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Cytokinin–auxin crosstalk

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Post-embryonic plant growth and development are sustained by meristems, a source of undifferentiated cells that give rise to the adult plant structures. Two hormones, cytokinin and auxin, are known to act antagonistically in controlling meristem activities. Here, we review recent significant progress in elucidating the molecular mechanisms through which these hormones interact to control specific aspects of plant development. For example, in the root meristem of *Arabidopsis thaliana*, cytokinin promotes cell differentiation by repressing both auxin signalling and transport, whereas auxin sustains root meristem activity by promoting cell division. The coordinated action of these two hormones is essential for maintaining root meristem size and for ensuring root growth.

Cytokinin and auxin: master regulators of plant development

Cytokinin and auxin have long been recognized as crucial signalling molecules controlling plant growth and development. In 1957, it was shown that root and shoot development in tobacco pith tissue cultures depends on the cytokinin:auxin ratio, and that organ differentiation can be regulated by changing the relative concentrations of these two growth factors in the culture medium: high levels of cytokinin supported shoot formation and high concentrations of auxin promoted rooting, whereas, at equal concentrations of cytokinin and auxin, the tissue tended to grow in an unorganized fashion [1]. In this classic paper, the concept of hormonal control of organ formation was suggested; however, little is still understood about the *in vivo* significance of these tissue culture experiments and of the molecular mechanisms through which the two hormones act in concert to exert these effects. In addition, the mode of interaction between cytokinin and auxin often depends upon the plant species and organ being studied. This has hampered the elaboration of a general model for the control of plant growth and development by these hormones.

Here, we summarize the state-of-the-art concerning cytokinin and auxin biosynthesis, transport and signalling. We also focus on recent progress in understanding the molecular mechanisms through which these hormones interact to control plant organ development.

Cytokinin biosynthesis, transport and signalling

Cytokinins came to light because of their ability to promote cell division in tobacco tissue culture [2] and, since their discovery, these N⁶-substituted adenine-based molecules

have been associated with important developmental roles, including shoot and root development [3,4]. Isopentenyladenine (iP), trans-zeatin (tZ) and dihydrozeatin (dZ) are the predominant cytokinins found in higher plants [5,6] and their activity *in planta* is thought to be controlled by a fine balance between synthesis and catabolism.

The rate-limiting step of cytokinin biosynthesis in *Arabidopsis thaliana* is catalyzed by the ATP/ADP-isopentenyltransferase (*AtIPT*) gene family [6]. Expression patterns of *IPT* genes indicate that cytokinin is produced at various sites in the plant, including roots, shoots and immature seeds [7]. Most metabolic cytokinin inactivation depends on the activity of the cytokinin oxidase/dehydrogenase (*CKX*) genes, which catalyze the irreversible degradation of cytokinins [6]. These genes, as well as *IPT* genes, show a spatially and temporally regulated pattern of expression during *Arabidopsis* development [7,8], confirming a fine regulation of cytokinin turnover.

By contrast the molecular basis of cytokinin transport is still unknown. The spatial expression patterns of cytokinin metabolic genes and the unequal distribution of tZ- and iP-type cytokinins in vascular transporting systems suggest that cytokinins act as both local and long-distance signals. Indeed, evidence for cytokinin acting as long distance signal [9,10] and local signal mediators [4,11] is available. Plants respond to cytokinin via a two-component signalling pathway. According to the current model in *Arabidopsis*, three *ARABIDOPSIS* HIS KINASE, AHK2, AHK3 and AHK4/WOL1/CRE1 [originally independently isolated as WOODENLEG 1 (WOL1) and CYTOKININ RESPONSE 1 (CRE1)] act as transmembrane cytokinin receptors [12–14] (Figure 1). These receptors transfer the signal via phosphorylation to the nucleus, activating two classes of primary *ARABIDOPSIS* RESPONSE REGULATORS (ARRs) denominated type-A and type-B response regulators [15,16] (Figure 1). Type-B ARR act as transcription factors [14,17] and induce the transcription of cytokinin primary response genes, including type-A ARR [14,16]. Whereas type-B ARR are positive regulators of the cytokinin response [14,17], type-A ARR are negative regulators of cytokinin signalling [14,16] (Figure 1). Thus, as in the case of auxin signal transduction, the cytokinin effectors network is regulated by a negative feedback loop to control the magnitude of subsequent responses (Figure 1). Recently, early components of the cytokinin response pathway have been discovered: the *CYTOKININ RESPONSE FACTORS* (CRFs), which belong to the *AP2 Arabidopsis* gene family and are transcriptionally induced through the cytokinin two-component signalling pathway, in tandem with the type-B ARR. It has been proposed that the activated CRFs, together with the activated type-B ARR, mediate

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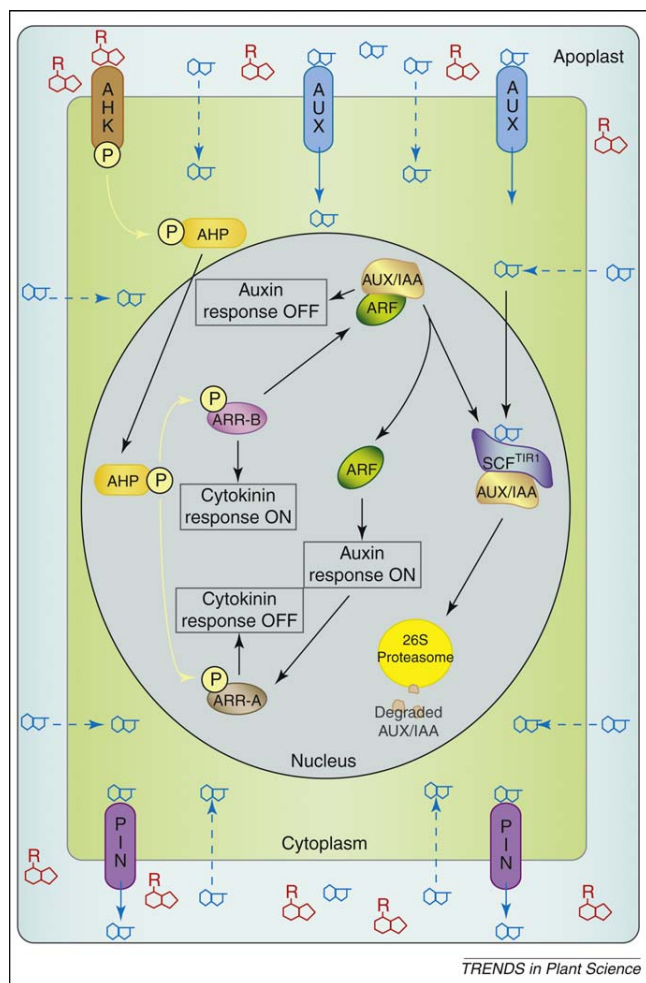


Figure 1. Cytokinin and auxin signalling interactions.

Cytokinins (red molecules) are perceived by the AHK receptors, which act as a histidine kinase. The phosphoryl group (P) on the His of the receptor is transferred via a conserved Asp residue on its receiver domain to a conserved His on an authentic *ARABIDOPSIS* HIS PHOSPHOTRANSFER PROTEIN (AHP) in the cytoplasm (yellow arrows indicate the phosphotransfer). The genome of *Arabidopsis thaliana* encodes for five authentic AHPs (AHP1–AHP5), which move into the nucleus and transfer the phosphoryl group to the type-A or type-B ARR (*ARABIDOPSIS* RESPONSE REGULATORS) cytokinin primary response gene [14]. The type-B ARRs act as transcription factors and their phosphorylation activates the transcription of the cytokinin-regulated genes, including the type-A ARRs (cytokinin response ON). Phosphorylated type-A ARRs activate negative regulation of cytokinin signalling through as yet unknown mechanisms (cytokinin response OFF) [14]. Auxin (blue molecules) can freely diffuse across the plasma membrane (blue broken arrows), and is also actively taken up from the apoplast by the action of influx transporters AUX/LAX (AUXIN-RESISTANT MUTATION 1/LIKE AUX1) and actively transported out of the cell by auxin efflux carriers, the PIN proteins. Auxin flux direction (solid blue arrows) depends on the PIN subcellular asymmetric localization [31]. When concentration of auxin in the cell is low, the Aux/IAA (auxin/indole-3-acetic acid) proteins heterodimerize with the ARF (AUXIN RESPONSE FACTOR) transcription factors, repressing the transcription of the auxin-response genes (auxin response OFF) [23]. At high auxin concentrations, auxin binds to the TIR1 (TRANSPORT INHIBITOR RESPONSE 1) receptor, stimulating the interaction of the Aux/IAAs proteins with the SCF^{TIR1} ubiquitin-ligase complex (SKP1, CUL1, CUL5, E-box), thus promoting their degradation by the 26S proteasome [23]. The consequent reduction in levels of Aux/IAA proteins releases the ARFs from their inhibition, inducing the expression of auxin-responsive genes (auxin response ON) [23]. The type-B ARRs can activate transcription of the Aux/IAA genes suppressing auxin signalling [44], whereas as yet unidentified auxin signalling components suppress cytokinin signalling, activating type-A ARRs [55].

cytokinin-regulated gene expression, by affecting an overlapping set of target genes [18].

However, little is known about how the cytokinin signalling components described above control specific devel-

opmental output. Cytokinin signalling and perception, mediated by the AHK4/CRE1 receptor and the AHP6 histidine phosphotransfer protein, are necessary for vascular tissue development [19], whereas activation of the type-B transcription factor ARR2 by the AHK3 receptor is necessary to control leaf longevity in *Arabidopsis* [20]. Furthermore, it has been shown that, in the root meristem, cytokinin perceived by an AHK3/ARR1, AHK3/ARR12 two-component signalling pathway acts in specific developmental domains to control cell differentiation rate, thus controlling root meristem size and root growth [4].

Auxin biosynthesis, transport and signalling

Auxin is the most studied plant hormone and has been shown to be involved in controlling fundamental aspects of plant development, such as cell fate determination, cell division and cell polarity [21–23]. The exact sites of auxin biosynthesis in the plant are unknown, whereas molecular components of the auxin biosynthesis pathway have been identified (reviewed in Ref. [24]). Among these molecular components, the *YUCCA* (*YUC*) gene family is the best characterized and encodes flavin monooxygenase-like enzymes that are necessary for the rate-limiting conversion of tryptamine into *N*-hydroxyl-tryptamine [25]. The overexpression of *YUC* genes and loss-of-function of multiple *yuc* mutants show many developmental defects that correlate well with the amount of auxin overproduction and reduction, respectively [26,27]. Interestingly, *yuc* multiple mutants are not rescued by exogenous auxin, demonstrating that spatially and temporally regulated auxin biosynthesis by the *YUC* genes is essential for correct development [26].

From its sites of biosynthesis, auxin is actively transported by influx and efflux carrier proteins to specific tissues, where it triggers a signalling cascade resulting in specific developmental responses. In *Arabidopsis*, the most-studied auxin transporters are the PIN genes, which form a small family of eight members, most of which appear to facilitate cellular auxin efflux [28] (Figure 1). Members of the PIN protein family are homologous and functionally redundant, as indicated by the increasingly severe phenotypes of multiple *pin* mutants [29,30]. The most distinctive aspect of PIN proteins is their asymmetric localization within auxin transport-competent cells [31]. The polarity of PIN localization correlates well with the direction of auxin transport and with the local accumulation of auxin in adjacent cells [22], suggesting that PIN polarity determines the direction of intercellular auxin flow (Figures 1,2).

Other probable auxin transporters in *Arabidopsis* belong to the multidrug resistance-like (MDR) p-glycoprotein (PGP) family of membrane proteins. In most cases, PGP proteins are localized within cells without pronounced asymmetric distribution, but, in specific cases, polar distribution has also been observed. Interestingly, in heterologous systems such as yeast or mammalian HeLa cells [32], some PGPs are capable of transporting different auxins across the plasma membrane out of (PGP1) or into (PGP4) the cell [33,34].

In addition to PIN- and MDR-type auxin transporters, a family of putative auxin influx carriers has been identified

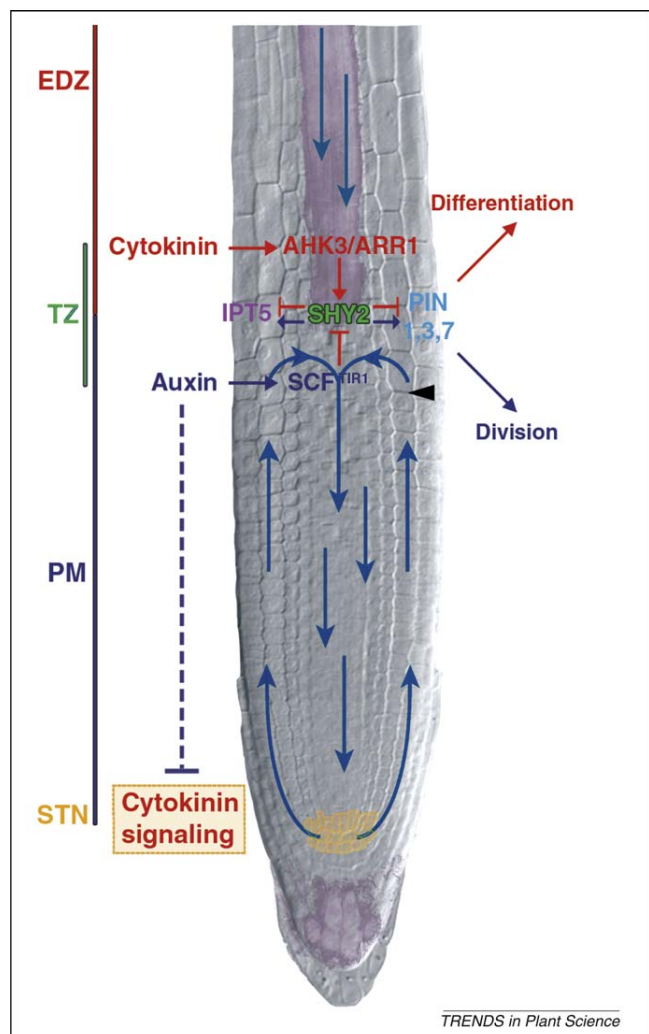


Figure 2. Cytokinin and auxin crosstalk during root meristem size determination. Longitudinal view of the *Arabidopsis* root meristem, where the stem cell niche (STN) [48], the proximal meristem (PM), the elongation differentiation zone (EDZ) and the transition zone (TZ) can be identified [4]. The TZ encompasses the boundary between the PM and the EDZ. The TZ is different for each cell type, giving a jagged shape to the boundary between dividing and expanding cells. Black arrowhead indicates the cortex tissue file TZ. Dark-green arrows represent PINs-mediated auxin flux direction, and purple labelling represents the cytokinin biosynthesis gene *IPT5* (ATP/ADP-isopentenyltransferase 5) expression. At the vascular tissue TZ, cytokinin-mediated activation of the *SHY2* (SHORT HYPOCOTYL 2) gene, through the AHK3/ARR1 two-component signalling pathway, leads to *PIN1*, *PIN3* and *PIN7* downregulation and cell differentiation [44]. By contrast, auxin mediates *SHY2* protein degradation through the SCF^{TIR1} complex, thus sustaining PINs activity and cell division [44]. The *SHY2* negative control on auxin transport on the one hand, and of cytokinin biosynthesis (through *IPT5* induction) on the other, confer robustness to the cytokinin-auxin feedback loop regulation [44]. In post-embryonic STN (cells highlighted in yellow), auxin might repress cytokinin signalling activating *type-A ARR*s (blue broken arrow), prompting cell division [55].

in *Arabidopsis*. Specifically, *AUX1* (AUXIN-RESISTANT MUTATION 1) and its related *LAX* (LIKE AUX1) proteins [35,36] are suggested to be involved in active cellular auxin uptake that is thought to occur in addition to diffusion [36] (Figure 1). Auxin influx and efflux carriers finely regulate auxin distribution in plant tissues and organs [31]. For example, in the root meristem, the *PIN* genes are responsible for accumulating auxin in a distal position and this auxin maximum was demonstrated to be instructive for patterning cell fate and cell polarity [21]. Recent use of

multilevel computational modelling of auxin diffusion and permeability demonstrated that a robust auxin maximum and an auxin gradient can be maintained in the root meristem and that the establishment of this gradient can have instructive functions in patterning and meristem zonation [37]. In this model, the main players are the polarly localized PIN proteins, which are necessary and sufficient to produce the auxin maximum and gradient [37].

As it reaches its site of action, auxin is perceived by receptors of the TRANSPORT INHIBITOR RESPONSE 1 (TIR1) family [38,39]. *TIR1* encodes for an F-box subunit of the ubiquitin ligase complex SCF^{TIR1} (SKP1, CDC53/CULLIN, F-box); when auxin binds to the TIR1 receptor, it stabilizes the interaction between TIR1 and the AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) proteins [40] (Figure 1). This interaction results in Aux/IAA ubiquitination and subsequent 26S-proteasome mediated degradation (Figure 1). The Aux/IAA proteins act as auxin-response inhibitors by forming heterodimers with the ARF (AUXIN RESPONSE FACTOR) transcription factors, thereby preventing activation of auxin-responsive genes [41] (Figure 1). The TIR1-mediated Aux/IAA protein degradation releases the ARFs from their inhibitory effect, prompting activation of the auxin-responsive genes, among which are also the *Aux/IAA* genes [23,42] (Figure 1). Degradation of the Aux/IAA proteins and consequent ARF activity depends on auxin concentration: high levels of auxin cause Aux/IAA degradation, whereas, at low auxin levels, these proteins are stable and interact with the ARFs [41] (Figure 1). This mechanism, together with regulated stability of Aux/IAA proteins, provides a self-regulatory loop for auxin-induced gene expression [23].

Cytokinin and auxin crosstalk

During the past few years, much pharmacological, genetic and transcriptomic evidence has accumulated that confirms the importance of cytokinin-auxin interactions during plant development. Auxin controls cytokinin biosynthesis [43] via the specific activation of *IPT5* and *IPT7* [7] and most auxin-resistant mutants also show changes in their cytokinin sensitivity [44,45]. Moreover, several transcriptome studies have shown that cytokinin and auxin mutually regulate their signalling factors and/or their metabolism [46,47]. Nevertheless, only recently have researchers begun to understand the molecular mechanisms through which these two hormones interact to produce a specific developmental output.

For example, cytokinin-auxin antagonistic interactions have been shown to be fundamental in controlling root development [44] and post-embryonic root growth is sustained by the root meristem (Figure 2). In *Arabidopsis*, stem cells localized in a stem cell niche in the apex of the root meristem [48] (Figure 2) generate daughter cells, which undergo additional division in the proximal meristem, and differentiate at the distal transition zone (TZ) that encompasses the boundary between dividing and differentiating cells of the different cell files [4] (Figure 2). For meristem maintenance and, therefore, to enable continuous root growth, the rate of cell differentiation must equal the rate of generation of new cells.

Cytokinin and auxin have a crucial role in the control of this balance.

It has recently been shown that cytokinin controls the rate of meristematic cell differentiation, thus contributing to the determination of the *Arabidopsis* root meristem size [4]. Application of cytokinin causes a decrease in root meristem size because of a progressive decrease in meristematic cell number, and cytokinin biosynthesis and signalling mutants display a larger root meristem owing to an accumulation of meristematic cells [4]. Furthermore, experiments of tissue-specific depletion of cytokinin, and the expression pattern of different cytokinin signalling genes, demonstrate that cytokinin specifically acts at the vascular tissue of the root meristem transition zone, where an AHK3/ARR1, AHK3/ARR12 two-component signalling pathway perceives cytokinin and controls the differentiation rate of all the other root cells [4] (Figure 2).

Experiments involving application of exogenous auxin (resulting in an increase of meristem size) and the effects of mutations in the *PIN* auxin efflux facilitators (which produce a shorter meristem compared with wild-type plants) are consistent with a role of auxin in controlling cell division [4,29]. It was therefore suggested that the balance between the antagonistic effects of cytokinin, which mediates cell differentiation at the transition zone, and auxin, which mediates cell division, establishes the size of the *Arabidopsis* root meristem [4]. The nature of this antagonistic interaction has recently been unveiled, showing that the balance between cell differentiation and cell division necessary for controlling root meristem size and root growth is the result of the crosstalk between cytokinin and auxin. This occurs through a simple regulatory circuit converging on the *SHY2* (SHORT HYPOCOTYL 2) gene, a member of the auxin repressor *Aux/IAA* gene family [44] (Figure 2).

Experiments using chromatin immunoprecipitation demonstrate that the primary cytokinin-response transcription factor ARR1 directly binds and activates the promoter of *SHY2* specifically at the vascular tissue transition zone [44]. In wild-type roots, transcription of *SHY2* is enhanced by cytokinin application, whereas no upregulation was observed when cytokinin was added to *arr1* mutant roots [44]. Furthermore, the *shy2-31* loss-of-function mutant allele [49] displays larger root meristems, mimicking the effects of auxin application, as also observed in cytokinin signalling mutants [4,44]. By contrast, the *shy2-2* gain-of-function mutant allele (which has a non-degradable version of the SHY2 protein [50]) has smaller meristems, mimicking the effects of cytokinin application [4,44]. These results indicate that the SHY2 protein is both necessary and sufficient for the cytokinin-mediated control of root meristem size.

Activation of *SHY2* results in repression of auxin signalling that negatively regulates the auxin transport facilitators *PIN* genes [44] (Figure 2). Accordingly, *PIN* mRNA levels are low in the *shy2-2* gain-of-function allele, whereas in the *shy2-31* loss-of-function allele, the domain of expression of the *PIN* genes is expanded specifically at the vascular transition zone [44]. Conversely, auxin mediates degradation of the SHY2 protein [41,44,50], thus sustaining the activity of the *PIN* genes, and positively regulating

root meristem size [44] (Figure 2). Hence, application of auxin to the *shy2-2* gain-of-function mutant allele did not trigger an increase in the *PIN* expression or root meristem size [44].

SHY2 also controls cytokinin biosynthesis (Figure 2), as the expression of the *IPT5* gene in the *shy2-31* and *shy2-2* mutants was shown to be higher and lower, respectively, than in wild-type plants [44]. Thus, cytokinin and auxin antagonistically interact at the vascular tissue of the transition zone to balance cell differentiation with cell division (and to determine root meristem size) by controlling in opposite ways the abundance of the SHY2 protein [44] (Figure 2). Cytokinin reduces auxin response by activating (via the AHK3/ARR1 two-component signalling pathway) transcription of the *SHY2* gene; the SHY2 protein, in turn negatively regulates the expression of the *PIN* genes [44] (Figure 2). The resulting redistribution of auxin leads to cell differentiation of all the other neighbouring tissues, thus reducing root meristem size. Conversely, auxin controls root meristem growth by directing degradation of the SHY2 protein, thus sustaining the activity of the *PIN* genes and cell division [44] (Figure 2). The SHY2 protein negatively controls auxin transport on the one hand and cytokinin biosynthesis on the other, thus conferring robustness to the auxin cytokinin feedback loop (Figure 2).

Beyond the maintenance of the root meristem, the negative effect of cytokinin on auxin signalling and transport might be of general significance in explaining the antagonistic interaction of cytokinin–auxin. The two hormones are known to have opposite effects on *de novo* auxin-induced organogenesis [51], lateral root formation [52] and leaf position determination [53,54]. In all these processes, the cytokinin–auxin antagonistic effects can be explained by a negative control of cytokinin on the PIN-dependent auxin distribution.

The cytokinin–auxin antagonistic interaction is also crucial for specifying the embryonic root stem cell niche during *Arabidopsis* embryogenesis [55]. The *Arabidopsis* embryonic root stem cell niche is initiated by the specification of a single cell, the hypophysis. By combining a new visualization tool for cytokinin two-component-output sensor and inducible genetic manipulations, it has been shown that an auxin maximum at the hypophysis activates transcription of the type-A *ARR7* and *ARR15* genes, two negative regulators of cytokinin signalling [55]. The consequent suppression of cytokinin output is necessary to enable normal embryonic stem cell niche formation. Accordingly, lack of both the *ARR7* and *ARR15* genes prevents the establishment of a normal embryonic root [55]. Thus, to specify root stem cell niche, auxin mediates cytokinin signalling suppression [55]. It is tempting to speculate that post-embryonic root growth depends on the coordinated activities of the cytokinin-mediated auxin-signaling suppression at the transition zone promoting cell differentiation, and on the auxin-mediated cytokinin-signalling suppression at the stem cell niche, stimulating cell division (Figure 2).

Concluding remarks and prospects

Important advances have been made in our understanding of the crosstalk between cytokinin and auxin. The root

meristem appears to be maintained and limited by different ratios of the two hormones in the meristem and at the borders of the meristematic zone. Auxin induces cell division in the meristem, whereas cytokinin controls the switch from meristematic to differentiated cell suppressing auxin signalling and transport at the transition zone. Thus, their antagonistic interaction is important for the formation and maintenance of the root transition zone. An important point to be clarified is whether the same mechanisms are also involved in determining the boundary of other organs; that is, whether this type of control could explain the positioning of the shoot meristem border as well as the growth of lateral organ. With the large data sets of hormone-responsive genes collected from genomic studies in different systems at hand, the search for the involvement of this remarkably flexible and simple circuit in different developmental contexts can now begin.

Acknowledgements

We thank Paolo Costantino, Pascal te Welscher, Serena Perilli and Raffaele Dello Ioio for helpful discussions and comments on the manuscript. This work was supported by The Giovanni Armenise-Harvard Foundation career development grant.

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