Cell-surface receptors enable perception of extracellular cytokinins

Ioanna Antoniadi 1,2, Ondřej Novák 2,3, Zuzana Gelová 4,5, Alexander Johnson 4, Ondřej Plíhal 3,6,7, Radim Simerský 3,8, Václav Mik 3,6, Thomas Vain 2,9,10, Eduardo Mateo-Bonmatí 2, Michal Karady 2,3, Markéta Pernisová 5, Lenka Plačková 3, Korawit Opasathian 1, Jan Hejátko 5, Stéphanie Robert 2, Jiří Friml 4, Karel Doležal 3,6, Karin Ljung 2,✉ & Colin Turnbull 1,✉

Cytokinins are mobile multifunctional plant hormones with roles in development and stress resilience. Although their Histidine Kinase receptors are substantially localised to the endoplasmic reticulum, cellular sites of cytokinin perception and importance of spatially heterogeneous cytokinin distribution continue to be debated. Here we show that cytokinin perception by plasma membrane receptors is an effective additional path for cytokinin response. Readout from a Two Component Signalling cytokinin-specific reporter (TCSn::GFP) closely matches intracellular cytokinin content in roots, yet we also find cytokinins in extracellular fluid, potentially enabling action at the cell surface. Cytokinins covalently linked to beads that could not pass the plasma membrane increased expression of both TCSn::GFP and Cytokinin Response Factors. Super-resolution microscopy of GFP-labelled receptors and diminished TCSn::GFP response to immobilised cytokinins in cytokinin receptor mutants, further indicate that receptors can function at the cell surface. We argue that dual intracellular and surface locations may augment flexibility of cytokinin responses.
Cytoxins are key hormones regulating cell division and differentiation, root and shoot architecture, senescence and responses to environmental stresses\(^3,2\). The active forms are the cytokinin free bases, which comprise a range of \(N^6\)-modified adenine molecules\(^3,4\), especially \(trans\)-zeatin (IZ) and isopentenyl adenine (iP). A cell-level map of cytokinins in roots indicates heterogeneous distribution between different cell types\(^5\). Homeoestatic regulation of active cytokinin pools occurs at the level of biosynthesis, and also through metabolic deactivation by glucosylation, phosphoribosylation or irreversible degradation by cytokinin dehydrogenase (CKX)\(^6,7\). Cytokinin signalling commences with perception of bioactive molecules by hybrid histidine kinases (HKs)\(^1,7,8\) that have much lower affinity for cytokinin precursor and glycosyl conjugate forms\(^7\). Several reports show GFP-fused Arabidopsis HKs (AHKs) mainly localised to the endoplasmic reticulum (ER) membrane\(^9,10\), yet the originally proposed extracellular site of cytokinin perception at plasma membrane receptors\(^11,12\) has never been disclosed, as highlighted in recent reviews\(^11,13,14\). Notably, several classes of cytokinin transporters facilitate movement of cytokinins in and out of the cell\(^15\). Cytokinin binding to receptors triggers a phosphorelay cascade, resulting in activation of B-type Arabidopsis Response Regulators (ARR-B) transcription factors\(^17\), which in turn upregulate type A-ARRs and Cytokinin Response Factors (CRF), a clade of transcription factors within the AP2/ERF superfamily\(^18\). The cytokinin-responsive synthetic promoter fusion \(TCSn::GFP\) was developed to reflect global \(ARR-B\) transcriptional activity\(^19\) and has facilitated in vivo monitoring of cytokinin responses, leading to new discoveries about cytokinin function\(^20,21\). However, it is unclear whether \(TCSn::GFP\) signal strength is quantitatively related to cellular cytokinin content and uncertainty remains about which active cytokinin(s) are responsible for different responses in the root tip and other tissues. Here, we used sorted protoplasts to examine the relationship between cell-level \(TCSn::GFP\) expression and cytokinin content, and also compared intracellular and extracellular cytokinin profiles. The presence of bioactive cytokinins in the latter then led us to test whether cytokinin signalling could operate from receptors at the cell surface. We show that, in addition to the known route of cytokinin signalling via ER receptors, plasma membrane receptors are able to initiate signalling in response to extracellular cytokinins.

We further tested the potential of \(TCSn::GFP\) to respond to perturbations in endogenous cytokinin pool sizes by applying the inhibitor INCYDE (2-chloro-6-(3-methoxyanilino)purine) to block cytokinin degradation by CKX enzymes\(^22\). Preliminary experiments indicated that the \(TCSn\) response to INCYDE was stronger than to exogenous cytokinin (Supplementary Fig. 2). In untreated cells, inactive cytokinin glucosyl conjugates were relatively abundant in the GFP\(^+\) cells, but IZ was the only active cytokinin significantly enriched in this population (Fig. 2a—Mock, light coloured bars). In contrast, iP, which has similarly high affinities to cytokinin receptors\(^9,23\), was not enriched. We therefore inferred that IZ, rather than iP, has a leading role in the cytokinin response reported by \(TCSn\). Indeed, inhibition of turnover by INCYDE resulted in both enhanced \(TCSn::GFP\) signal (Fig. 2b) and further elevation of IZ content in the GFP\(^+\) cells, but had no impact on iP content (Fig. 2a—INCYDE, darker coloured bars).

**Extracellular and intracellular cytokinin profiles differ.** Since cell walls and extracellular space were absent from protoplast samples used in cell sorting (Fig. 2a), we additionally analysed apoplastic and symplastic fractions from roots. LC–MS profiles revealed relative enrichment of cytokinin glucosyl conjugates in the symplast (Fig. 2c), consistent with high levels detected in root protoplasts (Supplementary Fig. 3). However, these conjugate forms are essentially inactive\(^2\) and unlikely to contribute directly to \(TCSn::GFP\) activation. Glucosyl-conjugate re-conversion to active forms during protoplast isolation was a possibility that was discounted by feeding labelled cytokinins (Supplementary Fig. 4). In contrast, cytokinin free bases and ribosides were either equally distributed between symplast and apoplast or relatively enriched in the latter (Fig. 2c). The presence of bioactive cytokinins in the apoplast led us to hypothesise that extracellular cytokinins could potentially initiate signalling.

**Extracellular cytokinins can activate cytokinin signalling.** Based on finding cytokinins in the apoplast, we next tested whether the bioactive compounds could be perceived by plasma membrane receptors\(^1,7,13,16,24\), by treating \(TCSn::GFP\) protoplasts with iP or IZ in free solution or covalently attached to Sepharose beads via flexible linkers designed to minimise steric hindrance to cytokinin binding (Supplementary Fig. 5). Since the beads are much larger than the protoplasts (Supplementary Fig. 6a, red arrows), the attached cytokinin ligands were unable to enter the protoplast, and could thus be considered as membrane-impermeant signals. \(TCSn\) fluorescence signal strength after treatment with bead-bound cytokinins provided in vivo evidence for activation of cytokinin response through perception of extracellular cytokinins (Fig. 3a, b, also Supplementary Fig. 7b for \(TCS::GFP\)). Since the beads are much larger than the protoplasts (Supplementary Fig. 6a, red arrows), the attached cytokinin ligands were unable to enter the protoplast, and could thus be considered as membrane-impermeant signals. \(TCSn\) fluorescence signal strength after treatment with bead-bound cytokinins provided in vivo evidence for activation of cytokinin response through perception of extracellular cytokinins (Fig. 3a, b, also Supplementary Fig. 7b for \(TCS::GFP\)). Further analysis showed that ~0.2–0.6% of cytokinins with their linkers and up to 0.2% of the free cytokinin ligands had potentially been detached from the beads (Fig. 3c, Supplementary Table 1). As predicted from their N9 substitutions, the cytokinins with linkers have substantially lower bioactivity both in \(TCSn\) activation (Supplementary Fig. 8a) and in receptor binding (Supplementary Fig. 8d) experiments. Because of their lower activity and because attachment internally to the bead matrix will hinder ligand access to the protoplast surface, we compensated by using a moderate excess of immobilised ligands to block cytokinin degradation by CKX enzymes\(^22\). Preliminary experiments indicated that the \(TCSn\) response to INCYDE was stronger than to exogenous cytokinin (Supplementary Fig. 2). In untreated cells, inactive cytokinin glucosyl conjugates were relatively abundant in the GFP\(^+\) cells, but IZ was the only active cytokinin significantly enriched in this population (Fig. 2a—Mock, light coloured bars). In contrast, iP, which has similarly high affinities to cytokinin receptors\(^9,23\), was not enriched. We therefore inferred that IZ, rather than iP, has a leading role in the cytokinin response reported by \(TCSn\). Indeed, inhibition of turnover by INCYDE resulted in both enhanced \(TCSn::GFP\) signal (Fig. 2b) and further elevation of IZ content in the GFP\(^+\) cells, but had no impact on iP content (Fig. 2a—INCYDE, darker coloured bars).

**Results**

**Cytokinin reporter signal output mirrors cytokinin content.** To test the relationship between endogenous cytokinin content and \(TCSn\) activity, we analysed cytokinins in root cell protoplasts isolated from \(TCSn::GFP\) seedlings using fluorescence-activated cell sorting (FACS) (Fig. 1a). Total cytokinin content was almost three times higher in the cytokinin-responsive (GFP\(^+\)) cells than in the non-responding (GFP\(^-\)) cells (Fig. 1b). These results are in accordance with evidence for a cytokinin gradient within the root tip\(^5\) that likewise matches the \(TCSn::GFP\) expression pattern\(^19\). We further showed a positive correlation between \(TCSn::GFP\) signal strength and cytokinin content within GFP\(^+\) cell subpopulations displaying higher (GFP\(^+\)\(_{max}\)) and lower (GFP\(^+\)\(_{min}\)) mean fluorescence (Fig. 1c and Supplementary Fig. 1). Active cytokinins and their riboside precursors were generally enriched in the more fluorescent cells, whereas inactive cytokinin glucosides were more equally distributed between the two subpopulations (Fig. 1d). We conclude that increases in \(TCSn::GFP\) readout, designed to approximate global \(ARR-B\) transcript levels, are indeed associated with elevated active cytokinin content as the input signal. Moreover, \(TCSn::GFP\) is not simply a binary sensor but instead can report dynamics of changes in cytokinin pool sizes within individual cells.
correspond to 4 nM free iP or iZ (Fig. 3c and Supplementary Fig. 9), concentrations that likewise would not lead to a significant TCSn::GFP response (Fig. 3d), let alone the very large responses seen with both free and immobilised cytokinins. Notably, extracellularly restricted iZ resulted in a TCSn::GFP response approaching that elicited with 2 μM free iZ, whereas the response to immobilised iP was substantially lower (Fig. 3a, b). These results suggest that apoplastic iZ can trigger cytokinin response, consistent with the increase in TCSn signal and iZ level when cytokinin degradation is impaired (Fig. 2a, b).

We further explored functional importance of signalling from extracellular cytokinins by analysing expression of members of the CRF clade within the AP2/ERF transcription factor superfamily, several of which are regulated via the canonical TCS signalling pathway. Transcript abundance of CRF6 measured by qPCR was significantly enhanced by both extracellular and free iP and iZ (Fig. 3g), whereas CRF3 was upregulated by both free cytokinins and by extracellular iP, but not by extracellular iZ (Supplementary Fig. 7e).

Although iP, unlike iZ, was not enriched in the cytokinin-responsive cells (Fig. 2a) nor in the apoplast (Fig. 2c), exogenous supplies of both compounds triggered cytokinin responses in protoplasts (Fig. 3a, b), consistent with previous studies. Cytokinin treatment of whole seedlings indicated similar significant enhancement of TCSn response by iP and iZ in roots, but a differential spatial regulation was also observed (Fig. 3e, f; also Supplementary Fig. 7a for corresponding TCSn::GFP responses). iP had the strongest effect on meristematic stele initials (3–28 μm in stele initials; Fig. 3e, f, highlighted in magenta), whereas iZ response was maximal in the transition zone in the stele (221–230 μm from stele initials; Fig. 3e, f, highlighted in green). We confirmed that minimal conversion of exogenous iP to iZ occurred (Supplementary Table 2) and therefore the response to iP was not due to increased iZ levels. Although the exact biological role for cytokinins in the stele initials remains to be determined, TCS signal in those cells was absent in ahk4 mutant roots (Supplementary Fig. 7c, d) providing genetic evidence for AHK4 being essential in cytokinin perception in the stele.

**Receptor dependence of extracellular cytokinin signalling.** To evaluate whether the TCSn response to extracellular cytokinins acted through one or more cytokinin receptors, we tested each of the three AHK receptors individually in the presence or absence of extracellular and free cytokinins, using TCSn::GFP lines mutated in the other two AHKs. Although absolute signal strength was diminished in these mutant lines compared with the wild type, as shown elsewhere, they all retained responsiveness to free iP and iZ (Fig. 4a, b). In root protoplasts, equivalent levels of response to both extracellular cytokinins were found for...
AHK2 (ahk3 ahk4), but interestingly AHK3 (ahk2 ahk4) and AHK4 (ahk2 ahk3) responded only to apoplastic IP or tZ, respectively. The application of IP and tZ to whole seedlings of the respective genotypes showed that AHK4 is not only essential, but also sufficient for cytokinin response in the stele, consistent with previous findings26 (Fig. 4b). In contrast, the same treatments of seedlings carrying only AHK3 or AHK2 receptors resulted in slightly enhanced TCSn::GFP response in some cell files within the stele (Fig. 4b bottom panels—vasculature). The baseline expression of TCSn::GFP in columella cells is constitutively high and did not show obvious further increases in response to exogenous cytokinins. As columella cells contain higher levels of endogenous cytokinins than found in other root tip cell types5, it may be that the columella cytokinin content is non-limiting or saturated in terms of TCSn signalling. Visualisation of AHK4–GFP and AHK3–GFP fusion proteins by 3D AiryScan microscopy indicated that a proportion of both cytokinin receptors was not co-localised with ER (Fig. 4c, Supplementary Fig. 10). Similar to previous11,12 and recent28 reports, some of the non-ER AHK signal was clearly at the cell surface.
indicating plasma membrane localisation. Across multiple imaged cells, 25% of AHK3 and 36% of AHK4 signals were localised in non-ER regions (Fig. 4d). These imaging experiments further support our functional evidence showing that extracellular cytokinins can be perceived by the sub-populations of AHK receptor proteins that reside on the cell surface.

**Discussion**

Our initial aim in this work was to explore the relationship between output strength of the TCSn cytokinin reporter and input signal level in terms of cytokinin content of individual cells. It can be argued that changes in TCSn signal could precisely reflect fluctuations in bioactive cytokinin levels, but equally it
could be that TCSn output is modified through variation in abundance of receptors or downstream signalling components. The evidence we provide here is that root cells with significant TCSn:GFP expression contain nearly threefold more total cytokinin than cells sorted into the GFP− category. Moreover, when we sorted the GFP+ cells into two pools representing higher and lower GFP signal, there was again a trend towards higher cytokinin in the GFPmax than in the GFPmin cells. When cytokinin catabolism was blocked with the inhibitor INCYDE, TCSn:GFP signal in whole roots was greatly enhanced especially in stele cells, and protoplasts showed higher levels of IZ, but not of the other bioactive cytokinins Z and iP. Although we did not attempt to provide a full calibration curve, it is clear that TCSn behaves as a cytokinin sensor with quantitative characteristics, and IZ is the bioactive cytokinin whose level most closely tracks the TCSn output.

In addition to assessing signalling strength from intracellular cytokinins, we also found substantial pools of extracellular cytokinins, leading us to test whether cell surface perception could occur. Several reports using AHK translational fusions to fluorescent proteins point to AHK receptors predominantly located on ER membranes10,11, and show that AHK interaction with their downstream AHP partners can also occur at the ER12. However, plasma membrane-localised receptors have never been excluded11,29 and AHK3 and AHK4 have been shown to at least partially reside in the plasma membrane10,11. Although we did not attempt to make detailed quantitation of relative proportions of AHKs on plasma membrane vs ER, our image analysis likewise clearly indicates that significant amounts of AHK are found in non-ER regions including the cell surface. Our data are strongly corroborated by independent experiments showing co-localisation of plasma membrane markers both with AHK4–GFP and with a fluorescently tagged iP derivative that preferentially binds AHK428. Moreover, Kubiasová et al.28 show that AHK4 and tagged iP are found in brefeldin A-dependent endocytic vesicles characteristic of trafficking to and from the plasma membrane. Despite the strong evidence for ER location of AHKs, recent reviews have highlighted the lack of direct substantiation of extracellular cytokinin perception14,15,16. In this context, our multiple strands of evidence for responses to extracellular cytokinins initiated via plasma membrane-bound receptors indicates that both sites of perception appear to exist. Previous studies have also detected a wide range of extracellular cytokinins30,31. However, the relative abundance of different forms varies substantially, possibly because of technological and experimental differences.

In particular, our in vivo data point to perception of apoplastic IZ being an important route for cytokinin response activation in roots (Figs. 2c, a, and 4a), while its endosomal degradation might act as a negative feedback loop in cytokinin signalling (Fig. 2a). These results are consistent with evidence showing that impaired cytokinin import/uptake results in induction of cytokinin response24 and with IZ-specific binding by AHK4 in outer membranes11. Moreover, selective extracellular degradation by CKX expression leads to diminished cytokinin responses, whereas intracellular targeting of CKX did not have such an effect24. Nonetheless, intracellular inactivation of IZ by endogenous CKX does appear to occur (Fig. 2a—INCYDE32. The spatially distinct tissue-level responses to iP and IZ (Fig. 4a, b) may relate to ligand preferences3,9,23, sites of maximal expression8 of each AHK type or and differentially localised and expressed CKX enzymes33. Indeed, given that there is so much heterogeneity between root cell types, the distribution of ER vs non-ER AHK proteins in the example cells shown in Fig. 4c will likely differ if other cell types are examined. Given that IZ cytokinins have previously been suggested to be dispensable for root function34, our evidence for a dominant role of IZ rather than iP is surprising. Moreover, although Kubiasová et al.28 did not test IZ, they show that a tagged version of iP can enter the secretory pathway, resulting in delivery to the apoplast. One explanation for the discrepancy could be that endogenous and exogenous iP compounds behave differently, for example, if the latter is present at non-physiological concentrations or is distributed into abnormal cellular compartments.

We found contrasting-specific extracellular cytokinin responses in different ahk double mutant protoplasts, where only a single AHK type remained functional. The response strengths to IZ and iP did not necessarily correspond to the known affinities of each AHK for these ligands3,9,23. One reason for the lack of correspondence may be that each AHK here is acting only as a homodimer, whereas in wild type plants heterodimerisation may occur. Although localisation-related selectivity of receptor–ligand interactions merits further exploration, here we have demonstrated the presence of functional receptors at the plasma membrane of the
cell, thus substantially resolving the lengthy debate on this issue. Dual location of receptors may potentially provide plants with additional flexibility in cytokinin responses. It remains to be ascertained whether different biological functions are associated with each location. Overall, many regulatory elements can influence cytokinin response including selective molecule-receptor affinities and influences of tissue-specific apoplastic pH, together with heterogeneous tissue- and cell-specific distribution of cytokinins, their cognate receptors and cytokinin inactivation enzymes.

**Methods**

**Plant material and growth conditions.** All experiments used Arabidopsis (*Arabidopsis thaliana*). The transgenic line TCSn::GFP was used for all cell-sorting experiments, TCSn::GFP and TCS::GFP were employed in protoplast and seedling treatment experiments, and the ecotype Col-0 was used for extraction of apoplast/ahk23*TCSn::GFP ahk24*TCSn::GFP ahk34*TCSn::GFP tZ iPMock a

![Fig. 4 Cytokinin responses when only one AHK receptor is active, and receptor localisation. a Quantification of GFP fluorescence in protoplasts derived from roots of 6-day-old TCSn::GFP seedlings in wild type (Col-0), ahk3,4, ahk2,3 and ahk2,4 backgrounds, after treatment for 16 h with or without free cytokinins (tZ or iP; 2 μM, denoted “free”) or immobilised cytokinins (tZ or iP, ligand mean density 10 μmol l⁻¹ equivalent, attached to Sepharose beads, denoted “bound”). Negative controls without added cytokinin were incubations with and without beads (control free and bound, respectively). Whiskers indicate entire range of values, box indicates first and third quartiles, and central line is mean; **p < 0.01; ***p < 0.001 by one-way ANOVA and Tukey’s test, indicating significant differences in fluorescence intensity between control and corresponding free or extracellular cytokinin treatments. Three independent experiments were performed with each comprising data from n > 20 images, corresponding to >1000 protoplasts. See also Supplementary Figs. 7 and 10. b Confocal images of roots from ahk3,4, ahk2,3 and ahk2,4 double mutants expressing TCSn::GFP, after treatment for 24 h with 100 nM tZ or iP. Scale bar is 39 μm. The inset panels show the respective root vasculature in 16-colour LUT (Look-Up Tables; https://imagej.net/) highlighting the gradations of fluorescence intensity. c 3D Airyscan images of Arabidopsis protoplasts expressing AHK3-GFP (green; above) and AHK4-GFP (below) and the ER maker RFP-p24δ5 (magenta). The left panel shows a zoomed in region of the cell (red rectangle). The right panel depicts the YZ orthogonal view of the Z-stack (orange dashed line). The bottom panel shows the XZ orthogonal view of the Z-stack (blue dashed line). Magenta arrows indicate regions of AHK-only signal on the cell surface. Scale bars, 5 μm. d Quantitative analysis of AHK-GFP signal co-localising or not co-localising with ER marker RFP-p24δ5. Whiskers represent entire range of values, boxes indicate first and third quartiles, central line is median and dots are values for individual cells. n = 3 for AHK3, n = 5 for AHK4.
sympat and for feeding experiments with labelled cytokinins. Crossed lines ahk x TCSn::GFP were published previously23,35 and the mutant lines cre1-2 (ahk3), ahk2-5, ahk2-3, and ahk2-2 were obtained previously (www.TCSn::GFP, TCSn::GFP ahk3,4) were crossed also with TCS::GFP. Homozygous lines were then identified and used in confocal experiments. In all experiments, the seeds were surface sterilized with 20% (v/v) dilution of bleach for 5 min (2 × 2.5 min) and then rinsed five times with sterile water. For cell-sorting experiments, the seeds were sown in three to seven square Petri dishes containing Murashige and Skoog medium (4.4 g/l Murashige and Skoog salt mixture, 1% sucrose, 0.5 g/MES, 1% agar and adjusted to pH 5.7 with KOH), covered with sterile mesh squares to facilitate the harvesting of the apical part of the primary root, and were stratified at 4 °C for 3 days. Seedlings were then grown on plates placed vertically, for 9 days at 23 °C under 150 μmol m−2 s−1 light with photoperiod of 16-h light and 8-h darkness. One standard cell-sorting experiment required 20 Petri dishes. For confocal microscopy experiments, sterilized seeds were sown (10 seeds/row), stratified and grown for 6 days as described above.

Fluorescence-activated cell sorting (FACS). The distal half of 9-day-old roots of TCSn::GFP seedlings were harvested, protoplasts were isolated in the presence or absence of 20 μM INCYDE and sorted using a BD FACS Aria I flow cytometer (BD Biosciences)3,37. The software used for data processing was BD FACSDiva version 6.1.2. Isolated protoplasts were loaded in the cell sorter (4 °C) and passed individually through a 100 μm nozzle (Becton Dickinson and Company) using 0.7% NaCl with sheath pressure 2 psig. Healthy protoplasts were initially selected using a 488 nm (blue) laser for excitation of their autofluorescence. For analysis of protoplasts’ relative size, the forward scatter detector was used while and for their respective granularity and complexity analysis, the side scatter detector was employed (bandpass filter 605/20, dichroic mirror 550) of the healthy protoplasts. The gated populations took place in a bi-plot of GFP fluorescence (bandpass filter 635/20, dichroic mirror 560) and auto-fluorescence emission (bandpass filter 610/20, dichroic mirror 595) of the healthy protoplasts. The gated populations were sorted, then frozen immediately in liquid nitrogen. Apoplastic and symplastic fractions were further collected by centrifugation with the freeze–thaw method. Each biological replicate represents an independent experiment.

Apoplast isolation. Protoplasts were isolated from roots of 9-day-old Arabidopsis seedlings37,38. The tissue was excised and rinsed in a 40-μm cell strainer (BD Falcon) with distilled water. The harvested tissue was then submerged into the protoplast isolation buffer (600 mM mannitol, 2 mM MgCl2, 10 mM KCl, 2 mM CaCl2, 2 mM MES and 0.1% BSA, pH 5.7) supplemented with pectolyase (0.3 units mL−1) and cellulysin (45 units mL−1). After 2 h incubation at 22 °C in darkness and with gentle stirring at 46 rpm, the protoplasts were isolated using a 40-μm cell strainer. Then they were centrifuged for 3 min at 1000 g at 4 °C and the resulting protoplast pellet was resuspended in 1 mL of cold sorting buffer (0.7% NaCl) and kept at 4 °C until further processing.

RNA isolation, cDNA synthesis and qRT-PCR. For qRT-PCR, total RNA was extracted using Neasy Plant Mini Kit (Qiagen) following the manufacturer instructions. Contaminating DNA was removed using TURBO DNA-free kit (Invitrogen). First-strand cDNA was synthesised using iScript cDNA Synthesis Kit (Bio-Rad). The housekeeping gene ACTIN2 (ACT2) was used as an internal control for relative expression analysis. Four biological replicates were analysed in triplicate. Reaction mixtures (10 µl) comprised 5 µl LightCycler 480 SYBR Green I Master (Roche), 4 µl of the corresponding primer pair (1.5 µM each) and 1 µl of cDNA template. Relative quantification of gene expression data was performed using the comparative Ct method (2−ΔΔCt) on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad). The primers used are the following: 5′-CTTCTTC CATGGGATGTTGAT-3′ (forward) and 5′-CAGCTGATCATCACCACACCA-3′ (reverse) for CKF6, 5′-GGTGGCATACGCGGTATAATAC-3′ (forward) and 5′-ACCACGTTTCATTACCCAGGA-3′ (reverse) for CRE1, 5′-GCCACC TTGCTCTTTAC-3′ (forward) and 5′-AACCCTCCTAGATTGCGACA-3′ (reverse) for ACT2.

AHK-GFP constructs for imaging. The genomic sequences of the AHK3 (At1g27320) and CRE1/AHK4 (At2g18830) genes were amplified from genomic DNA of Arabidopsis thaliana ecotype Columbia (Col-0) using the following primers: 5′-AATGTCGACGAGATGCTGGTGTTCA-3′ (forward) and 5′-GGCTGTTGGCATACGCGGTATAATAC-3′ (reverse) for AHK3 and 5′-ATTCTGACGATGAGAAGATGGTGTATCTAAATATATATGTC-3′ (forward) and 5′-ATGCGGCCGCAAGAGTTGAGATTGAGGGG-3′ (reverse) for CRE1/AHK4, creating Sal linker sequences at the 5′ end, and NotI linker sequences at the 3′ end. The amplified PCR fragments were inserted into SalI and NotI sites of pENTR/D-TOPO vector, for AHK3 and pENTR-2B-Dual-AHK4, respectively. Subsequently, enhanced GFP coding sequence was prepared using primers carrying NotI restriction site:
5′-AATGGGCGCAGGAGTTAGGTGGATCTATGTGACGAGACGTGGCAG
GAG-3′ (forward) and 5′-AATGGGCGCAGGAGTTAGGTGGATCTATGTGACGAGACGTGGCAG
GAG-3′ (reverse), and the resulting PCR fragment was inserted into the
unique NotI site of pENTR-2B-Dual-AHK3 or pENTR-2B-Dual-AHK4 to obtain
C-terminal GFP fusions with AHK3 and CRE1/AHK4, respectively. These entry
clones were recombined using the Gateway LR reaction (Invitrogen) into the
pCGW7.0 vector, containing the 35S promoter18, and the final constructs were used for
protoplast transformations.

3D AirScan sample preparation, imaging and analysis. Protoplasts were iso-
lated from 4-day-old Arabidopsis root suspension culture in enzyme solution (1%
cellulase (Yakult), 0.2% Macerozyme (Yakult) in B5-0.33 M Glc-mannitol solution;
4.4 g MS with vitamins, 30.5 g Glc, 30.5 g mannitol per l, adjusted to pH 5.5
with KOH), with slight shaking for 4 h, centrifuged at 12 000 x g for 5 min. The pellet
was washed with B5-0.34 M Glc-mannitol solution followed by one time wash
with B5-0.28 M sucrose buffer (4.4 g l−1 MS with vitamins, 9.6 g l−1 sucrose,
adjusted to pH 5.5 with KOH) and resuspended in B5-0.34 M Glc-mannitol
solution to a final concentration of 2 × 10^6 protoplasts per ml. Protoplasts were co-
transfected with 4 µg of 35S::AHK3–GFP or 35S::AHK4–GFP and 2 µg of ER
marker 35S::RFP-p2419. DNA gels were gently mixed together with 50 µl
of protoplast suspension and 150 µl of PEG solution (0.1 M Ca(NO3)_2, 0.45 M
mannitol, 25% PEG 6000) and incubated in the dark for 30 min. PEG was washed
by adding 0.275 M Ca(NO3)_2 solution in two steps of 500 µl each, centrifuged at
800 g for 7 min, then removing 240 µl of supernatant. The protoplast pellet was
resuspended in 300 µl of B5-0.34 M Glc-mannitol solution and incubated for 12 h
in the dark at room temperature. Protoplasts were transferred to 35 mm glass
bottom MatTek dishes (coverslip thickness #1.5) coated with poly-L-lysine
(Sigma) and imaged using a Zeiss LSM 880 inverted fast Airyscan microscope with
a Plan-Apochromat ×63 NA 1.4 oil immersion objective. Ten to 15.25 µm thick z-
stacks of transformed cells were taken using Nyquist sampling steps. Images were
then subjected to Airyscan processing. The channels were checked for correct
alignment. The ER marker channel was then filtered with a Gaussian blur and
converted to a mask in Fiji20. A custom Matlab script then determined the per-
centage signal present within and outside of the masked region in the channel
of interest.

Confocal microscopy. GFP expression patterns in 6-day-old seedlings or isolated
protoplasts of the transgenic Arabidopsis lines Col-0, ahk3, ahk4, ahk2, ahk3,2, ahk2,4 and
ahk3,4 carrying 35S::AHK3–GFP or AHK4::GFP were recorded using confocal laser scanning
microscopy (Zeiss LSM780). The 488 nm laser line was employed for the GFP and FM4-64
fluorescence detection, and emission was detected between 490 and 580 nm and between 620 and 670 nm,
respectively. Two tile scans were performed for root imaging and 5 × 5 tile scans for
protoplast imaging.

Semi-automated quantification using computer vision algorithm. Image
(http://imagej.nih.gov/ij/) was used to quantify GFP fluorescence intensity. Fluor-
escence profiles of the static and the full root were extracted using the profile
function. Profile plots represent quantification from ten roots per treatment and
two independent experiments were performed. For the calculation of overlap coefficients (Figs. 3a and
4a and Supplementary Fig. 6b) in treated protoplasts, semi-automated digital processing was performed with a semi-automated digital processing pipeline using iteration of morpho-filtermaths filters within ImageJ.
Raw images have been converted into 8-bit. Noise was reduced using a Median
filter. FM4-64 channel was converted to binary and Fill Holes function was used to
obtain the surface of the protoplasts as almost perfect circular structures. Proto-
plasts were counted and extracted from cellular debris using surface and circularity
filters within ImageJ.

Generation of cytokinins attached to Sepharose beads. iP and tZ ligands
possessing short linkers at the N9 position were synthesised according to the
scheme in Supplementary Fig. 5, and confirmed by 1H and 13C NMR spectra as
well as MS and MS/MS data (Supplementary Fig. 11). Ligands were coupled to
NHS-activated Sepharose™ 4 Fast Flow beads (GE Healthcare, United Kingdom).
Control beads blocked with ethanolamine were prepared in the same way, omitting
the ligand immobilisation step. Absorbance at 272 nm was used to determine the
concentration of the immobilised cytokinin ligand. Full details are in Supplemen-
tary methods.

References

1. Kieber, J. J. & Schaller, G. E. Cytokinin signaling in plant development. Development 145, 1–7 (2018).
2. Ha, S., Vankova, R., Yamaguchi-Shinozaki, K., Shinozaki, K. & Tran, L.-S. P. Cytokinins: metabolism and function in plant adaptation to environmental stresses. Trends Plant Sci. 17, 172–179 (2012).
3. Lomin, S. N. et al. Plant membrane assays with cytokinin receptors underpin the unique role of free cytokinin bases as biologically active ligands. J. Exp. Bot. 66, 1851–1863 (2015).
4. Sakakibara, H. Cytokinins: activity, biosynthesis, and translocation. Annu. Rev. Plant Biol. 57, 431–449 (2006).
5. Antoniadi, I. et al. Cell-type-specific cytokinin distribution within the Arabidopsis primary root apex. Plant Cell 27, 1955–1967 (2015).
6. Wang, J., Ma, X.-M., Kojima, M., Sakakibara, H. & Hou, B.-K. Glucosyltransferase UTG76C1 finely modulates cytokinin responses via cytokinin N-glucosylation in Arabidopsis thaliana. Plant Physiol. Biochem. 65, 9–16 (2013).
7. Inoue, T. et al. Identification of CRE1 as a cytokinin receptor from Arabidopsis. Nature 409, 1060–1063 (2001).
8. Nishimura, C. et al. Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in Arabidopsis. Plant Cell 16, 1365–1377 (2004).
9. Spichal, L. et al. Two cytokinin receptors of Arabidopsis thaliana, CRE1/AHK4 and AHK3, differ in their ligand specificity in a bacterial assay. Plant Cell Physiol. 45, 1299–1305 (2004).
10. Caesar, K. et al. Evidence for the localization of the Arabidopsis cytokinin receptors AHK3 and AHK4 in the endoplasmic reticulum. J. Exp. Bot. 62, 5571–5580 (2011).
11. Wulfetange, K. et al. The cytokinin receptors of Arabidopsis are located mainly to the endoplasmic reticulum. Plant Physiol. 156, 1808–1818 (2011).
12. Lomin, S. N. et al. Studies of cytokinin receptor–phosphotransmitter interaction provide evidences for the initiation of cytokinin signaling in the endoplasmic reticulum. Funct. Plant Biol. 45, 192–202 (2017).
13. Kim, H. J. et al. Cytokinin-mediated control of leaf longevity by AHK3 through phosphorylation of ARR2 in Arabidopsis. Proc. Natl. Acad. Sci. USA. 103, 814–819 (2006).
14. Ueguchi, C., Sato, S., Kato, T. & Tabata, S. The AHK4 gene involved in the cytokinin-signaling pathway as a direct receptor molecule in Arabidopsis thaliana. Plant Cell Physiol. 42, 751–755 (2001).
15. Romanov, G. A., Lomin, S. N. & Schmülling, T. Cytokinin signaling from the ER or from the PM? That is the question! N. Physiol. 218, 34–58 (2014).
16. Durán-Medina, Y., Díaz-Ramírez, D. & Marsch-Martínez, N. Cytokinins on the move. Front. Plant Sci. 8, 146 (2017).
17. Hwang, I. & Sheen, J. Two-component circuitry in Arabidopsis cytokinin signal transduction. Nature 413, 383–389 (2001).
18. Rashotte, A. M. et al. A subset of Arabidopsis AP2 transcription factors mediates cytokinin responses in concert with a two-component pathway. Proc. Natl. Acad. Sci. USA. 103, 10811–10815 (2006).
19. Zührer, E. et al. A robust and sensitive synthetic sensor to monitor the transcriptional output of the cytokinin signaling network in plants. Plant Physiol. 161, 1066–1075 (2013).
20. Marsch-Martínez, N. et al. The role of cytokinin during Arabidopsis gynoecia and fruit morphogenesis and patterning. Plant J. 72, 222–234 (2012).
21. Bencivenga, S., Simonini, S., Benková, E. & Colombo, L. The transcription factors BEI1 and SPL are required for cytokinin and auxin signaling during ovule development in Arabidopsis. Plant Cell 24, 2886–2897 (2012).
22. Zafouklak, M. et al. Novel potent inhibitors of A. thaliana cytokinin oxidase/ dehydrogenase. Bioorg. Med. Chem. 16, 9268–9275 (2008).
23. Romanov, G. A., Lomin, S. N. & Schmülling, T. Biochemical characteristics and ligand-binding properties of Arabidopsis cytokinin receptor AHK3 compared to CRE1/AHK4 as revealed by a direct binding assay. J. Exp. Bot. 57, 4051–4058 (2006).
24. Zührer, E., Liu, J., di Donato, M., Geiler, M. & Müller, B. Plant development regulated by cytokinin sinks. Science 353, 1027–1030 (2016).
25. Müller, B. & Sheen, J. Cytokinin and auxin interplay in root stem-cell specification during early embryogenesis. *Nature* **453**, 1094–1097 (2008).

26. Stožek, A. et al. The specificity of cytokinin signalling in *Arabidopsis thaliana* is mediated by differing ligand affinities and expression profiles of the receptors. *Plant J.* **67**, 157–168 (2016).

27. Pernisova, M. et al. Cytokinin signalling regulates organ identity via AHK4 receptors in Arabidopsis. *Development* **145**, dev163907 (2018).

28. Kubiakówna, K. et al. Cytokinin-induced upregulation of cytokinin oxidase activity in tobacco includes changes in enzyme glycosylation and secretion. *Physiologia Plant.* **117**, 11–21 (2003).

29. Schwartzenberg, K. V. et al. Cytokinins in the bryophyte *Physcomitrella patens*: analyses of activity, distribution, and cytokinin oxidase/dehydrogenase overexpression reveal the role of extracellular cytokinins. *Plant Physiol.* **150**, 786–800 (2007).

30. Werner, T., Motyka, V., Strnad, M. & Schmulling, T. Regulation of plant growth by cytokinin. *Proc. Natl. Acad. Sci. USA* **98**, 10487–10492 (2001).

31. Werner, T. et al. Cytokinin-deficient transgenic Arabidopsis plants show functions of cytokinins in the regulation of shoot and root meristem activity. *Plant Cell* **15**, 2532–2550 (2003).

32. Kiba, T., Takei, K., Kojima, M. & Sakakibara, H. Side-chain modification of cytokinins controls shoot growth in Arabidopsis. *Dev. Cell* **27**, 452–461 (2013).

33. Barbez, E., Dünser, K., Gaïdara, A., Lendl, T. & Busch, W. Auxin steers root cell expansion via apoplastic pH regulation in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **114**, E4884–E4893 (2017).

34. Riefler, O., Strnad, M. & Schmulling, T. Arabidopsis cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development and cytokinin metabolism. *Plant Cell* **18**, 40–54 (2006).

35. Yoo, S.-D., Cho, Y.-H. & Sheen, J. Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat. Protoc.* **2**, 1565–1572 (2007).

36. Novák, O., Antoniadi, I. & Ljung, K. High-resolution cell-type specificity of cytokinins in sorted root cell populations of *Arabidopsis thaliana*. *Methods Mol. Biol.* **1497**, 231–248 (2017).

37. Yu, Q., Tang, C., Chen, Z. & Kuo, J. Extraction of apoplastic sap from plant roots by centrifugation. *N. Phytol.* **143**, 299–304 (1999).

38. López-Millán, A. F., Morales, F., Abadia, A. & Abadía, J. Iron deficiency-associated changes in the composition of the leaf apoplastic fluid from field-grown pear (*Pyrus communis* L.) trees. *J. Exp. Bot.* **52**, 1489–1498 (2001).

39. Svačinová, J. et al. A new approach for cytokinin isolation from Arabidopsis tissues using miniaturized purification: pipette tip solid-phase extraction. *Plant Methods* **8**, 17 (2012).

40. Bolte, S. et al. FM-dyes as experimental probes for dissecting vesicle trafficking in living plant cells. *J. Microsc.* **214**, 159–173 (2004).

41. Karimi, M., Inze, D. & Depicker, A. GATEWAY vectors for Agrobacterium-mediated plant transformation. *Trends Plant Sci.* **7**, 193–195 (2002).

42. Montesinos, J. C. et al. Coupled transport of Arabidopsis p24 proteins at the ER–Golgi interface. *J. Exp. Bot.* **63**, 4243–4261 (2012).

43. Schindelin, J. et al. Fiji—an Open Source platform for biological image analysis. *Nat. Methods* **9**, 676–682 (2012).

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Author contributions

I.A., C.T. and K.L. conceived the project; I.A. performed most of the experiments; I.A., O.N., L.P. and M.K. conducted the purification and quantification of cytokinins; I.A. and S.R. discussed and performed the confocal experiments; T.V. and I.A. developed and trained semi-automated algorithms for confocal image quantification; R.S., V.M. and K.D. developed and produced the cytokinins attached to Sepharose beads; M.P. and J.H. provided the homologous lines of *ahk* mutant combinations with TCS::GFP and TCS::GFP; E.M.B. did the qRT-PCR assays; I.A. and K.O. did the apoplastic fluid experiments; O.P. generated the AHK-GFP constructs and Z.G., A.J. and I.F. performed the respective transfection assays and protoplast imaging. I.A., C.T. and K.L. analysed and interpreted the data; I.A. and O.N. made the figures; I.A., C.T. and K.L. wrote the paper.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to K.L. or C.T.

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