Cytokinins and shoot development

Stephen H. Howell, Sonia Lall and Ping Che

Plant Sciences Institute, Iowa State University, Ames, IA 50011, USA

Cytokinins promote the unfolding of a complex gene expression program in tissue culture that results in the formation of shoots. Much has been learned about cytokinin signaling in the past few years; the challenge now is to understand how known steps in cytokinin signaling interface with the process of shoot development in culture and *in planta*. Several ways have been found to block or bypass the requirement for cytokinins, and these findings have revealed key control steps in the shoot development process. Plant control is exercised not only in the promotion of shoot development, but also in its restraint under conditions where shoots are not ordinarily formed.

It is nearly 50 years since Folke Skoog and Carlos Miller [1] described the hormonal control of organ regeneration in plants, and yet developmental biologists continue to be fascinated by the capacity of plants to regenerate shoots. Despite the interest in regeneration as a developmental process, shoots are induced routinely in tissue culture for plant propagation and transgenic plant production purposes, typically by the addition of the hormone, cytokinin (CK).

Role of cytokinins in shoot development *in planta*

How do CKs act, and do they play the same role *in planta* as they do in tissue culture? These questions have been addressed in mutants and in transgenic plants in which the endogenous levels of CKs have been raised or lowered. For several years, investigators manipulated endogenous CK levels *in planta* by expressing bacterial isopentenyl transferase (*ipt*) genes in transgenic plants [2–22]. Overexpression of bacterial *ipt* genes in these studies is reported to elevate levels of zeatin (Z)-type CKs and affect a variety of traits including shoot development, leaf senescence and tolerance to stress. Typically, it was thought that Z-type CKs are synthesized by bacterial IPTs from precursor isopentenyl (IP)-type CKs (formed by conjugating IP-side chains to adenine nucleotide), which are then converted to Z-type CKs by stereo-specific hydroxylation on the IP side-chain (e.g. [23]). However, newer studies suggest that bacterial IPTs synthesize Z-type CKs directly through an IP-independent pathway, perhaps involving the conjugation of terpenoid side chains [24].

Recently, plant *ipt* genes have been discovered [25,26]. Nine *IPT* genes have been identified by sequence searches in the *Arabidopsis* genome; seven are CK biosynthetic genes related to bacterial *ipt* genes and two others are tRNA-*ipt* genes [27]. Multiple *IPT* genes suggest a high level of developmental and/or tissue-specific control of CK biosynthesis. Expression of some plant *ipt* genes has been manipulated experimentally. For example, an *ipt* gene, *Shooting* (*Sho*), in petunia has been activationally tagged resulting in a line with enhanced shoot formation in tissue culture and bushier plants (reduced apical dominance) when grown in soil [28]. Unlike, the overexpression of bacterial *ipt* genes, *Sho* expression enhances the accumulation of IP-type CKs in petunia and in transgenic tobacco expressing *Sho*. Estradiol-induced expression of *AtIPT8* (also known as *PGA22*) in *Arabidopsis* also results in elevated levels of IP-type CKs [29]. Thus, overexpression of some plant *ipt* genes results in the accumulation of a different spectrum of CKs than overexpression of bacterial *ipt* genes, which, in turn, appears to have somewhat different developmental consequences.

Other mutations have been found with high endogenous CK levels. The *Arabidopsis* mutant *altered meristem program 1* (*amp1*) shows pleiotropic phenotypes including elevated levels of endogenous CKs, altered shoot apical meristems, increased cell proliferation and cyclin (cycD3) expression, increased polycotyly, constitutive photomorphogenesis and early flowering time [30–33]. *amp1* is allelic to *primordia timing* (*pt*), *constitutively photomorphogenic 2* (*cop2*) and *hauptling* (*hpt*), and *AMP1* was found to encode a glutamate carboxypeptidase [34]. This finding was unexpected and given that *AMP1* might have effects other than CK metabolism, the phenotypes attributed to CK overproduction in the mutant need to be revisited. Another mutant called *high shoot-organogenic capacity* (*hoc*) also has high endogenous CK levels, produces shoots in culture in the absence of CK and has other whole plant characteristics typical of CK overproduction [35]. However, until the genes are identified, it is premature to associate the phenotype of *hoc* and other related mutants, such as *cristal1* (*cri1*) [36], with CK overproduction.

Others have attempted to reduce CK levels by overexpressing CK oxidase, an enzyme involved in CK catabolism and first cloned from maize [37]. The expression of an *Arabidopsis* CK oxidase in transgenic tobacco results in plants with reduced levels of IP and Z-ype CKs, stunted shoots with smaller apical meristems and much reduced leaf production [38]. Reduction in CKs has opposite effects on shoots and roots. Root meristems in CK oxidase-expressing plants enlarge, leading to the production of faster-growing and more branched roots. Thus, CKs appear to have important roles in shoot (and root) development *in planta* – confirming the observations made in tissue culture.

Corresponding author: Stephen H. Howell (shh@iastate.edu).

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However, it is not clear whether shoot development in tissue culture is fully comparable to that *in planta*. In tissue culture, hormone levels have been optimized to achieve desired effects and not necessarily to mimic physiological situations. The quantitative and qualitative differences between hormone levels in tissue culture and *in planta* might mean, for example, that receptors with different affinities for CKs are activated. Therefore, it is important to compare developmental phenomena in tissue culture with those *in planta*, if possible.

Cytokinin signaling

In the past few years, the basic structure of CK signaling pathways has been largely worked out in *Arabidopsis* (reviewed in [39–44]). The first major breakthrough was the identification by activational tagging of *CYTOKININ INDEPENDENT 1* (*CKI1*), a gene that confers CK-independent shoot formation from callus in *Arabidopsis* tissue culture [45]. *CKI1* encodes a histidine kinase (HK) related to sensory elements or receptors in two-component signaling pathways [46–48]. At first, *CKI1* was hypothesized to be a CK receptor [45] but it has not been shown to bind CKs at physiological CK levels, therefore its role in CK signaling is still unclear.

CYTOKININ RESPONSE 1 (*CRE1*), an *Arabidopsis* gene discovered in a search for a loss-of-function mutant defective in shoot formation in culture, is generally accepted to encode a bona fide CK receptor [49]. *CRE1* (or *AHK4*) encodes a hybrid HK with a sensor domain and two response regulator domains (Fig. 1). *CRE1* functions as a CK receptor when tested in a heterologous system [49–51] and is a member of a clade in the *Arabidopsis* HK gene family, which includes *AHK2* and *AHK3* [39,40], both of which have CK receptor activity [42]. *cre1-1* mutants do not have obvious shoot development defects *in planta*. However, a previously described mutant, *wooden leg* (*wol*), which was later recognized to be an allele of *cre1*, affects vascular formation in the root [52]. Thus, *CRE1* might be a CK receptor that is primarily expressed and/or functions in roots.

CK receptors are thought to transmit CK signals in a multi-step phosphorelay system [53] to the nucleus via nuclear shuttle proteins called histidine phosphotransfer proteins (HPts or AHPs in *Arabidopsis*) (Fig. 1). *Arabidopsis* encodes six AHPs [40], and a GFP-tagged form of AHP1 accumulates in the nucleus of *Arabidopsis* protoplasts treated with *trans*-zeatin [54]. Overexpression of *AHP2* (driven by the 35S promoter) has no immediate effect on plant development, However, in transgenic plants bearing 35S:*AHP2* constructs, inhibition of root elongation is more sensitive to CKs [55].

Nuclear CK effectors are response regulators (RRs) that come in two flavors – A- and B-type (Fig. 1) [39–41]. B-type RRs have putative transcriptional activator domains at their C-termini whereas A-type do not [56]. Transfection of protoplasts with constructs encoding B-type RRs transactivates target genes, demonstrating that B-type RRs can function as gene expression activators [54,57]. Some A-type RRs, in certain contexts, are thought to act as repressors, and others appear to function as activators [58]. For example, transfection of constructs

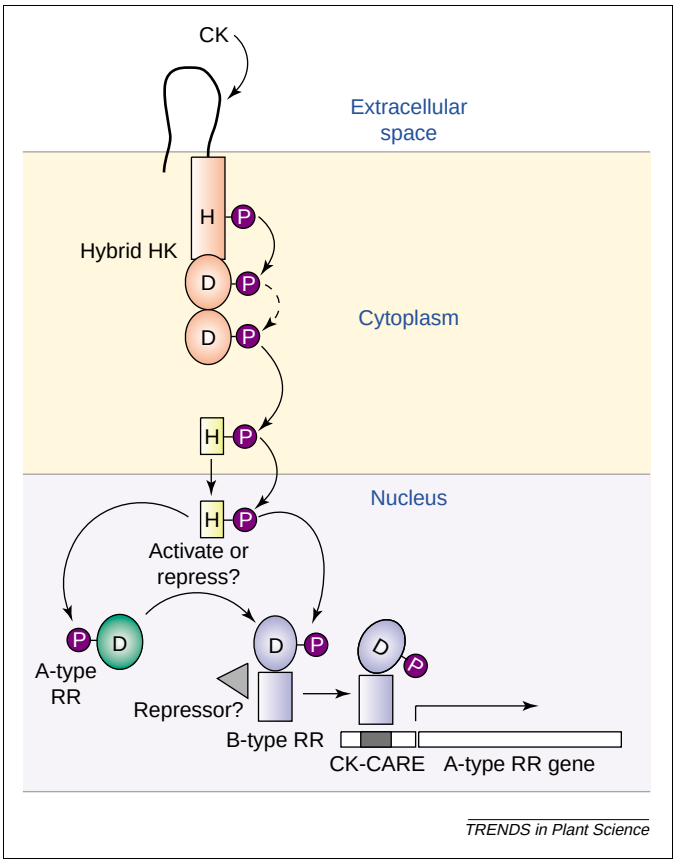

Fig. 1. Generic cytokinin (CK) signaling pathway model represented as a multi-step phosphorelay system involving several modular components. Membrane-localized sensory histidine kinases (HKs) serve as CK receptors and upon activation, transfer phosphoryl groups to histidine phosphotransfer elements (HPs or HPts). HPs are proposed to act as shuttles that transfer their phosphoryl groups and activate nuclear-localized response regulators (RRs). B-type RRs have DNA binding domains and have been reported to function as transcriptional activators for genes such as those encoding CK-activated A-type RRs. A-type RRs are thought to act as repressors and/or transcriptional activators, perhaps by interaction with B-type RRs. Other unidentified elements might function as repressors of B-type RRs, and the signal from an activated HP could serve to liberate a B-type RR from a repressor. Abbreviations: CARE, *cis*-acting regulatory element; D, aspartic acid; H, histidine.

encoding A-type RRs (*ARR4-7*) in *Arabidopsis* protoplasts interferes with the CK activation of target gene expression [54]. The *Arabidopsis* genome encodes several RRs, 10 to 11 A-type and 11 to 12 B-type RRs [39,40]. The large number of RR genes suggests that there is a diversity of functions and/or targets [27]. It will be interesting to determine in the future whether CK signals are transmitted by cognate interactions between specific HPts and their partner RRs or whether there are generic interactions and points of competition and crosstalk between signaling pathways [42].

Potential targets of B-type RRs have been identified through studies of genes that are rapidly upregulated by CKs. Ingrid Brandstatter and Joseph Kieber [59] found that some genes encoding A-type RRs are rapidly upregulated, and given that certain A-type RRs are thought to act as repressors, rapid activation of these genes might involve feedback repression of CK responses [54]. B-type ARRs bind to *cis*-acting regulatory elements (CAREs) bearing 5′(G/A)GAT(T/C)-3′ motifs [57], which are found in the promoters of some A-type ARR genes [54]. Although it has not yet been shown directly that these

elements are responsible for A-type ARR gene upregulation, identification of CK-responsive CAREs might help to identify promoters of other genes that are targets of B-type RR action.

Shoot development program

Given what we know about CK signaling, how do CKs set in motion a program of shoot development? CKs might activate a gene expression cascade in which B-type RRs upregulate a group of genes (encoding, for example, transcription factors and signaling components) that in turn might activate the expression of another wave of genes. However, this model is too simplistic in several ways because, for example, CKs do not trigger shoot development (e.g. in root explants) but are required until the time of 'shoot commitment' [i.e. the time when explants can form shoots when transferred onto non-hormone-containing basal medium (Fig. 2)]. Andrew Cary *et al*. [60] showed that shoot commitment occurred in *Arabidopsis* root explants midway through the 14-day incubation period on shoot induction medium (SIM).

In *Arabidopsis*, shoots can be induced from root explants in a two-step process – by preincubation on a hormonally balanced callus induction medium (CIM) followed by incubation on a CK-rich SIM [61–63]. During CIM preincubation, root explants 'acquire competence' to respond to the shoot induction signals (Fig. 2). What competence acquisition represents in molecular terms is not understood, but at a cellular level, competence is fully acquired when the pericycle cell layer of the root undergoes a single periclinal division [64].

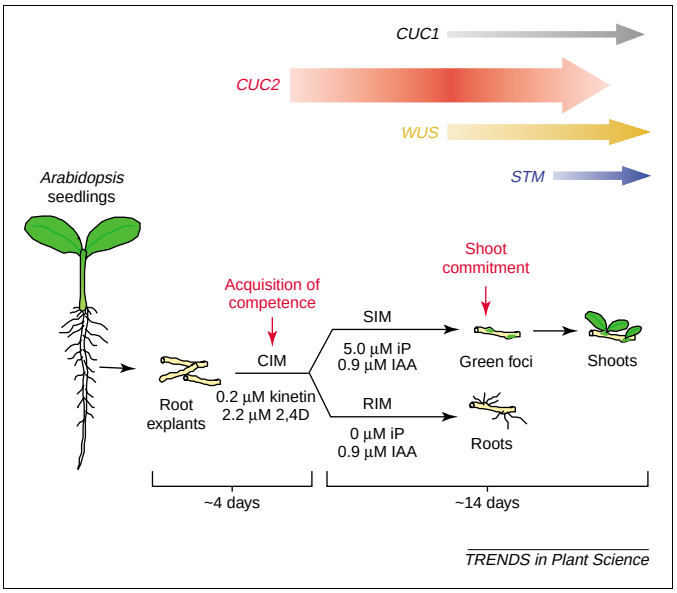

Fig. 2. Developmental events during shoot regeneration from root explants in *Arabidopsis* tissue culture. Explants acquire competence during preincubation on callus induction medium (CIM) to respond to hormone signaling on shoot induction medium (SIM). Midway through the SIM incubation period, shoot development becomes independent of the presence of cytokinins (CKs) (shoot commitment). Colored arrows represent the expression profiles of some of the more important genes involved in shoot meristem development. Arrow width and color gradients approximate relative gene expression intensities. Modified, with permission, from [64]. Abbreviations: 2,4 D, synthetic auxin 2,4-dichlorophenyoxyacetic acid; *CUC, CUP SHAPED COTYLEDON*; IAA, indole-3-acetic acid; iP, N^6-(Δ^2-isopentenyl) adenine; RIM, root induction medium; *STM, SHOOTMERISTEMLESS; WUS, WUSCHEL*.

Ping Che *et al*. [65] used oligonucleotide array analysis to describe the program of gene expression that unfolds in *Arabidopsis* root explants during preincubation on CIM and incubation on SIM. 2–3% of *Arabidopsis* genes are upregulated more than fourfold (compared with time 0) at any time in the shoot development process. The expression profiles that contribute most significantly to the overall variation in gene expression were identified by principal component analysis, which showed most genes were upregulated at only one developmental stage. Thus, the overall picture that emerges is one in which genes are progressively turned on and off during shoot development. The genes that are upregulated at single developmental stages largely fall into functional categories that seem to follow a pattern. Several hormone response genes, in particular, some of the AUX/IAA genes, were upregulated shortly after explanting root segments on CIM [65]. Soon after transfer to SIM, nearly half of the upregulated genes (with assigned functions) encode transcription factors or signaling components. As expected, many genes encoding chloroplast components were upregulated around the time of shoot emergence. Unlike the upregulated genes, it was difficult to generalize about the function of genes that were downregulated at different developmental stages.

Shoot meristems begin to organize (in the *Arabidopsis* shoot regeneration system described above) about the time of shoot commitment and when the gene *CUP SHAPED COTYLEDON 2* (*CUC2*) is highly upregulated [64] (Fig. 2). *CUC1* and *CUC2* encode putative NAC1-domain transcription factors and either one is required *in planta* for *SHOOTMERISTEMLESS* (*STM*) expression and shoot meristem formation [66,67]. Although *CUC1* and *CUC2* act redundantly *in planta*, their effects in tissue culture appear to be additive in that although single *cuc1* and *cuc2* mutants reduce shoot development, the double mutant reduces it even more [68]. *STM* and *WUSCHEL* (*WUS*) are also required for shoot meristem formation or maintenance and are upregulated as meristem organization proceeds [64]. *STM* (in *STM* promoter:GUS constructs) is expressed at presumptive sites of shoot formation in cotyledon explants from *Arabidopsis* [69]. In 35S promoter:*STM* constructs, *STM* is capable of restoring normal shoot meristem formation at the shoot apex in *stm* mutants, even though similar constructs do not promote ectopic shoot formation in wild-type *Arabidopsis* [70]. From this it has been argued that *STM* requires other functions normally expressed in the shoot apex to promote shoot meristem formation. Those functions could be provided or elicited by *WUS* because when *STM* and *WUS* are both expressed in *Arabidopsis* under the control of the 35S promoter, shoot meristems form ectopically on hypocotyls and cotyledons [70]. However, the activity of these ectopic meristems is transient, indicating that *STM* and *WUS*, on their own, cannot sustain functional meristems.

The roles in shoot development of CK and *KNOTTED-1 LIKE IN ARABIDOPSIS THALIANA* (*KNAT1*), a homeobox-containing gene related to *STM*, seem to be intertwined [71]. Recently, it was reported that CK levels regulate *KNAT1* expression, and *KNAT1* overexpression results in higher endogenous CK levels. So, for example, steady-state levels of *KNAT1* are elevated in transgenic

Arabidopsis overproducing CKs (through the action of *Drosophila* heat shock promoter:bacterial *ipt* constructs described above) [2]. Likewise, overexpression of *KNAT1* in *Arabidopsis* promotes ectopic meristem formation on leaves [72], and, in lettuce, leads to the accumulation of IP-type CKs and shifts leaf determinate growth to a shoot-like indeterminate growth [73]. In spite of the apparent interconnections between CK and *KNAT1*, the targets of *KNAT1* action are not known, and it is not understood how CK activates *KNAT1* expression.

Bypassing cytokinins

Other genes have been identified that bypass the requirement for CKs in shoot development. They appear to function in the primary CK signaling pathway or are more immediate effectors of hormone action (Fig. 3). Overexpression of *CKI1* promotes the CK-independent formation of shoots in tissue culture [45]. Overexpression of *ARR2* (a B-type RR in *Arabidopsis*) does the same [54]. The effects of *CKI1* and *ARR2* overexpression are surprising given the expectation that the activation, and not merely the presence of the components, is required for signaling. However, in a protoplast system, transfected *CKI1* stimulated the promoter of an A-type *ARR* gene in the absence of CK [54]. This finding suggests that *CKI1* is constitutively active, and is seemingly capable of signaling shoot development in the absence of added CK. Transfections with *ARR2* constructs basally activated the promoter of an A-type *ARR* gene in the absence CK; however, CK addition resulted in much higher activation [54]. Surprisingly ARR2 was activated even when its phosphoryl acceptor site was incapacitated leading to speculation that ARR2 might also be activated by release from a repressor, in addition to activation by phosphorylation at its phosphoryl acceptor site [54] (Fig. 3). This model provides an explanation to the paradox above – in that *ARR2* overexpression could overwhelm the capacity of a repressor that keeps ARR2 at bay.

Another gene that when overexpressed stimulates shoot development is *ENHANCER OF SHOOT DEVELOPMENT 1* (*ESR1*) (Fig. 3) [74]. *ESR1* was identified by screening a cDNA library for genes that enhance shoot formation in the absence of CKs. *ESR1* encodes an AP2 transcription factor (also known as EREBP), and when *ESR1* expression was controlled with an estradiol-inducible promoter, shoot formation was enhanced by the combination of inducer and CK [74]. Hence *ESR1* appears to interact synergistically with CK and does not bypass the need for the hormone altogether. The endogenous *ESR1* gene is upregulated in root explants one to two days following transfer to SIM, and its upregulation is dependent on prior CIM preincubation [74]. This represents one of the first molecular observations that helps to explain the role of CIM preincubation in *Arabidopsis* shoot development in culture.

Overexpression of either *CUC1* or *CUC2* (driven by the 35S promoter) also promotes shoot formation from callus in the presence of CK [68], much like *ESR1* does (Fig. 3). Hence, *CUC1* and *CUC2* are not able to bypass the requirement for CKs, but their overexpression enhances shoot development in the presence of CKs. It was interesting that overexpression of *CUC1* and *CUC2* in a *stm1* mutant background stimulated adventitious leaf formation but not shoot development [75]. Hence, these genes promote an organogenic process that culminates in the formation of shoots in the presence of active *STM*, but produces only leaves in the absence of *STM* function.

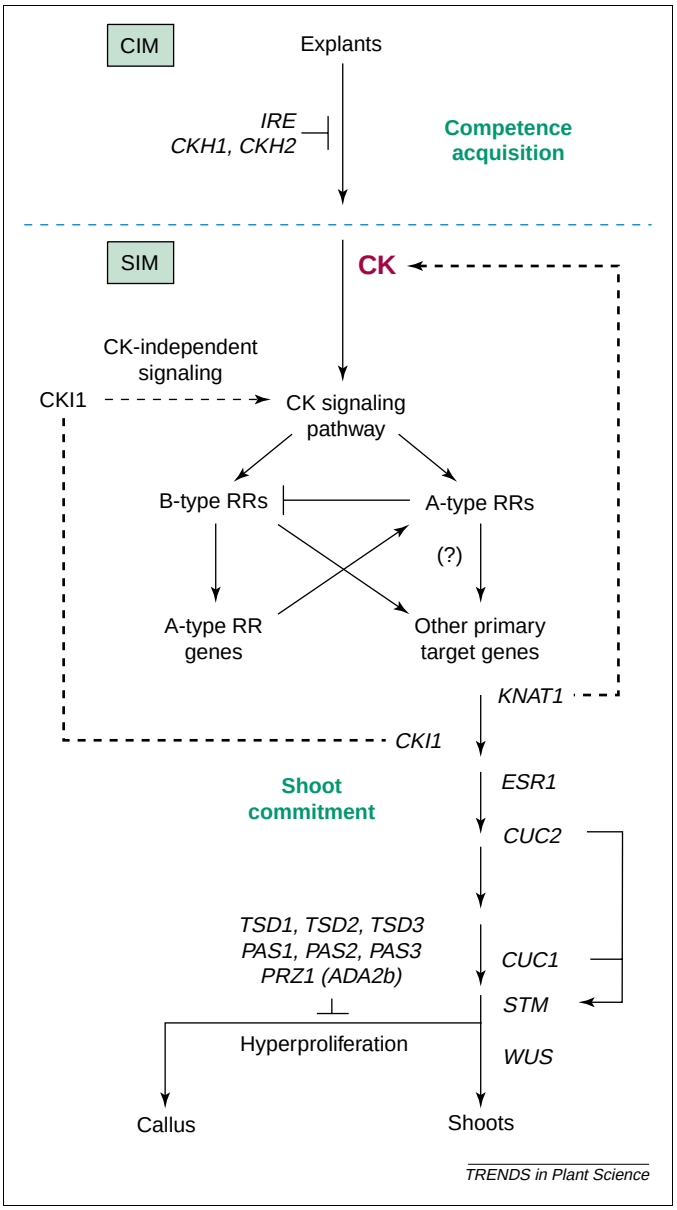

Fig. 3. Hypothetical interaction between cytokinin (CK) signaling and gene action pathways during shoot development. Events during preincubation on callus induction medium (CIM) are shown above the blue broken line, and events following transfer to shoot induction medium (SIM) are shown below the blue broken line. *CKI1* is capable of promoting CK-independent signaling in CKI1-overexpressing plants and is upregulated before shoot commitment. *KNAT*1 overexpression is correlated with higher levels of endogenous CKs. *CUC2* is highly upregulated about the time of shoot commitment and *CUC1* or *CUC2* expression activates *STM* function. The genes on the shoot development pathway are ordered by their patterns of expression, and, with the exception of *CUC1*, *CUC2* and *STM*, the order does not imply a causal relationship in gene action. *TSD*, *PAS* and *PRZ1* are thought to prevent the diversion of the shoot development signal to callus formation. However, in the absence of their function, callus hyperproliferates. Abbreviations: *CKH, CYTOKININ HYPERSENSITIVE*; *CKI, CYTOKININ INDEPENDENT*; *CUC, CUP SHAPED COTYLEDON*; *ESR, ENHANCER OF SHOOT DEVELOPMENT*; *IRE, INCREASED ORGAN REGENERATION*; *KNAT1, KNOTTED-1 LIKE IN ARABIDOPSIS THALIANA*; *PAS, PASTICCINO*; *PRZ, PROPORZ*; RRs, response regulators; *STM, SHOOTMERISTEMLESS*; *TSD, TUMOUROUS SHOOT DEVELOPMENT*; *WUS, WUSCHEL*.

Restraint of shoot development

The program of shoot development appears poised to run in many vegetative tissues, awaiting the appropriate hormone or environmental signals. Because of this, it is likely that certain gene functions restrain shoot development or hold it in check (Fig. 3). The loss-of-function mutation *increased organ regeneration* (*ire*) promotes more robust shoot formation at sub-optimal CK concentration in shoot regeneration from *Arabidopsis* root explants [60]. *ire* has modest effects on CK levels (more elevated Z-riboside-5′-monophosphate levels and somewhat reduced Z-N9-glucoside levels). The normal gene appears to restrain competence acquisition during preincubation on CIM because mutant root explants acquire competence more rapidly than the wild type do when responding to shoot induction signals [60]. *CK hypersensitive 1* and *CK hypersensitive 2* (*ckh1* and *ckh2*) were selected for more prolific callus production from cotyledon explants at sub-optimal CK levels [76]. The mutants did not have increased CK levels and their only major plant phenotype was shortened inflorescences.

Somewhat related are *Arabidopsis* mutants in genes that appear to prevent CK signals from being diverted toward the formation of undifferentiated callus (Fig. 3). Recessive *tumourous shoot development 1*, *2* and *3* mutants (*tsd1*, *2* and *3*) in *Arabidopsis* develop disorganized tumorous tissue instead of organized leaves and stems [77]. The green callus-like tumors that are produced are capable of unlimited growth *in vitro* on hormone-free medium. CK treatment of *pasticcino 1*, *2* and *3* mutants (*pas1*, *2* and *3*) in *Arabidopsis* causes hypertrophy (uncoordinated cell divisions) in developing aerial organs [78]. *PAS1* has been found to encode an immunophilin domain-containing protein [79] and *PAS2* (also named *PEPINO* or *PEP*) encodes an anti-phosphatase [80,81]. In both the *tsd* and *pas* mutants, the expression levels of some of the shoot meristem genes (*STM* and *KNAT1*, *KNAT2* or *KNAT6*) are elevated. No explanation has been offered for this phenomenon but we have represented it as upregulation of the genes in the shoot development pathway when the diversion of the CK signal leads to the hyperproliferation of callus, rather than to the formation of shoots (Fig. 3). Another mutant, *proporz1* (*prz1*) responds to organogenic signals, either auxin or CK, by the production of callus [82]. *PRZ1* is identical to *ADA2b*, a gene that encodes a transcriptional adaptor protein that interacts with HAT AtGCN5, a histone acetylase transferase [83]. The role of these genes in shoot development is not known but it possible that they are modifiers of the primary CK signaling pathway or represent components of undescribed response pathways.

Future directions

The challenge for the future is to understand how primary cytokinin signaling promotes shoot development and how the downstream steps of shoot meristem formation and shoot development are controlled. As yet, we do not even have a rough outline of the gene regulatory steps that give rise to events such as the acquisition of competence and shoot commitment. Understanding the developmental pathway should aid in improving strategies for regenerating recalcitrant plant species, cultivars or varieties.

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