WUS expression to the CZ (Yadav et al., 2011). This feedback loop thereby spatially defines the OC and stem cells (Perales and Reddy, 2012). In addition to acting as a transcriptional activator (e.g., inducing expression of *CLV3*), WUS can also function as a transcriptional repressor (Busch et al., 2010; Yadav et al., 2013). In

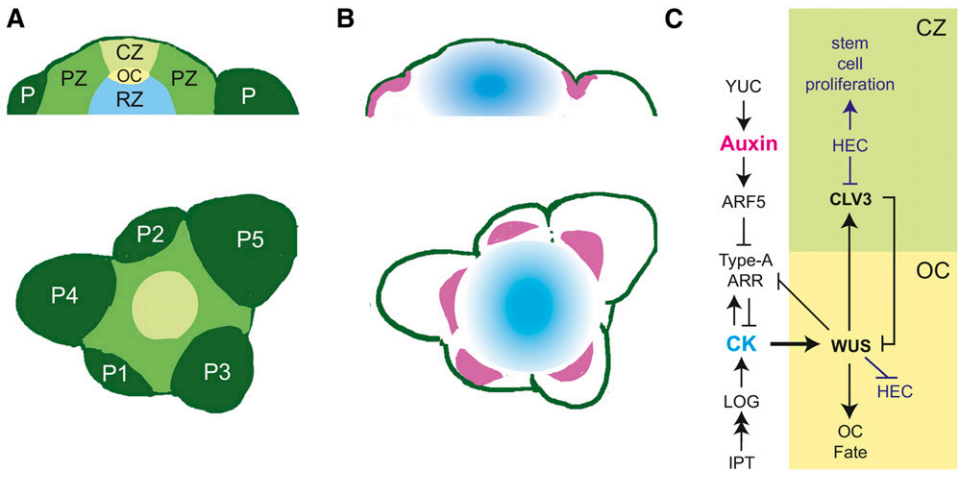

Figure 5. Patterning of the Shoot Apical Meristem.

(A) Organization of the SAM showing locations of the OC, CZ, PZ, and rib zone (RZ). A transverse cross section is shown along with a surface view to illustrate phyllotaxy with the primordia labeled P1 through P5.

(B) Auxin and cytokinin activity at the SAM. Maxima of auxin (purple) and cytokinin (blue) activity based on *DR5:VENUS* and *TCS:GFP* reporter analysis, respectively (Murray et al., 2012; Zürcher et al., 2013; Besnard et al., 2014). The cytokinin maximum is found at the OC, whereas auxin maxima are found at locations of primordia formation.

(C) A model of the circuitry by which auxin and cytokinin regulate *WUS* expression at the OC. The transcriptional regulator WUS promotes the expression of the signaling peptide CLV3, which interacts with the receptor kinase CLV1 in the CZ to negatively regulate *WUS* expression. The transcription factor HEC promotes stem cell proliferation in the CZ.

particular, WUS represses gene expression associated with differentiating cells by binding to the promoter regions of a variety of transcription factors, such as *KANADI1*, *KANADI2*, *ASYMMETRIC LEAVES2*, and *YABBY3*, that regulate early events in leaf differentiation.

Cytokinin activity is a key element in establishing the organization and regulating cell division in the SAM, with genetic studies indicating that cytokinin is a positive regulator of cell proliferation in the SAM (Werner et al., 2003; Giulini et al., 2004; Higuchi et al., 2004; Nishimura et al., 2004; Leibfried et al., 2005; Miyawaki et al., 2006; Bartrina et al., 2011; Kieber and Schaller, 2014). The location of maximal cytokinin biosynthesis and response differ in the SAM, and these are important for the positioning of the *WUS* expression domain (Yanai et al., 2005; Kurakawa et al., 2007; Gordon et al., 2009; Chickarmane et al., 2012; Zürcher et al., 2013). Cytokinin biosynthesis primarily takes place in upper layers of the SAM, but maximal sensitivity to cytokinin occurs at the OC. Active cytokinins diffusing downward from the tip of the SAM, together with WUS-induced CLV3 expression from below, regulate the dynamic positioning of the WUS expression pattern such that it corresponds to this maximum of cytokinin activity (Kurakawa et al., 2007; Chickarmane et al., 2012; Zürcher et al., 2013) (Figure 5B). The ERECTA family of leucine-rich repeat–receptor-like kinases appears to buffer the SAM against changes in cytokinin levels as disruption of multiple ERECTA family members results in an hyperinduction of *CLV3* expression in response to cytokinin (Uchida et al., 2013). *WUS* expression requires high levels of cytokinin activity, and this is maintained in part by WUS-mediated repression of type-A *ARR* expression (Figure 5C); WUS represses *ARR7* by direct binding to upstream regulatory sequences and also represses *ARR5*, *ARR6*, and *ARR15* (Leibfried et al., 2005).

Auxin also plays a role in enhancing the sensitivity of the OC to cytokinin (Figure 4C). Auxin produced by the YUCCA-dependent biosynthetic pathway downregulates expression of type-A *ARR*s (Zhao et al., 2010). The effect of auxin is transduced through *ARF5* (*MONOPTEROS*); *arf5* mutants exhibit ectopic expression of *ARR7* and *ARR15* throughout the central zone as well as the rib zone. These data support a regulatory circuit in which auxin, acting through ARF5, downregulates type-A ARRs and thereby increases cytokinin sensitivity; such a mechanism could play a significant role in maintaining the cytokinin activity maxima at the OC. It should be noted, however, that an *arr7 arr15* double mutant lacks a clear shoot meristem phenotype, just as it also lacks a root meristem phenotype during embryogenesis (Zhang et al., 2011), contrasting with phenotypes reported from microRNA-mediated silencing of *ARR7* and *ARR15*. Thus, the effects of auxin on type-A *ARR* expression are likely to involve more than just these two family members.

Recently, another element in this regulatory circuit was identified as HECATE1 (HEC1), a basic helix-loop-helix transcription factor previously implicated in development of the female reproductive organ system and now found to positively regulate stem cell proliferation in the SAM (Gremski et al., 2007; Schuster et al., 2014) (Figure 5C). In the OC, *HEC1* is a direct target for repression by WUS; misexpression of *HEC*1 in the OC interferes with OC activity and leads to loss of stem cell production (Schuster et al., 2014). However, HEC1 is expressed in the stem cells of the CZ, where it positively regulates cell proliferation by induction of genes involved in the cell cycle as well as by suppression of *CLV3*. HEC1 also ties into the cytokinin signaling circuitry as among its inductive targets are the type-A *ARRs*, *ARR5*, *ARR7*, and *ARR15*. The heightened expression of these type-A *ARRs* decreases cytokinin signaling activity outside the OC and therefore also result in decreased expression of *WUS* in the CZ.

Both cytokinin and auxin play positive roles in regulating cell division in the shoot, but we have only a limited understanding as to how this is accomplished. However, both hormones can affect cell cycle progression into the S-phase through the cyclin-dependent kinase A/D-type cyclin (CYCD) pathway. Cytokinin induces expression of the three *CYCD3* genes, and ectopic overexpression of *CYCD3;1* bypasses cytokinin requirements in tissue culture (Riou-Khamlichi et al., 1999; Dewitte et al., 2007; Scofield et al., 2013). The CYCD3s are not absolutely required for progression through cell cycle but maintain the undifferentiated state of the stem cells and suppress endoreduplication. Recently, it has been shown that SHOOT-MERISTEMLESS (STM) acts to inhibit cellular differentiation and endoreduplication by acting through cytokinin and through the cytokinin induction of *CYCD3* (Scofield et al., 2013). Auxin regulates additional elements of the CYCD/CDK pathway. One potential target of auxin, based on the analysis of lateral root initiation, is the cyclin-dependent kinase inhibitor KRP2/ICK2; auxin downregulates expression of *KRP2* and also promotes turnover of the KRP2 protein (Himanen et al., 2002; Sanz et al., 2011). A second potential target for auxin regulation is the E2F transcription factor family, which functions downstream of CYCD/CDK to regulate entry into S-phase; ectopic overexpression of E2FB in tobacco (*Nicotiana tabacum*) BY-2 cells bypasses their auxin dependence for division and, at least during lateral root initiation, auxin stabilizes E2FB protein (Magyar et al., 2005; Sanz et al., 2011). The effects of cytokinin and auxin on the cell cycle are not likely to be restricted to controlling S-phase entry. In fact, multiple lines of evidence suggest that cytokinin also plays a major role in controlling the G_2/M transition, but the underlying mechanisms are even less understood than those regulating the S-phase progression (reviewed in Schaller et al., 2014).

SAM Formation in Tissue Culture

Many plants are capable of regeneration through tissue culture, and this system has been used to study the developmental pathway by which a SAM is produced. In *Arabidopsis*, de novo production of shoots through tissue culture involves a two-step process. First, small pieces of plant tissue are treated with auxin-rich callus-inducing medium (CIM) to induce cell division and the formation of callus. Second, to induce tissue differentiation, the callus is transferred to either shoot- or root-inducing medium (SIM or RIM), which contain differing ratios of auxin to cytokinin, an elevated ratio of cytokinin to auxin favoring the SAM development and shoot formation (Sugimoto et al., 2010). The interplay of auxin and cytokinin is critical to both callus induction and de novo SAM development and involves both cooperative and antagonistic interactions. Here, we cover some of the major aspects of this crosstalk and refer readers to a recent excellent review by Motte et al. (2014) for a more complete

discussion of the molecular mechanisms controlling shoot regeneration from tissue culture.

Callus formation shares much in common with the initial steps leading to lateral root formation (Figure 3), regardless of whether the cultured tissue originates from roots or aerial tissues such as cotyledons and petals (Atta et al., 2009; Sugimoto et al., 2010; Motte et al., 2014). As occurs during the generation of lateral root primordia, the heightened auxin concentrations in CIM induce expression of the QC marker *WOX5* and anticlinal divisions of the pericycle, which are followed by periclinal divisions to create extra layers of cells. Callus differs from the lateral root in having a greatly expanded subepidermal layer of cells with QC-like identity. Transcriptomic analyses confirm a substantial overlap between the gene expression involved in lateral root initiation and that found in tissue incubated on CIM (Sugimoto et al., 2010; Motte et al., 2014). Based on these characteristics, callus tissue has an overall identity similar that of the root tip.

Both auxin and cytokinin signaling are required for callus formation. Mutations that inhibit auxin signaling, such as the *arf7 arf19* double mutant, impede callus formation on CIM (Fan et al., 2012). A key role for auxin is in the induction of the LATERAL ORGAN BOUNDARIES DOMAIN (LBD) transcription factors, some of which (*LBD16*, *LBD17*, *LBD18*, and *LBD29*) are direct targets of ARF7 and ARF19 and function in lateral root patterning (Fan et al., 2012). Ectopic expression of *LBD*s stimulates callus induction in the absence of auxin; conversely, their suppression inhibits callus induction on CIM. Although exogenous cytokinin is not necessary for callus formation, endogenous levels of cytokinin activity are required based on the severely reduced capacity of cytokinin-insensitive mutants to form callus (Nishimura et al., 2004; Mason et al., 2005; Hutchison et al., 2006; Riefler et al., 2006; Yokoyama et al., 2007; Argyros et al., 2008; Ishida et al., 2008; Hill and Schaller, 2013). The role of cytokinin in callus formation is of interest because cytokinin acts antagonistically with auxin in the formation of lateral roots, in part due to inhibition of the anticlinal divisions in the pericycle that initiate lateral root formation. Cytokinin's positive role in callus formation is thus likely to be on the subsequent periclinal divisions after the primordia are induced, which is supported by the analysis of auxin and cytokinin activity during callus formation (Gordon et al., 2007). Auxin activity is high during initial stages, but then decreases as cytokinin activity concomitantly increases in the dividing callus tissue. Interestingly, cytokinin-hypersensitive mutants appear to be not only more sensitive to cytokinin, but also more sensitive to auxin in callus formation, an observation at odds with the antagonism commonly observed for auxin-cytokinin interactions (Sakai et al., 2001; Mason et al., 2005; Ikeda et al., 2006; Kim et al., 2012; Hill et al., 2013). Cytokinin may positively regulate the expression of genes that directly affect auxin activity (e.g., *ARF*s) or downstream targets of auxin involved in callus formation (e.g., *LBD* family members).

Although callus has characteristics of the root tip, the primordia have not yet acquired organ identity and can give rise to either root or shoot meristems depending the subsequent hormone regime. Preincubation on CIM is required for the induction of SAMs, but extended callus growth on CIM irreversibly commits primordia to root identity (Christianson and Warnick, 1983). Among the genes induced on CIM that are likely to play a significant role during shoot organ formation are *CUP-SHAPED COTYLEDON1* (*CUC1*) and *CUC2*, along with *AHK4/CRE1*, which encode NAC transcription factors and a cytokinin receptor, respectively (Gordon et al., 2007, 2009; Motte et al., 2011). Transfer of callus to cytokinin-rich SIM results in partitioning of cell identity in the callus such that *CUC2* expression decreases overall and becomes localized to regions capable of forming shoot precursors. Significantly, cytokinin also induces the expression of *WUS*, initially in the regions underlying the *CUC2*-enriched shoot primordia (Gordon et al., 2007; Z.J. Cheng et al., 2013). Although cytokinin activity is coincident with regions of high auxin activity during callus growth on CIM, the transfer to SIM results in more restricted cytokinin activity, corresponding to the region of *WUS* expression.

Continued incubation on SIM results in formation of a patterned SAM that is dependent on the crosstalk between cytokinin and auxin. Incubation on SIM results in localized auxin biosynthesis based on upregulation of *YUC1* and *YUC4* and polarized auxin transport within the shoot primordia mediated by PIN1 (Gordon et al., 2007; Atta et al., 2009; Z.J. Cheng et al., 2013). Auxin activity localizes to a ring of cells that is apical and peripheral to a restricted zone of *WUS* expression, with the auxin ring exhibiting increased expression of the *ARF* family genes that mediate the auxin response (Z.J. Cheng et al., 2013). At this point, auxin and cytokinin exhibit the antagonism often associated with their crosstalk. Auxin suppresses cytokinin activity within the apical ring by directly suppressing expression of the *IPT* family of cytokinin biosynthetic genes (Z.J. Cheng et al., 2013); the expression of *IPT5* is restricted to the zone directly above that expressing *WUS*, thereby establishing the gradient of cytokinin production characteristic of the SAM. The key meristematic regulators *CLV3* and *STM* also exhibit changes in expression during the process of SAM patterning. *CLV3* is expressed in the new meristem apex soon after *WUS* expression becomes restricted (Gordon et al., 2007; Chatfield et al., 2013). *STM* expression varies such that initially, when *CUC2* expression is high throughout the shoot primordia, it is found at the outer boundary of the primordia; later, after a decrease in *CUC2* expression, *STM* becomes more generally expressed in the meristem (Gordon et al., 2007).

MicroRNAs also play a significant role in shoot regeneration, acting through control of the auxin response to affect SAM development (Qiao et al., 2012). In particular, the microRNA *miR160*, which targets several *ARF* family members and has been previously implicated in control of organ development, negatively regulates shoot development in vitro (Liu et al., 2007; Qiao et al., 2012). *miRNA160* is normally downregulated after transfer of callus to SIM, and its overexpression inhibits shoot regeneration, pointing toward the need for *ARF* gene expression in shoot development (Qiao et al., 2012). Consistent with this hypothesis, expression of a *miR160*-resistant form of *ARF10* increased shoot regeneration and resulted in the increased expression of *CUC1*, *CUC2*, *WUS*, and *CLV3*. These results reinforce how incubation on a cytokinin-rich media can result in the upregulation of specific genes that mediate the auxin response and furthermore indicate that this may be controlled through microRNA-dependent mechanisms.

The finding that SAMs are induced in tissue culture by passing through a lateral root primordia-like stage has recently been exploited to develop a high-throughput methodology to study

SAM formation (Chatfield et al., 2013). For this purpose, lateral root primordia are induced in seedlings by incubation on auxin-rich media, and these primordia then synchronously converted to SAMs by transferring the seedlings to cytokinin-rich media. As with callus, the transfer to cytokinin-rich media induces expression of *WUS* followed by *CLV3* and *WUS* expression appearing in lateral root primordia within 1 d of cytokinin treatment and *CLV3* expression in cell layers above and overlapping the cells expressing *WUS*. Within 5 d, the roots of *Arabidopsis* are densely covered with tiny shoots. This system has been used for transcriptomic studies and identified both known and novel *WUS*-regulated genes (Chatfield et al., 2013).

Taken together, these studies indicate that cytokinin and auxin interact both negatively and positively to control shoot initiation in tissue culture. On auxin-rich CIM, cytokinin activity is induced in response to auxin and occurs in regions also high in auxin activity. On cytokinin-rich SIM, genes involved in auxin biosynthesis and transport are expressed. However, there are also antagonistic relationships revealed during the establishment of the SAM, with auxin and cytokinin activity becoming localized to separate and distinct zones, the zone of auxin activity serving a critical role in suppressing and restricting the cells engaged in cytokinin biosynthesis.

Organogenesis and the Establishment of Phyllotaxy

The SAM maintains a balance between cell proliferation and differentiation (Kerstetter et al., 1997; Peris et al., 2010; Besnard et al., 2011; Su et al., 2011). Cells in the CZ slowly divide, with stem cells providing a renewable resource for undifferentiated cells in the CZ; cytokinin is the major hormonal signal involved in establishing and maintaining the CZ (Figure 5). Differentiation is initiated once cells move from the CZ into the PZ, at which point the rate of cell division increases and organ primordia are formed. Furthermore, organogenesis occurs in distinctive patterns, this repeated arrangement of organs such as leaves and flowers being referred to as phyllotaxis (Besnard et al., 2011; Murray et al., 2012). Organogenesis and phyllotaxis are primarily controlled through the action of auxin (Figure 5). These differing roles of cytokinin and auxin in the SAM can be distinguished based on phenotypes uncovered through genetic studies. Whereas mutations affecting cytokinin action can alter SAM size due to effects on cell division, mutations affecting auxin action can have pronounced effects on organogenesis. In particular, loss-of-function mutations in *PIN1*, which encodes an auxin efflux carrier, exhibit a pointed inflorescence stem that fails to produce flowers, emphasizing not only the role of auxin, but of auxin transport in this process (Okada et al., 1991; Gälweiler et al., 1998). Significantly, external application of auxin to the *pin1* inflorescence rescues the mutant phenotype and induces floral organ formation (Reinhardt et al., 2003).

The flux of auxin at the SAM plays a central role in patterning (Benjamins and Scheres, 2008; Murray et al., 2012). This cell-to-cell movement of auxin is mediated by the PIN family of efflux carriers as well as by influx carriers such as the AUX1/LAX and MDR families. Both families of carriers are enriched in the outermost cell layer of the SAM, also called the L1 layer, which gives rise to the epidermal cells in the organ primordia. The AUX/LAX carriers appear uniformly distributed at the cell periphery and are likely to increase auxin concentration within the L1 layer. The PIN transporters, in contrast, are polarly localized at the cell membranes and situated such that they direct an auxin flux toward a maxima or convergence point, which becomes the site for organ primordia initiation. Interestingly, this polar transport at the L1 surface layer is sufficient not only for phyllotactic patterning but also for differentiation of the primordia inner tissues that do not arise from the L1 layer (Kierzkowski et al., 2013). The new primordia are auxin sinks, reducing auxin levels in the neighboring cells, such that additional primordia are not formed in their immediate vicinity (Reinhardt et al., 2003). Changes in the subcellular PIN localization at the developing primordia, regulated through PIN endocytosis, facilitate the formation of these auxin sinks (Furutani et al., 2014). This results in a consistent repositioning of auxin maxima that establishes the characteristic phyllotaxy around the SAM. Significantly, the positioning of organ primordia can be computationally modeled based on their induction at auxin maxima and the positioning of the PIN auxin effluxers (Smith et al., 2006; Stoma et al., 2008).

The topological arrangement of additional auxin metabolism and signaling components at the SAM lend insight into how auxin mediates the formation of organ primordia. First, the ARFs and IAAs involved in auxin transcriptional output are localized to the SAM periphery, indicating that auxin-mediated changes in gene expression are primarily associated with organogenesis (Vernoux et al., 2011). Second, the *YUCCA* family of genes involved in auxin biosynthesis are expressed in upper cell layers of the apical dome as well as in the organ primordia (Cheng et al., 2006). Expression of the *YUCCA* genes is dependent on the PLETHORA family of transcription factors, with *YUCCA* or *PLETHORA* mutations resulting in aberrant phylotaxis (Cheng et al., 2006; Gallavotti et al., 2008; Pinon et al., 2013). Thus, without auxin production, even within the context of normal auxin transport, auxin maxima of sufficient strength cannot be induced for proper regulation of phyllotaxis. Furthermore, auxin biosynthesis occurs at a locality away from its site of transcriptional action, a mechanism likely to facilitate the production of distinct auxin maxima via the localized PIN efflux network. Third and finally, the TIR1 family of auxin coreceptors is also expressed broadly in the apical meristem, in the CZ and the PZ, indicating that these can potentially regulate transcriptional and nontranscriptional events across the SAM (Vernoux et al., 2011).

Crosstalk between auxin and cytokinin plays a significant role in the ability of auxin to establish the local maxima needed for organogenesis. Changes in meristem size, such as those found in *Arabidopsis* cytokinin signaling mutants, can potentially alter phyllotaxy indirectly (Leibfried et al., 2005; Argyros et al., 2008; Bartrina et al., 2011). Similarly, a mutation in rice that reduces cytokinin sensitivity results in a larger shoot meristem and altered leaf phyllotaxy (Itoh et al., 2012). In *Arabidopsis*, the *ERECTA* family of genes, which are implicated in buffering the SAM against changes in cytokinin levels (Uchida et al., 2013), also play a role in organ initiation and phyllotaxy through effects on auxin flux, apparently due to a role in establishing the appropriate PIN expression pattern (Chen et al., 2013). These effects of ERECTA correlate with changes in the SAM size, but also suggest that the ERECTA family may play a significant, but largely unexplored role, in mediating the crosstalk between auxin and cytokinin.

Additional studies point to more specific hormonal interactions directly related to the establishment of robust auxin maxima and the developing primordia. Of particular significance in this regard has been characterization of the *ABERRANT PHYLLOTAXY1* (*ABPH1*) mutant of maize (*Zea mays*), which encodes a type-A ARR (Giulini et al., 2004; Lee et al., 2009). Loss of *ABPH1*, which is predicted to increase cytokinin activity, results in a change of phyllotaxy such that leaves of the *abph1* mutant are initiated in 180° pairs rather than alternating as in wild-type plants. The expression of *ABPH1* and *PIN* genes at organ initiation sites suggests a more direct effect on organogenesis than resulting solely from a change in meristem size. Interestingly, expression of *ABPH1* depends not only on cytokinin but also on auxin transport at the meristem, as its expression decreases in the presence of an auxin transport inhibitor. The auxin dependence suggests that this type-A *RR* might be induced as a means to antagonize cytokinin signaling and thereby facilitate the formation of an auxin maximum. However, this explanation does not jibe with additional findings, which point to a positive role of ABPH1 in establishing the auxin flux needed for primordia initiation. Specifically, the *abph1* mutant exhibits reduced auxin levels as well as reduced levels of the maize PIN1, suggesting that the altered phyllotaxy may arise due to a combination of factors including delayed leaf initiation and an enlarged meristem.

The recent discovery that AHP6, a negative regulator of cytokinin signaling in *Arabidopsis*, plays a role in establishing phyllotaxy in *Arabidopsis* points to yet another mechanism by which auxin-cytokinin crosstalk can be modulated (Besnard et al., 2014). Mutations of *AHP6* result in altered phyllotaxis during inflorescence development, the most obvious phenotype being in the divergence angle for siliques. Irregular silique placement has also been previously observed in *Arabidopsis* plants with increased cytokinin levels as well as in cytokinin-insensitive type-B ARR mutants, but in these cases is probably due to the change in meristem size (Argyros et al., 2008; Bartrina et al., 2011). In the case of the *ahp6* mutants, no significant change in meristem size was observed, suggesting a more specific role in the regulation of phyllotaxy. Analysis of the expression patterns for *AHP6* as well as auxin and cytokinin activity reporters supports a model in which AHP6 regulates the spatio-temporal pattern of cytokinin activity at the SAM periphery, accentuating the cytokinin peaks and valleys of activity. The cytokinin inhibitory fields help create less noisy auxin fields, thereby facilitating a robust phyllotactic patterning.

Organogenesis does not proceed at a constant rate. For example, light serves as an important morphogenic signal to control SAM activity. The requirement of light for leaf initiation was observed over 40 years ago in pea (*Pisum sativum*; Low, 1970) and was recently confirmed in tomato (*Solanum lycopersicum*) and *Arabidopsis* and found to be due to regulation of both cytokinin and auxin activity (Yoshida et al., 2011). Cytokinin is essential for leaf initiation: Cytokinin activity in the central zone decreases in the dark and application of cytokinin to dark-grown apices is sufficient to induce the formation of new leaf primordia. However, induction of the new leaf primordia is also dependent on the distribution of auxin at the SAM, with tip growth but no organ initiation occurring when the SAM is treated with cytokinin and the auxin transport inhibitor 1-*N*-naphthylphthalamic acid. The distribution of auxin needed for organ initiation is dependent on PIN1, which exhibits increased internalization from the plasma membrane in the dark.

Gynoecium and Female Gametophyte Development

The *Arabidopsis* silique is derived from the gynoecium, which displays apical-basal patterning consisting of, in order, the stigma, the style, the ovary (which contains the ovules), and the basal gynophore (Roeder and Yanofsky, 2006). Auxin plays a critical role in this apical-basal patterning, with high levels of auxin at the apical part of the developing gynoecium (Nemhauser et al., 2000; Hawkins and Liu, 2014). A recent study examined the spatio-temporal pattern of auxin and cytokinin responses in developing gynoecia using the DR5rev:GFP and TCS:GFP reporters, respectively. As in several other developmental context, auxin and cytokinin displayed complementary patterns of activation. For example, TCS:GFP expression was observed in the center of the gynoecium in the early stages of development, while DR5rev:GFP was expressed in the cells surrounding this (Marsch-Martínez et al., 2012a, 2012b). Interesting, there was a strong cytokinin signal in the valve margins in developing fruit, and this signal was dependent on functional INDEHISCENT (IND), SHATTERPROOF1 (SHP1), and SHP2, which are key regulators of valve development and function. Consistent with this, localized application of cytokinin could restore valve margin formation and dehiscence in the *ind* and *shp1 shp2* mutants (Marsch-Martínez et al., 2012a), suggesting that cytokinin acts downstream of IND and SHP1/2 to mediate their effects on valve margin function.

Disruption of polar auxin transport or of auxin biosynthesis or signaling causes severe defects in apical-basal patterning of *Arabidopsis* gynoecia (Zúñiga-Mayo et al., 2014). Intriguingly, exogenous application of cytokinin causes phenotypes similar to 1-*N*-naphthylphthalamic acid treatment, and this effect was enhanced in auxin signaling and transport mutants (e.g., *pin3-4* or *tir1*). Moreover, treatment with cytokinin altered the pattern of expression of a PIN1 promoter-protein GFP fusion (Zúñiga-Mayo et al., 2014). Together, these results suggest that cytokinin and auxin act together to specify the apical basal patterning of the developing gynoecium, acting in complementary tissues. Furthermore, cytokinin likely acts in part by affecting polar auxin transport in the developing gynoecium. This is similar in many respects to the interaction of cytokinin and auxin in various aspects of root development as described above.

Auxin and cytokinin have also been linked to the development of the female gametophyte, which develops within the ovule of the gynoecium. Genetic studies indicated that the CKI1 histidine kinase, which is related to AHK cytokinin receptors but lacks a cytokinin binding CHASE domain, is required for female gametophyte development (Pischke et al., 2002; Hejátko et al., 2003), as are the downstream AHPs (Deng et al., 2010). Genetic studies of the cytokinin receptors in *Arabidopsis* indicated that cytokinin is required in the sporophytic tissue for female gametophyte development (Nishimura et al., 2004; Kinoshita-Tsujimura and Kakimoto, 2011), in contrast to CKI1, which acts in the gametophytic tissues (Pischke et al., 2002; Hejátko et al., 2003). A more recent study found that while some allelic combinations of the AHK cytokinin receptors are able to form viable seeds, the strongest combination (*ahk2-7 ahk3-3 cre1-12*) displayed nearly complete female gametophyte arrest, and the defect occurs at a much earlier stage than that observed in *cki1* mutants (C.-Y. Cheng et al., 2013). Furthermore, in a fraction of *ahk2-2tk ahk3-3 cre1-12* triple mutant female gametophytes, integument initiation was impaired and finger-like ovule structures were observed (Bencivenga et al., 2012). This integument defect was

at least partially the result of a downregulation of *PIN1* expression in the ovules of the triple receptor mutant (Bencivenga et al., 2012). The transcription factors SPL/NZZ and BEL1, which play important roles in ovule development, are involved in the cytokinin regulation of PIN1 in the developing ovule.

Cytokinin appears to provide positional information for the development of the female gametophyte. Cytokinin signaling is enriched in the chalaza in the maternal, sporophytic tissue via localized expression of genes involved in cytokinin biosynthesis and perception (AHKs) (C.-Y. Cheng et al., 2013). In what is now a familiar theme, this gradient of cytokinin mirrors an auxin gradient present in the sporophytic tissue early in female gametophyte development (Pagnussat et al., 2009) that is likely formed through the action of PIN1 (Ceccato et al., 2013). This auxin gradient was suggested to define cell-type specification in the female gametophyte (Pagnussat et al., 2009), though Lituiev et al. (2013) suggested, on theoretical grounds and an analysis of a degron-based auxin sensor, that there was no auxin gradient in the female gametophyte itself, though they did observe a gradient in the surrounding sporophytic tissue. This suggests that in the sporophytic tissues, there are opposing auxin and cytokinin gradients that play complementary roles during early stages of female gametophyte development, including the specification of the functional megaspore cell from the four products arising from meiosis of the megaspore mother cell (C.-Y. Cheng et al., 2013).

CONCLUSIONS AND FUTURE PROSPECTS

One emerging theme from the interactions between auxin and cytokinin in development is that, like yin and yang, the response to these hormones is often expressed in complementary patterns to impart distinct fates on neighboring cells. This is achieved through interactions at the level of signaling and biosynthesis, though it is clear that there remains much to learn. For example, no studies have described all aspects of the interaction between these hormones (i.e., biosynthesis, transport, signaling, and degradation); indeed, additional points of regulation are known, such as the induction of several *LOG* and *CKX* genes by auxin, that have not as yet been linked to a particular developmental process (Figure 1) (Bhargava et al., 2013). Mathematical modeling has helped reveal the circuitry of these interactions and will likely continue to contribute to our understanding of how auxin and cytokinin act to bring about integrated responses. The studies described in this review focus almost exclusively on *Arabidopsis*. It is likely that the circuitry connecting these hormones regulating development may have unique features in other species and in other context. Finally, it will be crucial to elucidate how auxin and cytokinin interact with other hormonal and developmental signals in order to fully understand how these hormones act within the context of the whole organism to contribute to an integrated circuit regulating plant growth and development.

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AUTHOR CONTRIBUTIONS

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