Two-component circuitry in *Arabidopsis* cytokinin signal transduction

Ildoo Hwang & Jen Sheen

Department of Molecular Biology, Massachusetts General Hospital, Department of Genetics, Harvard Medical School, Boston, Massachusetts 02114, USA

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Cytokinins are essential plant hormones that are involved in shoot meristem and leaf formation, cell division, chloroplast biogenesis and senescence. Although hybrid histidine protein kinases have been implicated in cytokinin perception in *Arabidopsis*, the action of histidine protein kinase receptors and the downstream signalling pathway has not been elucidated to date. Here we identify a eukaryotic two-component signalling circuit that initiates cytokinin signalling through distinct hybrid histidine protein kinase activities at the plasma membrane. Histidine phosphotransmitters act as signalling shuttles between the cytoplasm and nucleus in a cytokinin-dependent manner. The short signalling circuit reaches the nuclear target genes by enabling nuclear response regulators ARR1, ARR2 and ARR10 as transcription activators. The cytokinin-inducible *ARR4, ARR5, ARR6* and *ARR7* genes encode transcription repressors that mediate a negative feedback loop in cytokinin signalling. Ectopic expression in transgenic *Arabidopsis* of ARR2, the rate-limiting factor in the response to cytokinin, is sufficient to mimic cytokinin in promoting shoot meristem proliferation and leaf differentiation, and in delaying leaf senescence.

Cytokinins have long been recognized as essential plant hormones that are involved in diverse processes of plant growth and development. These processes include cell division, shoot initiation, leaf and root differentiation, chloroplast biogenesis and senescence[1,2]. The cellular, molecular and biochemical mechanisms underlying the actions of cytonkinins have not been elucidated. However, recent genetic and molecular studies in various plant species have suggested the involvement of two-component signalling proteins in cytokinin signal transduction[3–7].

Two-component systems—consisting of a histidine protein kinase that senses the input and a response regulator that mediates the output—control signal transduction pathways in many prokaryotes and in some eukaryotes. The signalling pathway is initiated when a histidine protein kinase sensor, modulated by an environmental cue, phosphorylates its own conserved histidine residue and transfers the phosphate to a conserved aspartate residue of a response regulator. In some cases, additional phosphotransfer steps may intervene between the histidine protein kinase and response regulator, mediated by a histidine phosphotransmitter[8–10]. The completion of the *Arabidopsis* genome sequence has revealed over 40 genes encoding putative AHK, AHP and ARR proteins, suggesting an important involvement of the ancient and conserved signalling mechanism in many facets of plant cell regulation[11,12]. The identification of conserved histidine protein kinase signature motifs in the photoreceptor phytochrome, a putative osmosensor, and the ethylene and cytokinin receptors of *Arabidopsis* further supports this view[6,13–15].

In eukaryotes, a two-component circuit often provides a link between the sensing of an environmental cue and a MAP kinase (MAPK) signalling cascade. For example, the SLN1/YPD1/SSK1 phosphorelay in yeast translates a change in osmolarity into the phosphorylation state of the response regulator SSK1, which then modulates the HOG1 MAPK cascade in the cytosol to control gene expression[16–18]. Plant histidine protein kinases, such as the ethylene receptor (ETR1) and a putative osmosensor (AHK1), may also use phosphorelays to transmit signals to MAPK cascades[14,15,19,20]. These eukaryotic sensor kinases seem to have negative roles as their inactivation results in downstream signalling. However, the implication of the putative AHK proteins, CKI1 and CRE1 (refs 3, 6, 21), in cytokinin signalling raises the possibility that a two-component system may activate cytokinin signal transduction in *Arabidopsis*. The lack of a physiological and genetically manipulable plant cell

system has made it difficult to discern the mechanism by which a histidine protein kinase may mediate cytokinin signalling or any other plant response.

We have developed a transient expression assay on the basis of the transcriptional activation of a cytokinin primary response gene, *ARR6*, using mesophyll protoplasts of *Arabidopsis* leaves. This assay has enabled us to perform functional genomic analysis of the two-component regulators, and to decipher a cytokinin signalling pathway in *Arabidopsis*. The pathway does not follow the established eukaryotic histidine protein kinase and MAPK cascade model, but rather integrates multiple histidine protein kinase activities to common AHP proteins, which serve as cytoplasm/nuclear shuttles, and to distinct ARR proteins in the nucleus. The *Arabidopsis* cytokinin signal transduction pathway consists of four principal steps: histidine protein kinase sensing and signalling; AHP translocation; ARR-dependent transcription activation; and a negative feedback loop through cytokinin-inducible *ARR* genes. Analyses of transgenic tissues and plants support the importance of this central signalling pathway in diverse cytokinin responses.

Cytokinin-inducible transcription in leaf cells

To elucidate the regulatory circuitry in cytokinin signal transduction, we developed a leaf cell assay on the basis of cytokinin-inducible transcription in *Arabidopsis* mesophyll protoplasts. The 2.4-kilobase (kb) promoter of an *Arabidopsis* cytokinin primary response gene—encoding the response regulator 6 (ARR6)—was fused to the firefly luciferase (LUC) coding sequence to create a reporter construct, *ARR6–LUC*. In transfected protoplasts, the activity of *ARR6–LUC* was specifically induced by cytokinin but not by other plant hormones such as abscisic acid (ABA) or auxin (Fig. 1a). In the same system, the *GH3* promoter was activated only by auxin, whereas the *RD29A* promoter was induced only by abscisic acid (ABA), demonstrating the specificity of three plant hormone responses in protoplasts (Fig. 1a). The activity of *ARR6–LUC* was induced by physiological concentration of a natural cytokinin *trans*-zeatin (*t*-zeatin) from 1 to 100 nM (Fig. 1b). To further show the specificity of the cytokinin response, various active and inactive cytokinin analogues were examined. Only active cytokinins, *t*-zeatin, 2-isopentenyladenine (2-IP) and 6-benzyladenine (BA), induced the reporter gene *ARR6–LUC* (Fig. 1c). The response in the protoplast system is similar to the cytokinin activation of various *ARR* genes shown *in planta*[4,5]. Thus, we have

established a reliable and physiological system to dissect the regulatory components in the cytokinin signal transduction pathway in *Arabidopsis*.

Cytokinin signalling by distinct AHK receptors

CKI1, a hybrid histidine protein kinase with a conserved receiver domain, has been implicated in cytokinin responses[3]. The enhanced CKI1 expression in *Arabidopsis shooty callus* mutants also supports its role in cytokinin signalling[22]. However, the biochemical mechanism of CKI1 function and its role in cytokinin signalling are still unknown. To determine whether CKI1 mediates cytokinin responsive transcription, the full-length *CKI1* gene was cotransfected with

ARR6–LUC into *Arabidopsis* protoplasts. Notably, wild-type CKI1 activated the *ARR6* promoter without exogenous *t*-zeatin (Fig. 1d). It is possible that overexpression of CKI1 renders protoplasts hypersensitive to endogenous cytokinin and/or exceeds the capacity of negative regulators. Alternatively, CKI1 could encode a constitutively active histidine protein kinase connected to the cytokinin signal transduction pathway. Cytokinin treatment did not significantly enhance the reporter gene activity in the presence of CKI1 (Fig. 1d). To see whether the histidine protein kinase activity and phosphoryl transfer is required for CKI1 activation of the *ARR6* promoter, the conserved His 405 and Asp 1050 residues in the histidine protein kinase and receiver domains, respectively, were
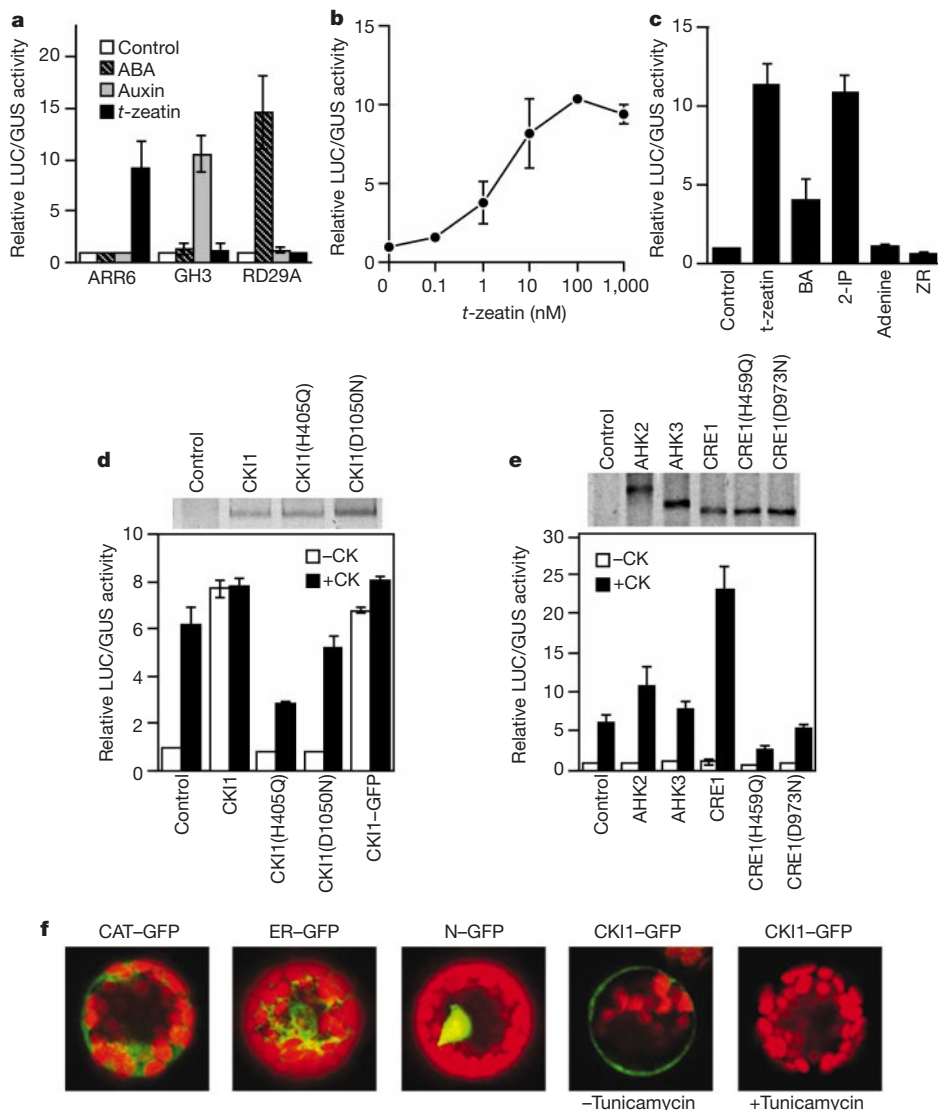

Figure 1 Cytokinin signalling is initiated by multiple histidine protein kinase receptors. **a**, Specificity of plant hormone responses in the *Arabidopsis* mesophyll protoplast transient expression system. Protoplasts were transfected with *UBQ10–GUS* (internal control) and *ARR6–LUC* (ARR6), *GH3–LUC* (GH3) or *RD29A–LUC* (RD29A) plasmid DNA. Transfected protoplasts were incubated without (control) or with 100 nM *t*-zeatin, 1 μM IAA, or 100 μM ABA. **b**, Cytokinin dose response for the *ARR6* promoter induction. **c**, Induction of the *ARR6* promoter by different cytokinins. All chemicals are at 100 nM. BA, 6-benzyladenine; 2-IP, 2-isopentenyladenine; ZR, zeatin ribose. **d**, CKI1 activation of *ARR6–LUC* requires histidine protein kinase activity and phosphoryl transfer. Protoplasts were cotransfected with the *ARR6–LUC* reporter and an effector plasmid expressing CKI1, CKI1(H405Q), CKI1(D1040N) or CKI1–GFP. Vector DNA was used as a control. The top panel shows the ^{35}S-methionine labelled CKI1, CKI1(H405Q) and

CKI1(D1050N) proteins after immunoprecipitation. Transfected protoplasts were treated without (−CK) or with (+CK) 100 nM *t*-zeatin. **e**, CRE1 confers cytokinin hypersensitivity on the activation of the *ARR6–LUC* reporter. Protoplasts were cotransfected with the *ARR6–LUC* reporter and an effector plasmid expressing AHK2, AHK3, CRE1(AHK4/WOL), CRE1(H459Q) or CRE1(D973N). The top panel shows the expression of AHK2, AHK3, CRE1(AHK4/WOL), CRE1(H459Q) and CRE1(D973N) proteins. **f**, CKI1 is localized at the plasma membrane. Protoplasts were transfected with CKI1–GFP or various GFP marker plasmid DNA. Tunicamycin (1 μg ml⁻¹) treatment was for 12 h. The subcellular GFP markers are CAT–GFP for the cytosol, ER–GFP[47] for the endoplasmic reticulum, and N–GFP (DOF–GFP)[48] for the nucleus. The red fluorescence is from chloroplasts.

mutated. Despite comparable expression of the CKI1(H405Q) and CKI1(D1050N) mutants with that of wild-type CKI1, neither mutant could activate the expression of *ARR6–LUC* (Fig. 1d). Furthermore, the CKI1(H405Q) mutant could exert dominant negative effects and diminish the activation of the *ARR6* promoter by exogenous *t*-zeatin. The CKI1(1050N) mutant was less potent. The results suggest that histidine protein kinase activity and phosphoryl transfer from His 405 to Asp 1050 are required for the CKI1 function in activating cytokinin signalling. The dominant negative CKI1(H405Q) mutant might interfere with cytokinin perception and/or disturb downstream signalling.

Another *Arabidopsis* hybrid histidine protein kinase, CRE1/AHK4/WOL, has been shown to be a cytokinin receptor. The evidence is based on the inability of the *cre1* mutant to respond to cytokinin in the shoot induction assay, and the ability of CRE1 to complement histidine protein kinase mutants of budding and fission yeast and *Escherichia coli* in a cytokinin-dependent manner[6,21,23]. As *CRE1/AHK4/WOL* is predominantly expressed in roots[23,24] and the *wol(cre1)* recessive mutants lack an obvious leaf phenotype[6,23,24], it is possible that other closely related *Arabidopsis* histidine protein kinase genes, such as *AHK2*, *AHK3* and *CKI1* (refs 3, 6, 24), provide cytokinin receptor functions. To test directly the function of these histidine protein kinases in cytokinin signalling, a construct expressing AHK2, AHK3 or CRE1(AHK4/WOL) was cotransfected into *Arabidopsis* protoplast with the cytokinin-inducible reporter *ARR6–LUC*. In contrast to CKI1, neither AHK2, AHK3 nor CRE1(AHK4/WOL) caused the activation of *ARR6–LUC* in the absence of exogenous cytokinin. However, cytokinin treatment resulted in further activation of the reporter in the presence of AHK proteins, especially CRE1(AHK4/WOL) (Fig. 1e). We then tested the requirement of the conserved His 459 and Asp 973 residues in the histidine protein kinase and receiver domains, respectively, for the CRE1 activity. CRE1(H459Q) and CRE1(D973N) mutants lost their ability to further enhance *ARR6–LUC* expression in the presence of exogenous cytokinin (Fig. 1e). Both mutants also imposed a dominant negative effect and reduced cytokinin signalling. Similar to the CKI1 mutants, the His mutant is more potent than the Asp mutant, perhaps owing to the bimolecular phosphoryl transfer process between mutant and wild-type histidine protein kinases. These results indicate that cytokinin signals are sensed by multiple histidine protein kinase receptors with different or overlapping expression patterns[24]. Although CRE1 is predominantly expressed in roots[23,24], it can certainly function in leaf cells as a cytokinin receptor. However, CKI1 and CRE1 represent two different types of cytokinin receptors that require histidine protein kinase activity and phosphoryl transfer to initiate cytokinin signalling, but have different sensing mechanisms.

To gain an insight into where cytokinin signalling is initiated, the subcellular localization of CKI1 was examined using the CKI1–green fluorescent protein (GFP) fusion. We first confirmed that CKI1–GFP acted in a similar way as CKI1-haemagglutinin (HA) in the protoplast assay based on *ARR6–LUC* activation (Fig. 1d). Confocal microscopy revealed that CKI1 is mainly localized to the plasma membrane but not in the cytosol, endoplasmic reticulum or nucleus, indicated by various GFP markers (Fig. 1f). As the amino terminus of CKI1 is loaded with putative glycosylation sites, we examined the effect of a glycosylation inhibitor, tunicamycin[25], in CKI1–GFP expression, localization or function. Although tunicamycin did not affect the expression of other GFP constructs (not shown), the expression of CKI1–GFP was completely abolished (Fig. 1f). This result suggests that the glycosylation of CKI1 is probably important for its stability, processing and/or trafficking in plant cells. Whether the N-terminal domain of CKI1 is important for cytokinin binding, proper folding, and/or receptor dimerization awaits further analysis.

AHP proteins as cytoplasm and nucleus shuttles
The analysis of the *Arabidopsis* genome has revealed at least five genes encoding putative AHP proteins with unknown physiological functions[26,27]. To determine whether AHP proteins are involved in cytokinin signal transduction, AHP1, AHP2 and AHP5 were cloned into the plant expression vector and tested in the protoplast cytokinin response assay. AHP1, AHP2 and AHP5 had little effect on the expression of *ARR6–LUC* with or without *t*-zeatin (Fig. 2a).
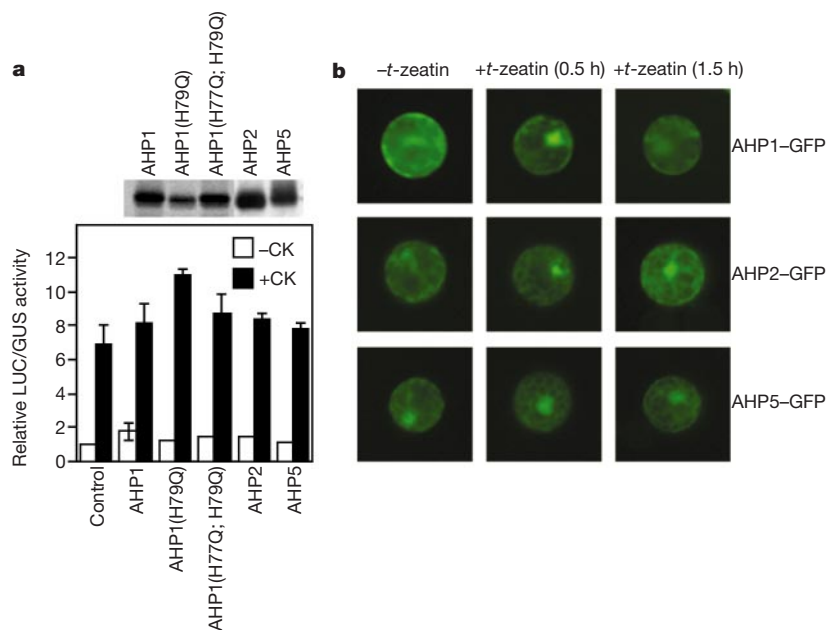
Figure 2 AHP acts as a shuttle between the cytoplasm and nucleus in cytokinin signalling. **a**, AHP overexpression does not affect cytokinin signalling. Protoplasts were cotransfected with the *ARR6–LUC* reporter and an effector plasmid expressing AHP1, AHP2, AHP5 or AHP1 mutant protein. Transfected protoplasts were treated without (−CK) or with (+CK) 100 nM *t*-zeatin. The top panel shows the expression of the AHP proteins. **b**, Cytokinin induces AHP translocation. Protoplasts were transfected with *AHP1–GFP*, *AHP2–GFP* or *AHP5–GFP* plasmid DNA. Transfected protoplasts were observed before and after *t*-zeatin (100 nM) treatment for 30–90 min.

The AHP1 mutant—where the conserved His 79 was replaced with Gln—did not repress the cytokinin activation of *ARR6–LUC*. Even the AHP1(H77Q; H79Q) double mutant was unable to exert dominant negative effect and inhibit the *ARR6–LUC* activation by cytokinin. AHP proteins and AHP1 mutants were expressed at comparable levels in the protoplast system (Fig. 2a). This result indicates that AHP proteins are not the limiting factor, or their action as potential mediators may require cytokinin-dependent modification. To further understand the role of AHP proteins in cytokinin signalling, we followed the action of AHP1–GFP in transfected protoplasts in the absence or presence of cytokinin using fluorescence microscopy. AHP1–GFP was mainly localized in the cytoplasm without cytokinin stimulation, but was translocated into the nucleus after treatment of the protoplast with *t*-zeatin. The cytokinin-dependent translocation of AHP1–GFP was transient, occurring within 30 min after cytokinin treatment (Fig. 2b). AHP2–GFP showed similar cytokinin-dependent translocation, but not AHP5–GFP, suggesting that they have different functions in plant cells.

Opposite functions of two types of nuclear ARR proteins

The requirement of histidine protein kinase activity in the initiation of cytokinin signalling from CKI1 and CRE1, the cytokinin-inducible expression of *ARR6*, the cytokinin-dependent translocation of AHP proteins into the nucleus, and the physical interaction of AHP and ARR proteins in yeast[28–31] strongly suggest that some ARR proteins may act downstream in cytokinin signal transduction. There are two principal subfamilies of *ARR* genes in the *Arabidopsis* genome: the cytokinin-inducible A type and the DNA-binding B type[11,12,31,32]. Their physiological functions are currently unclear. To investigate whether any *Arabidopsis* ARR proteins are involved in cytokinin signalling, we cloned four representative A-type *ARR* genes and three B-type *ARR* genes into the plant expression vector fused to a HA tag or GFP. As shown in Fig. 3a, A-type ARR proteins, such as ARR4, ARR5, ARR6 and ARR7, repressed *ARR6–LUC*

activity induced by 100 nM *t*-zeatin. Although ARR6 seemed to be expressed at lower abundance, its repression activity was the strongest. In contrast, the B-type ARR proteins, such as ARR1, ARR2 and ARR10, markedly activated *ARR6–LUC* expression. Ectopic expression of ARR1, ARR2 and ARR10 was sufficient to activate cytokinin signalling at different levels in the absence of exogenous cytokinin. ARR1 and ARR2 activated *ARR6–LUC* about 40- and 400-fold, respectively. Cytokinin treatment further enhanced the effect of ARR2 on *ARR6–LUC* to over 1,000-fold. The lower activation by ARR1 could be due to its lower expression level in transfected protoplasts. In the absence of cytokinin, ARR10 activated *ARR6–LUC* about 10-fold. Cytokinin enhanced the effect of ARR10 on the *ARR6–LUC* activity another 10–20-fold.

The differential effect of ARR2 and ARR10 on activating cytokinin signalling could be attributed to differences in their intrinsic activities in DNA binding and/or transcription activation. It could also be due to their distinct affinity to endogenous repressors, which probably exist in leaf protoplasts to prevent cytokinin signalling under unstimulated condition. Surprisingly, mutations of the conserved Asp residue in the receiver domains of ARR4(D95N), ARR6(D86N) and ARR2(D80N) did not alter their abilities to reduce or enhance *ARR6–LUC* expression. (Fig. 3a). This result indicates that phosphorelay stimulated by cytokinin may be involved in liberating the positive regulators such as ARR1, ARR2 and ARR10. Ectopic expression of these ARR proteins probably bypasses the negative regulation and causes constitutive cytokinin responses without the signal.

To further test the role of ARR2 in cytokinin signalling, we designed a dominant negative mutant of ARR2 (ΔARR2), which contained only the DNA-binding domain but not the putative transcription activation and the receiver domains[32]. If ΔARR2 can compete with the endogenous ARR proteins in binding to the *ARR6* promoter without transcription activation, it may block cytokinin- or CKI1-dependent activation of *ARR6–LUC*. As shown in Fig. 3b, the dominant negative mutant of ARR2 reduced *ARR6–LUC*
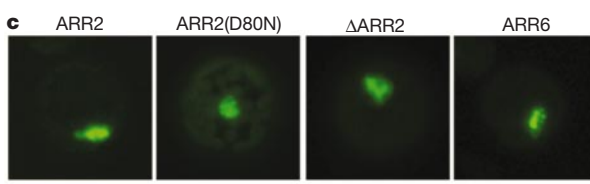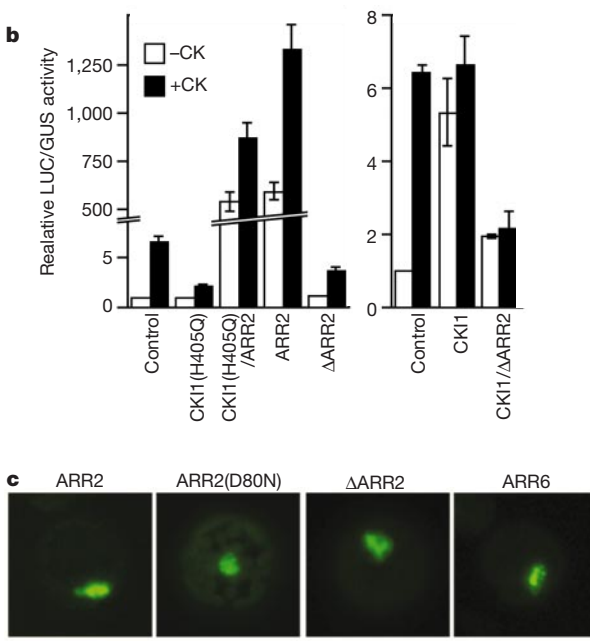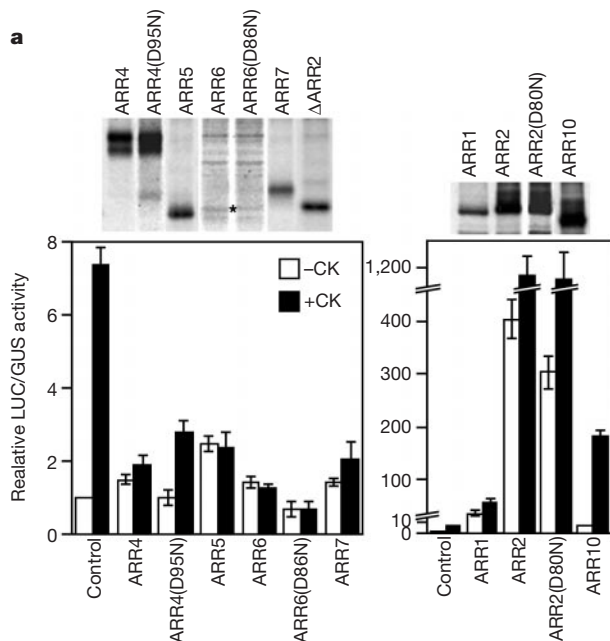

Figure 3 Opposite regulations of cytokinin primary response gene transcription by two types of ARR protein. **a**, Negative and positive regulation by ARR proteins. Protoplasts were cotransfected with the *ARR6–LUC* reporter and an effector plasmid expressing ARR1, 2, 4, 5, 6, 7, 10 or various mutant proteins, ARR2(D80N), ARR4(D95N) or ARR6(D86N). The transfected protoplasts were treated without (–CK) or with (+CK) 100 nM *t*-zeatin. The top panel shows the expression of ARR proteins. The expression of

ARR6 and ARR6(D86N) was lower than the other proteins and required longer exposure. **b**, ARR proteins act downstream of CKI1 in cytokinin signalling. Protoplasts were cotransfected with the *ARR6–LUC* reporter alone or with an effector plasmid as indicated. ΔARR2 is a dominant negative version of ARR2. **c**, ARR proteins are localized in the nucleus. Protoplasts were transfected with *ARR2–GFP*, *ARR2(D80N)–GFP*, *ΔARR2–GFP* or *ARR6–GFP* plasmid DNA and observed with a fluorescence microscope.

expression that was elicited by 100 nM *t*-zeatin or CKI1. Furthermore, the dominant negative effect of the CKI1(H405Q) mutant on the protoplast response to exogenous cytokinin could be bypassed by ectopic expression of wild-type ARR2 (Fig. 3b). This epistatic relationship places ARR2 and/or ARR2-like proteins downstream of multiple histidine protein kinase receptors in the cytokinin signal transduction pathway. The finding of multiple ARR2 binding motifs, (G/A)GAT(T/C), in the promoter regions of *ARR6* and other cytokinin-inducible genes[32] suggests that ARR2 could be a master regulator in cytokinin signalling. To test further the idea that the A-type and B-type ARR proteins are transcription repressors and activators, respectively, in cytokinin signalling, we examined the subcellular localization of ARR6–GFP and ARR2–GFP. Both ARR proteins are exclusively localized in the nucleus regardless of the cytokinin treatment (Fig. 3c), consistent with nuclear localization of ARR1 and ARR2 in onion epidermal cells and parsley protoplasts[31,32]. Their nuclear localization is probably independent of the phosphorylation state as ARR2(D80N)–GFP was found in the nucleus. The ARR2 dominant negative mutant (ΔARR2) was also localized in the nucleus (Fig. 3c). These results indicate that the cytokinin-dependent phosphorelay does not have a role in the nuclear localization and DNA-binding of ARR proteins or their intrinsic transcription activation or repression activities.

Ectopic expression of ARR2 mimics cytokinin responses

In tissue culture, induction of cell proliferation and subsequent shoot formation require cytokinin. To determine whether the same cytokinin signalling pathway is responsible for transcription regulation, cell proliferation, shoot meristem initiation, and leaf formation, we developed a seedling cytokinin response assay. *Arabidopsis* seedlings were stably transformed using *Agrobacterium* carrying *GFP*, *CKI1*, *CKI1(H405Q)*, *ARR2* and *ARR6* constructs in the mini-binary vector pCB302 (ref. 33). Transformation with the pCB301 vector that lacks a phosphinothricin acetyl transferase (*bar*) gene did not result in viable tissues on the selection medium. The transformed seedlings with the GFP control proliferated on the indole-3-acetic acid (IAA) medium. The addition of cytokinin enhanced both cell proliferation and ectopic shoot formation; however, both CKI1 and ARR2 promoted extensive cell proliferation and shoot and leaf formation in the absence of exogenous cytokinin (Fig. 4a). Compared with the GFP control, the CKI1(H405Q) mutant and ARR6 slightly inhibited cell proliferation on the same medium with or without exogenous cytokinin (Fig. 4a). The results of this seedling cytokinin assay are consistent with those from protoplast transient expression analyses based on transcription. As the CKI1(H405Q) mutant did not promote shoot initiation and leaf formation, it is clear that the histidine protein kinase activity of CKI1 is required to initiate cytokinin signalling.

To observe the consequences of constitutive cytokinin signalling *in planta*, the binary vectors expressing CKI1, CKI1(H405Q), ARR2 and ARR6 were introduced into *Arabidopsis* plants. Ectopic expression of ARR2 in transgenic *Arabidopsis* plants delayed leaf senescence (Fig. 4b). Transgenic *Arabidopsis* plants carrying the CKI1 construct showed similar phenotype, but not the CKI1(H405Q) and ARR6 transgenic plants, which served as negative controls. Thus, CKI1 and ARR2 are positive regulators in the cytokinin signal transduction pathway that mediate transcription, cell proliferation, shoot initiation and leaf formation, as well as delaying leaf senescence. ARR2 could be one of the direct downstream targets of CKI1 and CRE1, and serve as a central regulator in diverse cytokinin responses.

Discussion

Although the conserved signature motifs of histidine protein kinases have been found in the photoreceptor phytochromes, a putative osmosensor, and ethylene and cytokinin receptors in flowering plants, the importance of histidine protein kinase activity

and phosphorelay in signal transduction remains unclear in plant cells[6,11,13,15]. Here, we provide evidence that both histidine protein kinase activity and phosphorelay are essential for cytokinin-mediated transcription, cell proliferation, leaf formation and leaf longevity, on the basis of protoplast transient expression and transgenic tissue and plant assays. Quantitative transcription analyses (Fig. 1d, e) suggest that CKI1 and CRE1 histidine protein kinase receptors act through different cytokinin perception mechanisms. The action of CKI1 without exogenous cytokinin implies that CKI1 could be constitutively active and/or could sense endogenous signals (Fig. 5). CRE1 and perhaps AHK2 and
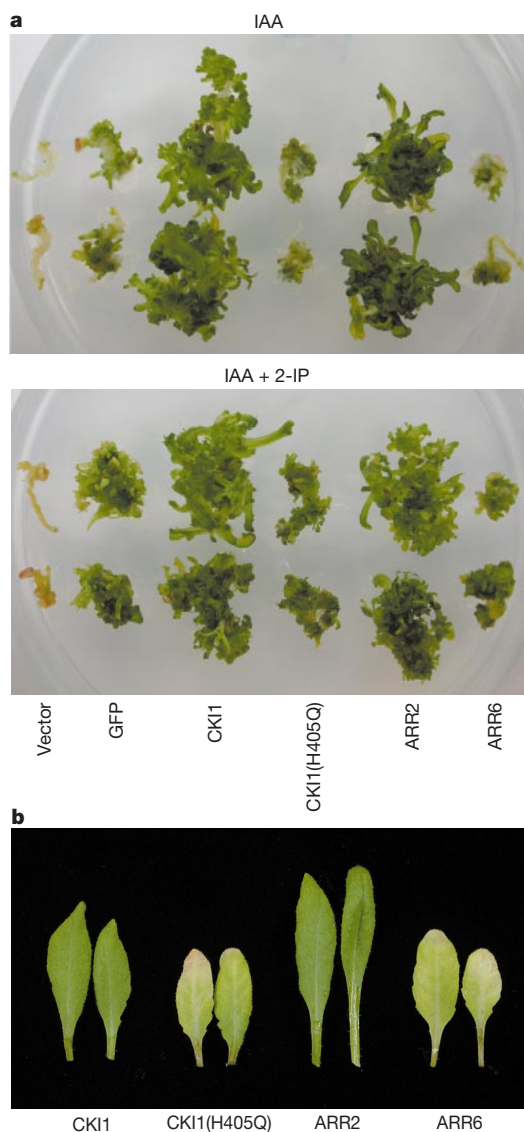
Figure 4 Ectopic expression of ARR2 is sufficient to promote cytokinin responses in transgenic tissues and plants. **a**, ARR2 stimulates cell proliferation and shoot formation in the absence of cytokinin. *Arabidopsis* seedlings were transformed with *Agrobacterium* carrying a binary vector with various constructs, including *CKI1*, *CKI1(H405Q)*, *ARR2*, *ARR6* and *GFP*. Seedlings that were transformed with the *Agrobacterium* carrying the binary vector pCB301 without a bar selection marker were used as a negative control. The transformed seedlings were maintained on the selection medium with auxin (IAA) only (top) or with auxin and cytokinin (2-IP) (bottom) for 14 days. The GFP control showed green callus formation with exogenous IAA but promoted shoot formation with exogenous cytokinin. **b**, Analysis of dark-induced senescence in transgenic *Arabidopsis* plants. The fully expanded fourth leaves from the transgenic plants expressing CKI1, CKI1(H405Q), ARR2 or ARR6 were detached and floated on distilled water in the dark for 4 days.

AHK3 require extracellular cytokinin for their activation (Fig. 5). Further analyses of cytokinin binding and chimaeric histidine protein kinases with swapped domains should clarify the underlying mechanism of each histidine protein kinase action in cytokinin signalling. Detailed expression analysis of each histidine protein kinase at the cellular level and the systematic isolation of knockout mutants[34] will help define their precise roles *in planta*.

As histidine protein kinase activity and phosphoryl transfer are required for CKI1 and CRE1 action, these histidine protein kinases probably converge on AHP proteins that serve as shuttles and phosphorelay carriers between the cytokinin receptors and the downstream nuclear responses (Fig. 5). Although other mechanisms have not been ruled out, the involvement of AHP proteins in cytokinin signalling is supported by previous studies of CKI1 interaction with AHP proteins *in vitro*[29,35] and AHP interactions with ARR proteins in the yeast two-hybrid assay[27–29,31]. Our analysis of AHP–GFP fusions provides the first visual and *in vivo* evidence that AHP1 and AHP2, but not AHP5, are translocated into the nucleus in a cytokinin-dependent manner (Fig. 2b). The action of AHP proteins seems to be passive and not rate-limiting because overexpression of AHP proteins does not affect cytokinin signalling (Fig. 2a). With the exception of CKI1, our studies show that simple overexpression of AHK and AHP proteins cannot markedly disturb cytokinin responses. The potential functional redundancy of AHK, AHP and ARR proteins may explain the difficulty in isolating related cytokinin response mutants based on classical genetic screen. Combining multiple knockout mutants may be necessary to reveal further cytokinin functions *in planta*.

By analogy with the SLN1 and HOG1 osmosensing pathway in yeast, it has been proposed that the ethylene receptor and osmosensor histidine protein kinases transmit signals through a MAPK cascade in *Arabidopsis*[14,19]. The completion of the *Arabidopsis*

genome sequence has revealed a large number of *AHP* and *ARR* genes that have potential signalling roles in physiological responses of plant cells[11,12,26–28]. Our studies unravel a cytokinin signalling circuit mediated by distinct functions of AHP and ARR proteins. The cytokinin phosphorelay mechanism is unique in that AHP proteins act as cytoplasm and nuclear shuttles, and in that enabling ARR proteins with DNA-binding domains is the nuclear consequence (Fig. 5). As the expression of ARR1, ARR2 and ARR10 is not regulated by cytokinin[4], we propose the presence of putative repressors in controlling cytokinin responses (Fig. 5) based on the fact that ectopic expression of ARR1, ARR2 and ARR10 confers constitutive cytokinin signalling (Figs 3 and 4). The transcriptional activation of genes that encode the repressor-type of ARR proteins (Fig. 3a) possibly provides a negative feedback loop in controlling the transient induction of cytokinin primary response genes, and allows resetting and/or fine-tuning of the physiological state of the cells.

The cellular assay based on cytokinin-inducible transcription has identified the transcription activators ARR1, ARR2 and ARR10 as the central rate-limiting step in cytokinin signalling. These factors could potentially be regulated by signals other than cytokinin[36] to provide a crosstalk mechanism in plant signalling networks. ARR2 seems to act as a master regulator, manifested by its ability to mimic a broad spectrum of cytokinin actions in transgenic *Arabidopsis*. Other B-type ARRs could share a similar role. Further experiments will be required to elucidate the details in cytokinin perception and protein–protein interactions that are essential in cytokinin signalling. The expression analysis of an *ARR5–GUS* transgene in *Arabidopsis* has shown that cytokinin responses can occur in many different cell types, including leaf cells[5]. The short cytokinin signalling circuit, elucidated by using the mesophyll protoplast transient expression assay, could represent a conserved core signalling pathway in different cell types in response to cytokinin. This hypothesis is supported by the similar effects of CKI1, ARR2 and their mutants on cytokinin signalling analysed in mesophyll protoplasts and in stably transformed tissues and plants. However, additional cell-type-specific components probably have important roles for cytokinin responses in different cell types and tissues, for example dividing and non-dividing cells. The expression patterns of various histidine protein kinases, AHP and ARR proteins may also contribute to their unique or redundant roles in cytokinin responses[24,26,31,32,37]. It will be of interest to investigate how this cytokinin signal transduction pathway influences cell cycle[5,38,39], leaf senescence[40–42], and shoot initiation and leaf patterning controlled by transcription factors[43]. The combination of the complete *Arabidopsis* genome sequence and the development of the physiological leaf cell assays will provide useful tools to elucidate other histidine protein kinase-mediated signal transduction pathways in plants. □
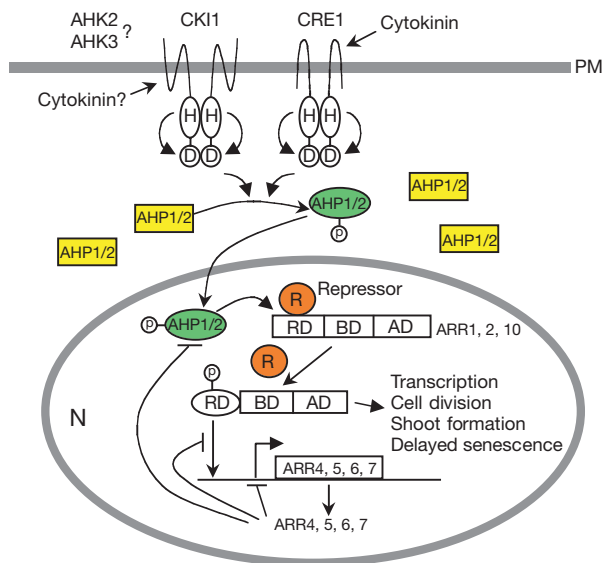
Methods

Plasmid constructs

The 2.4-kb *Arabidopsis ARR6* promoter was amplified by polymerase chain reaction (PCR) and fused to the firefly luciferase gene to create the *ARR6–LUC* reporter construct. We amplified *CKI1* and *AHK2* genes from the *Arabidopsis* genomic DNA by PCR. The *AHK3*, *CRE1(CRE1/WOL)*, *AHP1*, *2*, *5* and *ARR1*, *2*, *4*, *5*, *6*, *7* and *10* coding regions were obtained by PCR from an *Arabidopsis* complementary DNA library[44]. All of the mutants were generated by QuickChange site-directed mutagenesis (Stratagene). The coding regions of all proteins were tagged with either two copies of the haemagglutinin epitope (DHA) or GFP and inserted into a plant expression vector that contained the *35SC4PPDK* promoter[44] and the *NOS* terminator. We confirmed all PCR products and the mutations by DNA sequencing.

Arabidopsis protoplast transient expression assay

Arabidopsis Bensheim protoplasts were isolated and transfected as described with some modifications[45]. Typically, 2×10^4 protoplasts were transfected with 20 µg plasmid DNA with different combinations of reporter, effector and internal control. Transfected protoplasts were incubated at 1×10^4 per ml with or without 1–100 nM *t*-zeatin for 3–6 h under light conditions at 23 °C. The *UBQ10–GUS* construct was used as an internal

Figure 5 Model of the cytokinin signal transduction pathway in *Arabidopsis*. Cytokinin signal is perceived externally or internally by multiple histidine protein kinases at the plasma membrane. On perception of the cytokinin signal, histidine protein kinases initiate a signalling cascade through the phosphorelay that results in the nuclear translocation of AHP proteins from the cytosol. Activated AHP proteins interact with sequestered ARR proteins or ARR complexes, and release the activation type of ARR proteins from putative repressors in the nucleus. The liberated ARR proteins bind to multiple *cis* elements in the promoter of target genes. The transcription activation is essential for cell proliferation, shoot formation and delayed leaf senescence. Activation of the repressor-type of *ARR* genes as cytokinin primary response genes provides a negative feedback mechanism. RD, response domain; BD, DNA-binding domain; AD, transcription activation domain; PM, plasma membrane; N, nucleus; R, putative repressor; H, histidine; D, aspartate.

control to normalize the variations of each transfection in cell numbers, transformation efficiency and cell viability. The results are shown as the means of relative LUC activities from duplicate samples with error bars. All transient experiments were repeated at least three times with similar results. GFP fluorescence was observed by either Nikon TE200 fluorescent microscopy or Leica TCSNT confocal microscopy.

Protein immunoprecipitation

Transfected protoplasts were incubated with ^{35}S-labelled methionine (200 μCi ml^{-1}) for 6 h. The effector proteins were immunoprecipitated as described[44], analysed by SDS–polyacrylamide gel electrophoresis (PAGE) (10 or 12.5%) and visualized by fluorography.

Seedling cytokinin response assay

Four-day-old *Arabidopsis* Bensheim seedlings were placed on the callus induction medium, containing 0.5 mg l^{-1} 2,4-dichlorophenoxy acetic acid, 0.05 mg l^{-1} BA, and 0.05 mg l^{-1} kinetin, for 4 days. The seedlings were co-cultivated and transformed with *Agrobacterium* GV3101 carrying pCB302 with either *CKI1*, *CKI1(H405Q)*, *ARR2*, *ARR6*, *GFP* under control of the *35SC4PPDK* promoter, or the pCB301 binary vector[33] alone for three days. Transformed seedlings were then selected on the glufosinate ammonium-containing medium (with either 0.15 mg l^{-1} IAA alone or with 0.15 mg l^{-1} IAA and 5 mg l^{-1} 2-IP) and observed up to 4 weeks. The transformed proliferating tissues were mostly derived from the apical shoot meristem of the seedlings.

Transgenic plant analysis

The same constructs that were analysed in the *Arabidopsis* protoplast transient expression and seedling assays, including *CKI1*, *CKI1(H405Q)*, *ARR2* and *ARR6*, were used to generate *Arabidopsis* transgenic plants using the floral dip method and bar selection as described[46]. We obtained more than 200 transgenic plants with each construct. For senescence assay, fully expanded fourth leaves from representative transgenic plants were detached and floated on distilled water in the dark for 4 days.

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