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Research Report

MIZU-KUSSEI 1 regulates root hydrotropism and cytokinin signal transduction by interacting with cytokinin receptors

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Abstract

Roots exhibit hydrotropism in response to moisture gradients to avoid drought stress. Several proteins have been reported to regulate this process, with MIZU-KUSSEI 1 (MIZ1) being identified as a pivotal regulator. Although most studies on the regulatory mechanisms of root hydrotropism have focused on MIZ1, the molecular mechanisms of MIZ1 are poorly understood. Here, we report that MIZ1 plays an essential role in regulating cytokinin signal transduction by interacting with cytokinin receptors, ARABIDOPSIS HISTIDINE KINASEs (AHKs), in Arabidopsis (*Arabidopsis thaliana*). The *miz1-2* mutant exhibited a decreased response to cytokinins, whereas overexpressors of *MIZ1* showed an increased response to cytokinins. The expression levels of 2 Type-A Arabidopsis response regulators (*ARR*s) of cytokinins, *ARR16* and *ARR17*, were downregulated, and their upregulation by cytokinins was substantially attenuated in *miz1-2* compared with those in Col-0. Overexpression of *MIZ1* partially rescued the decreased response of the *ahk2-5 ahk3-7* double mutant to cytokinins. MIZ1 can physically interact with AHKs, as revealed by yeast 2-hybrid, bimolecular fluorescence complementation (BiFC), and co-immunoprecipitation (co-IP) assays. Mutants of cytokinin signal transduction, such as *ahk2-5 ahk3-7 ahk4-2* and *arr3 arr4 arr5 arr6 arr8 arr9 arr16-C arr17-C*, showed a greatly reduced hydrotropic response, similar to *miz1-2*. Additionally, MIZ1 also regulates the homeostasis of cytokinins by controlling the expression of genes encoding their biosynthetic and catabolic enzymes. Our results reveal the critical role of MIZ1 in regulating the cytokinin signaling response, which is essential for the root hydrotropic response.

Introduction

Root tips can sense moisture gradients and grow into areas with higher water potential. This process is referred to as root hydrotropism and was first reported about 270 years ago in the literature (Knight 1811; Bonnet 1754). Inhibition of root gravitropism can significantly enhance root hydrotropic response, suggesting that the regulatory mechanisms governing root hydrotropism differ from those controlling root gravitropism (Kaneyasu et al. 2007; Shkolnik et al. 2016). Although the underlying molecular mechanisms are poorly understood, several proteins, such as MIZU-KUSSEI 1 (MIZ1), MIZ2/GNOM (GN), ER-TYPE Ca^{2+}-ATPASE 1 (ECA1), NF1-RELATED PROTEIN KINASEs (SnRKs), and Arabidopsis H^+-ATPase 2 (AHA2), have been found to regulate root hydrotropism in Arabidopsis within the last 2 decades (Kobayashi et al. 2007; Miyazawa et al. 2009; Dietrich et al. 2017; Miao et al. 2018; Shkolnik et al. 2018; Yuan et al. 2018). Only the single mutants of *MIZ1* exhibit no hydrotropic response (Kobayashi et al. 2007). However, *MIZ1* mutants do not exhibit any obvious developmental defects under normal growth conditions. These findings suggest that MIZ1 specifically regulates root hydrotropism. MIZ1 is a soluble protein associated with the cytosolic side of the endoplasmic reticulum (ER) membrane. It contains a conserved domain of uncharacterized function (the DUF617 domain), and its functional role remains to be elucidated (Kobayashi et al. 2007; Yamazaki et al. 2012). In one of our previous studies, we revealed that the asymmetric distribution of cytokinins plays a key role in root hydrotropism (Chang et al. 2019). In the presence of a moisture gradient, cytokinins accumulated at the side of the root tips facing to the lower water potential medium. As a result, 2 Type-A response regulators (ARRs), ARR16 and ARR17, are accumulated and promote cell division on the lower water potential side of the root tips, leading the roots to grow towards to the area with higher water potential. This process relies on the function of MIZ1. However, the mechanisms by which MIZ1 modulates the distribution and signal transduction of cytokinins have not been elucidated.

Cytokinins are a group of plant hormones that play crucial roles in diverse aspects of plant growth and development (Kieber and Schaller 2014). Natural cytokinins are adenine derivatives and can be classified into aromatic or isoprenoid cytokinins, with isoprenoid cytokinins more prevalent in plants (Sakakibara 2006). The isoprenoid cytokinins are composed of isopentenyl adenine (iP), *trans*-zeatin (tZ), *cis*-zeatin (cZ), and dihydrozeatin (DZ), among which isopentenyl adenine and *trans*-zeatin are the primary active and most abundant cytokinins in Arabidopsis. The signaling pathway of cytokinins is mediated via a multistep phosphorelay (Hwang et al. 2012). In Arabidopsis, cytokinins bind to their receptors, the ARABIDOPSIS HISTIDINE KINASES (AHK2, AHK3, and AHK4; Ueguchi et al. 2001; Inoue et al. 2001). After the binding to a cytokinin, AHK is autophosphorylated on a conserved histidine residue in the kinase domain. The phosphate

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group is then transferred first to an aspartic acid residue within the receiver domain of AHKs and then to ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEINs (AHPs; Hwang and Sheen 2001). The AHP transfers the phosphate group to ARRs, which are classified into 3 types: Type-A, Type-B, and Type-C (To and Kieber 2008). Type-B ARRs are transcription factors that contain a receiver domain at their amino (N)-termini to receive the phosphate group from AHPs and a GARP-like DNA-binding domain at their carboxyl (C)-termini to activate the transcription of their target genes (Argyros et al. 2008; Hwang et al. 2012). Both Type-A and Type-C ARRs have a receiver domain and a differing C-terminal extension sequence but lack a DNA-binding motif (Hwang et al. 2012). The transcription of Type-A *ARRs* but not Type-C *ARRs* is rapidly induced by cytokinins (Pils and Heyl 2009).

In a previous study, we revealed that cytokinins play critical roles in MIZ1-mediated root hydrotropism (Chang et al. 2019). However, whether MIZ1 regulates the cytokinin signaling response and the molecular mechanisms underlying this regulatory function remain to be elucidated. In this report, we demonstrate that MIZ1 modulates the cytokinin signaling response by directly interacting with cytokinin receptors, AHK2, AHK3, and AHK4.

Results and discussion

A *MIZ1* mutant exhibits a decreased sensitivity to cytokinins

To determine whether MIZ1 is involved in cytokinin signal transduction, the root lengths of Col-0, *miz1-2*, 35S::*MIZ1*, and 2 independent lines of transgenic seedlings harboring 35S::*MIZ1* in the *miz1-2* background were measured after the treatment with various concentrations of *trans*-zeatin. Compared with the roots of wild-type (Col-0), the roots of *miz1-2* seedlings showed a reduced sensitivity to *trans*-zeatin, whereas the roots of *MIZ1*-overexpressing seedlings exhibited an increased sensitivity to *trans*-zeatin (Fig. 1, A to D and Supplementary Fig. S1). The reduced response of *miz1-2* roots to cytokinins was confirmed by the treatment of 6-benzylaminopurine (BAP), a bioactive form of aromatic cytokinins (Supplementary Fig. S2; Sakakibara 2006). The decreased sensitivity of *miz1-2* roots to cytokinins can be rescued by the expression of *MIZ1* in a dosage-dependent manner (Fig. 1, A to D). These results indicated that the reduced sensitivity of *miz1-2* roots to cytokinins is indeed caused by the loss-of-function of MIZ1.

We previously reported that lower concentrations of cytokinins (i.e. *trans*-zeatin) can increase cell division activity in the root meristem of Arabidopsis, whereas higher concentrations of cytokinins can inhibit cell division activity (Chang et al. 2019). Consistent with this finding, the cell number in root meristematic cortex was increased after the treatment with a lower concentration of *trans*-zeatin (1 nM) and reduced after the treatment with a higher concentration of *trans*-zeatin (100 nM) in Col-0 and 35S::*MIZ1* seedlings (Fig. 1, E to K). However, compared with that in Col-0 roots, cell division activity in the meristematic region of *miz1-2* roots exhibited a reduced response to *trans*-zeatin (Fig. 1, E to K). The expression of *CYCLIN B1;1* (*CYCB1;1*), encoding an effector of growth control at the G2/M transition of cell cycle, was induced in the root meristematic region in Col-0 seedlings by the application of lower concentrations of *trans*-zeatin and inhibited by the application of higher concentrations of *trans*-zeatin (Fig. 2A and Supplementary Fig. S3) (Hemerly et al. 1992; Schnittger and De Veylder 2018). The expression of *CYCB1;1* in the root meristematic region of *miz1-2*, however, was nearly unchanged by *trans*-zeatin treatments (Fig. 2A and Supplementary Fig. S3).

MIZ1 regulates the response of *ARR16* and *ARR17* to cytokinins

We previously reported that the expression levels of *ARR16* and *ARR17* are induced at the lower water potential sides and inhibited at the higher water potential sides of the wild type root tips when growing in the medium with a moisture gradient (Chang et al. 2019). In the root tips of *miz1-2*, however, the expression levels of these 2 *ARRs* were substantially decreased and could not be altered by moisture gradients. Given the fact that ARR16 and ARR17 are Type-A response regulators of cytokinin signaling, whose expression levels are induced by cytokinin signaling (Hwang et al. 2012), we therefore investigated whether the expression levels of Type-A *ARRs* are also regulated by MIZ1. The expression levels of Type-A *ARRs* in the root tips and seedlings of Col-0 and *miz1-2* treated with various concentrations of *trans*-zeatin were measured via RT-qPCR. Compared with those in Col-0, the expressional levels of *ARR16* and *ARR17* were decreased in both the seedlings and the root tips of *miz1-2*. They also showed a greatly reduced response to various concentrations of *trans*-zeatin in *miz1-2* (Fig. 2, B to C and Supplementary Fig. S4). The expression levels of other Type-A *ARRs*, including *ARR3*, *ARR4*, *ARR5*, *ARR6*, *ARR7*, *ARR8*, *ARR9*, and *ARR15* in both the seedlings and the root tips of *miz1-2*, however, showed no obvious differences from those of Col-0 with or without the treatment of *trans*-zeatin (Supplementary Fig. S4 and Supplementary Fig. S5). These results suggest that MIZ1 functions as the upstream regulator of ARR16 and ARR17. Previous studies suggested that the expression of *ARR16* depends on the function of AHK4 and ARR1 (Kiba et al. 2002; Ramireddy et al. 2013). In one of our previous publications, we found that the expression level of *ARR16* is down-regulated in the mutants of biosynthesis and signal transduction of cytokinins (Chang et al. 2019). Although its expression level is also down-regulated in the mutants of biosynthesis and the receptors of cytokinins, *ARR17* is up-regulated in 2 triple mutants, *ahp1 ahp2 ahp3* and *ahp2 ahp3 ahp5*, which is different from that of *ARR16*. The mechanisms underlying the specific regulation of these 2 Type-A ARRs by MIZ1 remain to be further investigated.

Consistent with the reduced response of an *ahk3-7 ahk4-2* double mutant to cytokinins, the expression and cytokinin response of *ARR5*, *ARR16*, and *ARR17* were greatly reduced in the root tips of the double mutant compared with those in Col-0 (Fig. 2, D to F) (Riefler et al. 2006). The expression and cytokinin response of *ARR5*, *ARR16*, and *ARR17* in *ahk3-7 ahk4-2* double mutant were partially restored by the overexpression of *MIZ1*, suggesting that MIZ1 functions as a downstream regulator of AHKs in the cytokinin signaling pathway (Fig. 2, D to F).

MIZ1 physically interacts with AHKs

Our genetic and physiological results strongly suggest that MIZ1 plays a role in cytokinin signaling pathway. In addition, the genetic data suggest that MIZ1 functions as a component of cytokinin signaling, acting as a regulatory component between AHKs and ARRs. MIZ1 is a soluble protein associated with the cytoplasmic side of the ER membrane (Yamazaki et al. 2012). On the other hand, AHKs are localized to both the ER membrane and the plasma membrane (Caesar et al. 2011; Wulfetange et al. 2011; Antoniadi et al. 2020; Kubiasová et al. 2020). Therefore, we investigated whether MIZ1 can physically interact with AHKs by utilizing a number of experimental approaches. Our yeast 2-hybrid assay demonstrated that MIZ1 can directly interact with AHK2, AHK3, and AHK4 (Fig. 3A). We also used the split yellow fluorescent protein (YFP)-based bimolecular fluorescence complementation

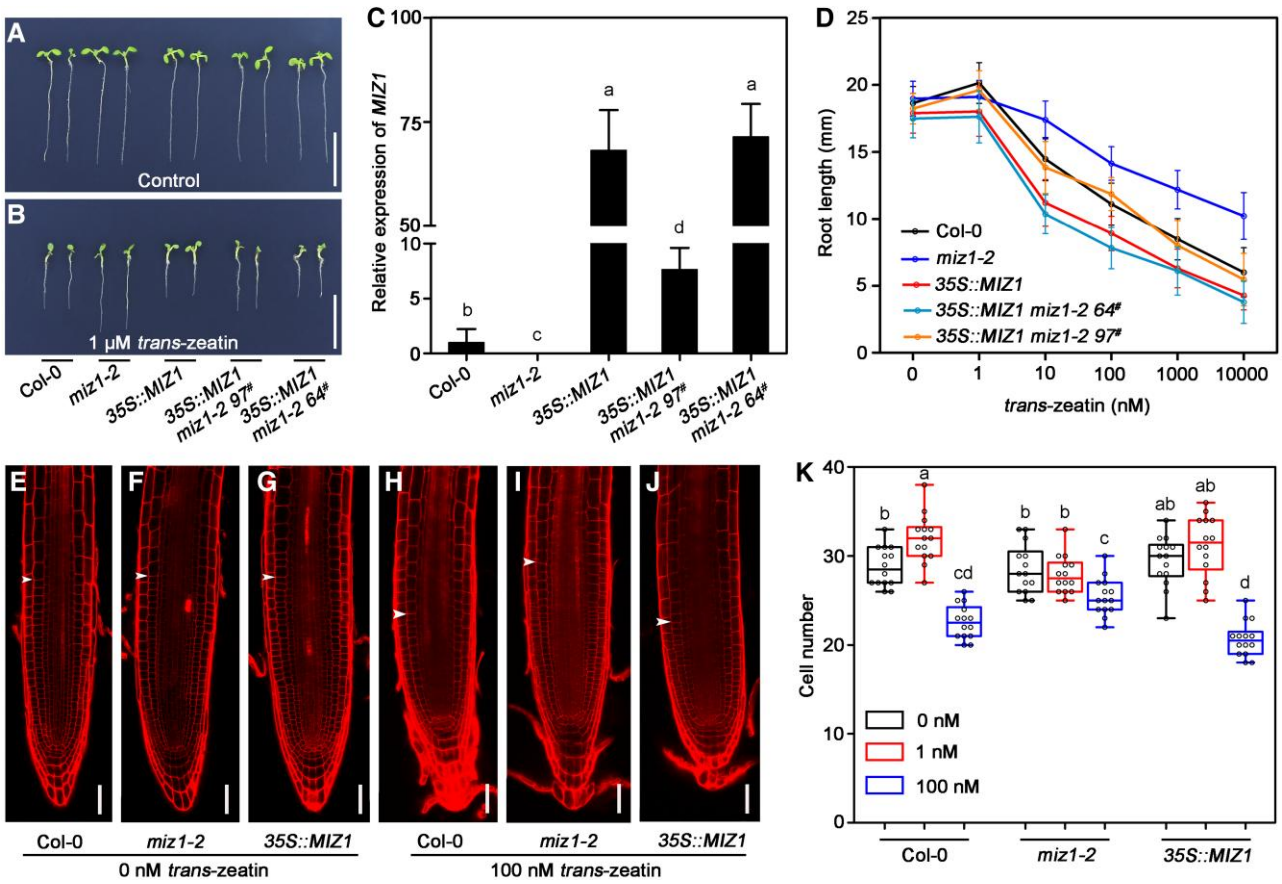

Figure 1. The roots of *MIZ1* mutant exhibited a decreased response to *trans*-zeatin. **A** and **B)** Four-day-old seedlings of the indicated genotypes were grown on a half-strength MS medium supplemented with or without 1 μM *trans*-zeatin. **C)** Relative expression levels of *MIZ1* in the root tips of the indicated seedlings were quantified via RT-qPCR. The data are presented as the means ± SEMs of 3 biological replicates. **D)** Measurement of the length of roots from the indicated genotypes after treatment with various concentrations of *trans*-zeatin. The data are presented as the means ± SEMs ($n \geq 27$). **E** to **J)** Response of the size of root meristem in wild-type and *miz1-2* to 100 nM *trans*-zeatin. The white arrows indicate the boundary between the meristematic and the transition zones. **K)**, Quantification of cells in the meristematic cortex of roots as shown in **E)** to **J)** ($n = 14$). Each circle represents a measurement from an individual root. The boxes in the plot span the first to the third quartiles of the data. The whiskers indicate the minimum and maximum values. The lines inside the boxes represent the means. Scale bars represent 10 mm in **A)** and **B)** and 50 μm in **E)** to **J)**. Three independent biological replicates were carried out and similar results were obtained. The data from a single experiment are presented. One-way ANOVA with Tukey's multiple comparison test was used for statistical analysis with $P < 0.05$.

(BiFC) assay to confirm the interactions between MIZ1 and AHKs (Fig. 3B). MIZ1 and AHKs proteins expressed in and extracted from *Nicotiana benthamiana* were further used for coimmunoprecipitation (Co-IP) assays. Consistent with the above results, MIZ1 were able to interact with AHK2, AHK3, and AHK4 in vivo (Fig. 3C). These results demonstrate that MIZ1 physically associates with AHKs.

Mutants of cytokinin signaling showed a decreased hydrotropic response

MIZ1 plays a key role in the regulation of root hydrotropism. Roots of *MIZ1* mutants exhibit no hydrotropic response (Kaneyasu et al. 2007). We found that MIZ1 can physically interact with AHKs to control the response of *ARR16* and *ARR17* to cytokinins. An upcoming question is whether *AHKs* and *ARRs* mutants exhibit a defect in root hydrotropic response. *ARR16* and *ARR17* single mutants exhibits a hydrotropic response comparable to that of wild type (Fig. 4, A and B). A double mutant *arr16 arr17* and a sextuple mutant *arr3 arr4 arr5 arr6 arr8 arr9* exhibited a significant decreased hydrotropic response compared with Col-0 (Fig. 4, C and D). These results suggest that in addition to ARR16 and ARR17, other Type-A ARRs also play some roles in root hydrotropism and MIZ1 may regulates these ARRs through post-transcriptional mechanisms. A triple mutant *ahk2-5 ahk3-7 ahk4-2*, generated via genetic crossing using T-DNA insertion mutant alleles of *AHKs*, was reported to exhibit no response to cytokinins (Riefler et al. 2006). This triple mutant exhibited no hydrotropic response similar to that of *miz1-2* (Fig. 4, E and F, Supplementary Video S1, and Supplementary Video S2). We also generated an octuple mutant *arr3 arr4 arr5 arr6 arr8 arr9 arr16-C arr17-C*, which showed no hydrotropic response as well (Fig. 4, G and H). The octuple mutant was generated by partial sequence deletion or insertion of *ARR16* and *ARR17* in *arr3 arr4 arr5 arr6 arr8 arr9* sextuple mutant background via a CRISPR-Cas9 system (Supplementary Fig. S6 and Supplementary Video S3; Chang et al. 2019). These results indicate that cytokinin signaling plays essential roles in root hydrotropism, the biological functions of MIZ1 in regulating root hydrotropism rely on cytokinin signal transduction.

MIZ1 modulates the homeostasis of cytokinins

To examine the possible signaling pathways regulated by MIZ1, we compared the gene expression profiles of Col-0 and *miz1-2*. Total RNA from 5-mm-long root tips of Col-0 and *miz1-2* plants was extracted, and RNA-seq analysis was conducted. We identified 704 differentially expressed genes (DEGs) in the root tips of

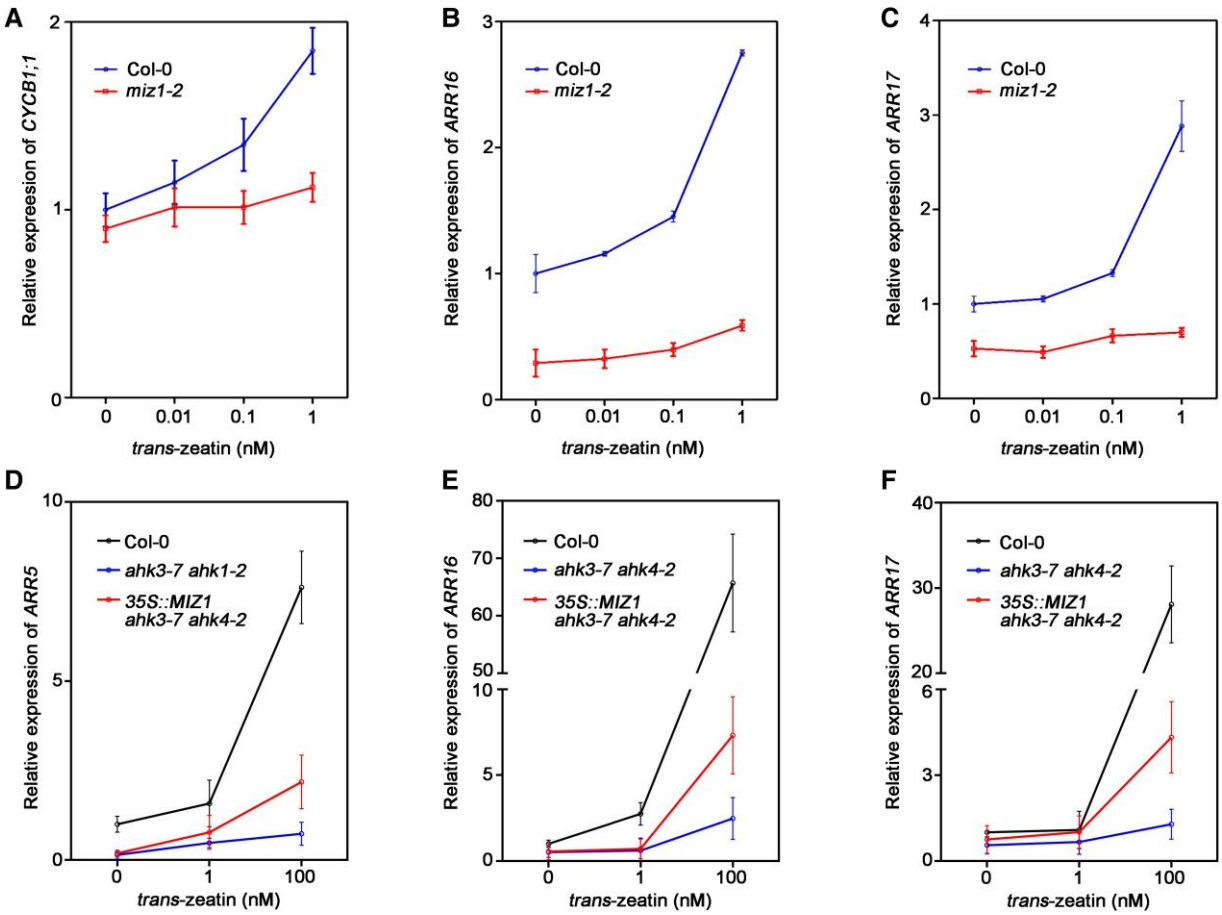

Figure 2. MIZ1 mediates the response of *ARR16* and *ARR17* to cytokinins. **A** to **C)** Relative expression levels of *CYCB1;1*, *ARR16*, and *ARR17* upon the treatments of various concentrations of *trans*-zeatin in the root tips of Col-0 and *miz1-2*. **D** to **F)** The relative expressions of *ARR5*, *ARR16*, and *ARR17* upon the treatments of various concentrations of *trans*-zeatin in the root tips of Col-0, *ahk3-7 ahk4-2*, and *35S::MIZ1* in *ahk3-7 ahk4-2* background. Four-day-old seedlings were transferred from a 1/2 MS medium to a 1/2 MS medium supplemented with or without *trans*-zeatin. After 6 h of incubation, the root tips were collected for RNA extraction and RT-qPCR analysis. The data are presented as the means ± SEMs of 3 biological replicates.

miz1-2 compared with those in Col-0. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis revealed that some of the DEGs are enriched in plant hormone signal transductions, such as *ARR16* and *ARR17* in cytokinin signaling (Fig. 5A). In addition, some DEGs exhibited enrichment in the cytokinin biosynthesis. We next performed RT-qPCR analysis of 25 genes known or predicted to encode enzymes involved in the biosynthesis and catabolism of cytokinins (Kamada-Nobusada and Sakakibara 2009). Most of these genes were significantly downregulated in the root tips of *miz1-2* compared with those of Col-0 (Fig. 5B). These data suggest that MIZ1 can regulate the expression of genes encoding enzymes to modulate the homeostasis of cytokinins. In a previous study, we demonstrated that the asymmetric distribution of cytokinins is one of the driving forces of root hydrotropism (Chang et al. 2019). The asymmetric distribution of cytokinins depends on the function of MIZ1. Here, we discovered that MIZ1 controls the expression of genes involved in cytokinin biosynthesis and catabolism. These results suggest that MIZ1 mediates the asymmetric distribution of cytokinins is likely through control the expression of genes involved in cytokinin biosynthesis or catabolism in Arabidopsis during root hydrotropic response. The mechanisms involved these processes will be investigated in detail in future studies.

A previous report from Takahashi's group showed that, in comparison with Col-0, a *MIZ1* mutant (*miz1-1*, a single-base substitution mutant (G704A)) displayed a reduced response to BAP (Moriwaki et al. 2011). Overexpression of *MIZ1*, on the other hand, led to root hypersensitivity to BAP. Moreover, we previously revealed that the roots of *miz1-2* exhibited reduced sensitivity to *trans*-zeatin (Chang et al. 2019). Although these findings suggest that MIZ1 regulates cytokinin response, the molecular mechanisms underlying this process have not been elucidated.

In this study, we demonstrated that MIZ1 regulates the cytokinin response through direct interaction with the cytokinin receptors of AHKs to specifically control the expression of *ARR16* and *ARR17*. Our findings indicate that cytokinin signal transduction plays an essential role in root hydrotropic response.

Materials and methods

Plant materials and growth conditions

The Columbia accession (Col-0) ecotype was used as the wild-type. The T-DNA insertion mutant of *miz1-2* (SALK_076560) was obtained from the Arabidopsis Biological Resource Center (ABRC). mutants of *ahk3-7 ahk4-2*, *ahk2-5 ahk3-7 ahk4-2* and *arr3 arr4 arr5 arr6 arr8 arr9* were kindly provided by professors Guangqin Guo and Yurong Bi (Lanzhou University, China). The single and double mutants of *ARR16* and *ARR17* were obtained from Jianru Zuo's lab (Institute of Genetics and Developmental

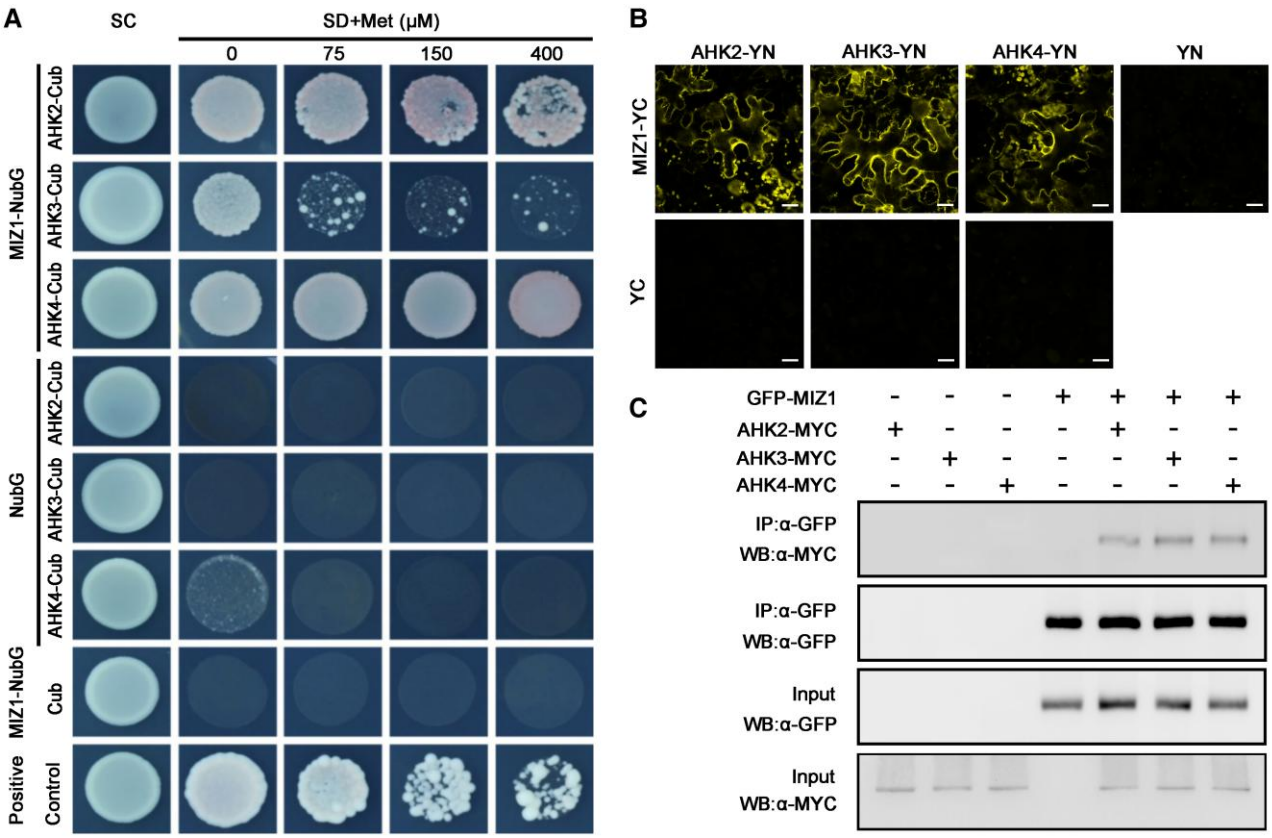

Figure 3. MIZ1 physically interacts with cytokinin receptors of AHKs. **A)** The interactions between MIZ1 and AHK2, AHK3 as well as AHK4 were detected via an mbSUS yeast 2-hybrid system. The MIZ1 protein was fused to the N-terminal domain of ubiquitin, the protein of AHK2, AHK3 and AHK4 were fused to the C-terminal domain of ubiquitin. Yeast cells were grown on a medium to select for diploid cells (SC + Ade + His medium) or to detect interactions (SD medium with 4 different methionine concentrations). **B)** A BiFC assay was performed in *Nicotiana benthamiana* leaves to visualize the interactions between MIZ1 and AHK2, AHK3 or AHK4. MIZ1 was fused to the C-terminal domain of YFP (YC), AHK2, AHK3 and AHK4 were fused to the N-terminal domain of YFP (YN). The interactions between MIZ1-YC and YN or between YC and AHK2-YN, AHK3-YN, or AHK4-YN were employed as negative controls. Scale bars represent 50 μm. **C)** In vivo co-immunoprecipitation of GFP-MIZ1 with AHK2-MYC, AHK3-MYC or AHK4-MYC in *N. benthamiana*. Three independent biological replicates were carried out, and similar results were obtained. The data from a single experiment are presented.

Biology, Chinese Academy of Sciences). The seeds of the transgenic plants harboring *pCYCB1;1::GUS*, was provided by Professor Chuanyou Li (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China). The transgenic plants of *35S::MIZ1* were generated by introducing the coding sequence (CDS) of *MIZ1* into the vector *pEarleyGate104* (*35S::GFP-GWR*). The constructed vectors were subsequently transformed into *Agrobacterium tumefaciens* strain GV3101 and then into *Arabidopsis thaliana* via the floral dip method. Homozygous transgenic lines were selected and used for analyses. Information regarding primers used for genotyping and DNA cloning can be identified in supplementary information (Supplementary Tables S1 and S2).

For seedling preparation, surface-sterilized Arabidopsis seeds were incubated at 4 °C for 48 h for stratification and subsequently germinated vertically on half-strength Murashige and Skoog (1/2 MS) agar plates (1% w/v) supplemented with 1% sucrose (w/v). The plates were maintained in a growth chamber set at 22 °C under a 16-hour light/8-hour dark photoperiod.

Cytokinin treatment

Seeds were germinated on 1/2 MS media supplemented with various concentrations of *trans*-zeatin or BAP and incubated in a growth chamber for 7 or 15 days before being photographed. For confocal imaging, 4-day-old seedlings were transferred from a 1/2 MS medium to a 1/2 MS medium supplemented with various concentrations of *trans*-zeatin or BAP. After 24 h of treatment, the roots were stained with propidium iodide (PI) for 3 min before being imaged with a confocal laser scanning microscope (A1R + Ti2-E, Nikon, Japan). To detect changes in gene expression in response to cytokinins, 4-day-old seedlings were transferred from a 1/2 MS medium containing 1% agar and 1% sucrose (w/v) to the same 1/2 MS medium supplemented with various concentrations of *trans*-zeatin. After 16 h of incubation, 5-mm-long root tips were collected for total RNA extraction and RT-qPCR analysis.

Staining and microscopy

Root tips of 4-day-old seedlings were stained with propidium iodide (PI, 10 μg/mL in distilled water) for 3 min and examined using a confocal laser scanning microscope (A1R + Ti2-E, Nikon, Japan). The image acquisition was performed with the following program: laser power, 10.2; emission wavelength, 595 nm; excitation wavelength, 561 nm; line average mode, average; line average/integrate count, 4.

For GUS staining, 4-day-old homozygous transgenic seedlings harboring *pCYCB1;1::GUS* were transferred from a normal 1/2 MS medium supplemented with 1% agar (w/v) and 1% sucrose (w/v) to the plates with varying concentrations of *trans*-zeatin. After a 2-hour treatment, the seedlings were then transferred to ice-cold

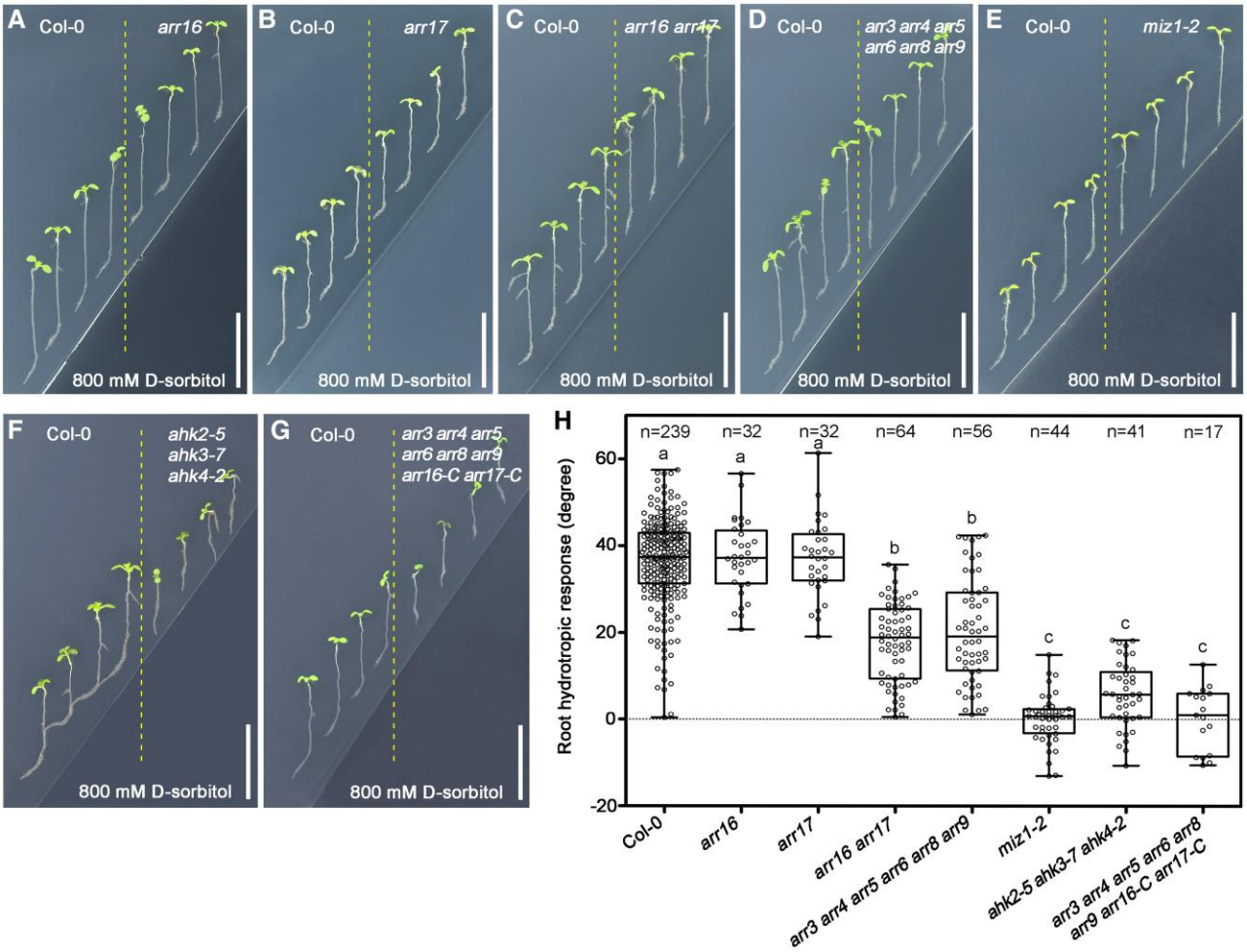

Figure 4. Mutants of cytokinin signaling exhibited a decreased hydrotropic response. **A** to **G)** The hydrotropic response of representative wild-type (Col-0) and indicated mutants. Four-day-old seedlings were transferred from a 1/2 MS medium to a hydrostimulation medium (800 mM D-sorbitol at the bottom-right side) with the root tips 0.5 cm away from the border, and the seedlings were then vertically grown on the medium for 36 h before being photographed. **H)** Measurements of root growth curvature in response to hydrostimulation. Each circle represents a measurement from an individual root. The boxes in the plot span the first to the third quartiles of the data. The whiskers indicate the minimum and maximum values. The lines inside the boxes represent the means. "*n*" indicates the number of roots used in this experiment. Scale bars represent 10 mm. Three independent biological replicates were carried out and similar results were obtained. The data from a single experiment are presented. One-way ANOVA with Tukey's multiple comparison test was used for statistical analysis with $P < 0.05$.

90% acetone and incubated for 15 min. The acetone was removed and the rinse solution (50 mM sodium phosphate buffer pH 7.2, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$) was added. Incubation with the rinse solution was performed at room temperature for 5 min. The rinse solution was then discarded, and the staining solution (the rinse solution plus 2 mM X-gluc) was added. The samples were then incubated at 37 °C in the dark for 30 min. The samples were then washed with a series of ethanol solutions (15%, 30%, 50%, 70%, 80%, 95%, 100%, and 85%) for 30 min each at room temperature. The samples were then stored in 70% ethanol overnight. Ethanol was replaced with a chloral hydrate solution (80% chloral hydrate solution (w/v), 20% glycerin (v/v), and 20% purified water (v/v)). After an incubation period of 48 h, root tips were visualized under a microscope. GUS staining intensity was measured via ImageJ software according a previously reported protocol (Chang and Li 2022).

RT-qPCR analysis

Four-day-old seedlings were mock treated or treated with various concentrations of *trans*-zeatin. The seedlings or root tips (approximately 5-mm long) were subsequently harvested for total RNA extraction and subsequent RT-qPCR analysis. RNA was extracted using the RNeasy Plant Mini Kit (QIAGEN, 74903). The first-strand cDNA synthesis was performed using the Hifair II reverse transcriptase (Yeasen, China). qPCR was conducted using the Hieff qPCR SYBR Green Master Mix (11203ES08; Yeasen, China). The expression of *ACTIN2* was used as an internal reference. Three biological replicates were analyzed. The primers used for RT-qPCR are listed in the supplementary information (Supplementary Table S3).

Yeast 2-hybrid assay

A mating-based split ubiquitin system (mbSUS) was used to examine the interactions between MIZ1 and AHKs (Grefen et al. 2009; Asseck and Grefen 2018). The full-length coding sequences of MIZ1 and AHKs were cotransferred into THY.AP5 yeast cells via the vector *pX-NubWTgate* (linearized by the enzymes of EcoRI and SmaI) or into THY.AP4 yeast cells via the vector *pMetYCgate* (linearized by the enzymes of PstI and HindIII). Recombinant THY.AP4 and THY.AP5 cells were selected on appropriate medium

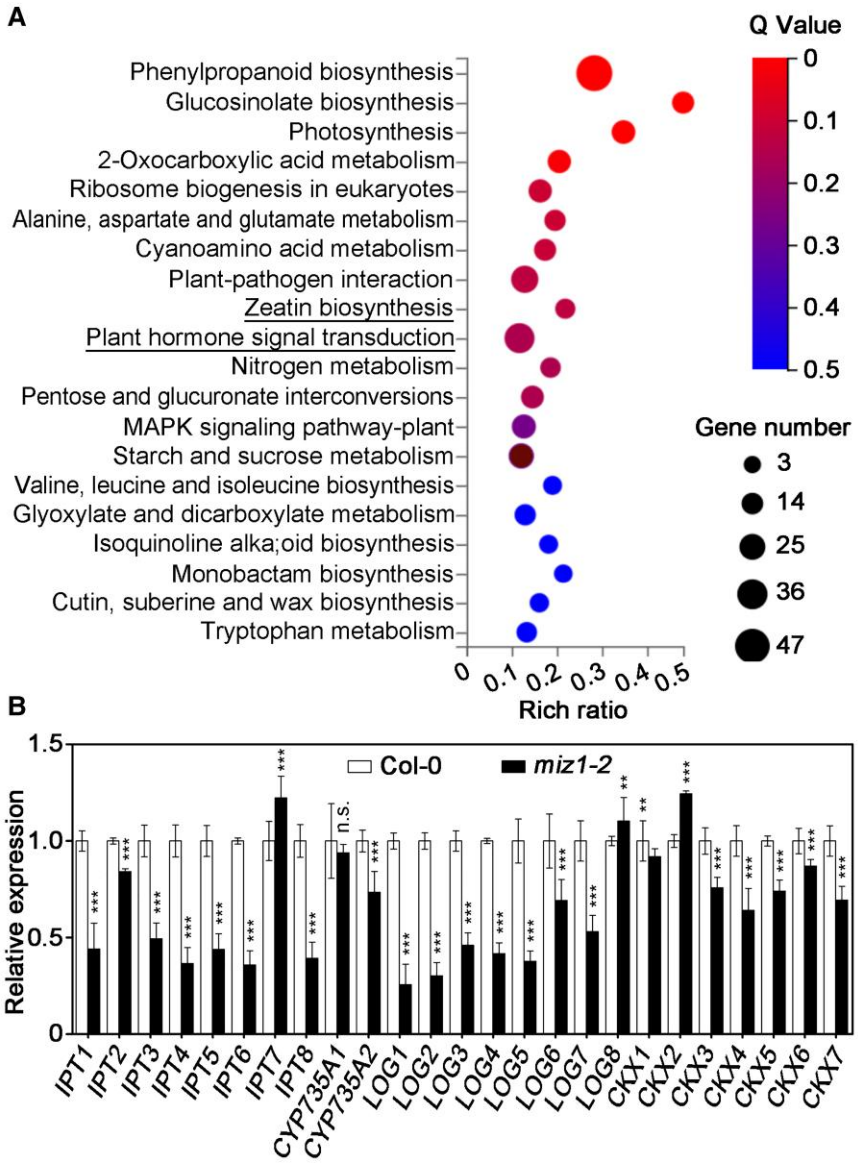

Figure 5. MIZ1 modulates the homeostasis of cytokinins. **A)** Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of the differentially expressed genes (DEGs) in the root tips of *miz1-2* compared with those in Col-0 identified via RNA-seq analysis. KEGG pathway enrichment was analyzed via KEGG Mapper. Zeatin biosynthesis and plant hormone signal transduction pathways are underlined. The source data of RNA-seq are included in the Source Data file. The "Rich ratio" value represents the degree of enrichment of DEGs in each pathway. The number of enriched DEGs is represented by the size of each circle, with a larger circle indicating a greater number. The Q value is the adjusted *P* value after multiple comparison testing, with a lower Q value indicating more significant enrichment. **B)** RT–qPCR analysis showing the expression levels of genes encoding enzymes involved in cytokinin biosynthesis and catabolism in the root tips of *miz1-2* relative to those in Col-0. The data are presented as the means ± SEMs of 3 biological replicates. Statistical significance was determined by 2-tailed unpaired t tests without correction for multiple comparisons with $P < 0.01$.

without leucine or tryptophan. The cells generated from each cross were mixed and mated on a yeast extract–peptone–dextrose (YPD) plate for 6 to 8 h at 30 °C. Diploid cells were then selected on synthetic complete medium (SC) plates supplemented with adenine and histidine (SC + Ade + His) and transferred onto synthetic minimal medium (SD) plates supplemented with different concentrations of methionine. Growth was recorded after incubation for 3 days.

BiFC assay

The coding sequences of MIZ1 and AHKs were fused with either the N-terminal or C-terminal domain of YFP in the vectors *pEarleygate201-YN* or *pEarleygate202-YC*, respectively. The constructed plasmids were subsequently transformed into *A. tumefaciens* strain GV3101. Bacterial cultures were grown overnight in Luria–Bertani (LB) medium supplemented with 20 μM acetosyringone and resuspended to an optical density at 600 nm (OD600) of 0.4 in injection buffer (liquid MS medium containing 150 μM acetosyringone, 10 mM $MgCl_2$ and 10 mM 2-(*N*-morpholino)ethanesulfonic acid [pH 5.7]). Equal volumes of resuspended cultures of MIZ1-YC and AHK2-YN, AHK3-YN, or AHK4-YN were co-injected into 3-week-old *N. benthamiana* leaves. The interactions between MIZ1-YC and YN, or between YC and AHK2-YN, AHK3-YN, or AHK4-YN were employed as negative controls. At 48-h post-injection, the reconstituted YFP fluorescence was visualized using a confocal laser scanning microscope (A1R + Ti2-E, Nikon, Japan). The image acquisition was performed with the following program:

laser power, 25.2; emission wavelength, 540 nm; excitation wavelength, 513 nm; line average mode, average; line average/integrate count, 4.

Co-IP assay

The coding sequence of MIZ1 was introduced into the vector *pEarleyGate104* (*BASTA-35S-GFP-GWR*), and the coding sequences of AHK2, AHK3, and AHK4 were inserted into the vector *pBIB-BASTA-35S-GWR-MYC*. The constructed plasmids were subsequently transformed into *A. tumefaciens* strain GV310. The cultures were grown overnight in LB medium containing 20 μM acetosyringone and resuspended to an OD600 of 0.4 in injection buffer (liquid MS medium containing 150 μM acetosyringone, 10 mM $MgCl_2$ and 10 mM 2-(*N*-morpholino)ethanesulfonic acid [pH 5.7]). Equal amounts of resuspended cultures of GFP-MIZ1 and AHK2-MYC, AHK3-MYC, or AHK4-MYC were coinjected into 3-week-old *N. benthamiana* leaves. Leaves were collected and ground to a fine powder in liquid nitrogen and were then lysed with extraction buffer (10 mM HEPES [pH 7.5], 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100 and 1:100 complete protease inhibitor cocktail [04693132001, Roche]) 48 h after injection. After solubilization with extraction buffer and vortexing for 5 min, the samples were cleared at 16,000 × *g* for 10 min at 4 °C, and the supernatant was then incubated with anti-GFP beads (AlpalifeBio, KSTM1301) for 3 hours at 4 °C with gentle shaking. The beads were then collected and washed 5 times with extraction buffer containing 0.5% Triton X-100. The proteins bound to the beads were boiled with 1× SDS loading buffer for 5 min and were then analyzed by immunoblotting with anti-GFP (Roche, 11814460001) and anti-MYC (Abmart, m20002L) antibodies.

Root hydrotropism treatment

To prepare a split-agar medium with a moisture gradient for the hydrostimulation assay, 1/2 MS medium supplemented with 1% sucrose and 1% agar (w/v) was added to a Petri dish. After solidification, the right bottom half of the medium was excised and replaced with 1/2 MS medium supplemented with 1% sucrose, 1% agar (w/v), and 800 mM D-sorbitol. Four-day-old seedlings were transferred from 1/2 MS medium to the hydrostimulation medium with the root tips 0.5 cm away from the border, and the seedlings were then vertically grown on the medium for 36 h before being photographed. The root growth curvature, indicating the hydrotropic response, was measured via ImageJ software.

Statistical analysis

The data were analyzed using Student's *t* test or 1-way ANOVA. Student's *t* test, by 2-side without making any adjustments for multiple comparisons, was used to determine whether 2 datasets were significantly different. "***" indicates $P < 0.001$, "**" indicates $P < 0.01$, and "*" indicates $P < 0.05$, while n.s. indicates no significant difference between 2 datasets. ANOVA was used if >2 datasets were being compared. One-way ANOVA with Tukey's multiple comparison test was applied to each dataset based on a *P* value of 0.05. Letters were used to indicate the significance levels between all the datasets. The same letter indicates no significant difference. However, different letters indicate significant differences between the datasets. Figures with box plots, boxplots span the first to the third quartiles of the data, and whiskers indicate the minimum and maximum values. The line in the box represents the mean. The exact *P*-value was included in the Source Data file.

Accession numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers of *At2g41660* (*MIZ1*), *At5g35750* (*AHK2*), *At1g27320* (*AHK3*), and *At2g01830*_(*AHK4*).

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Author contributions

J.C. and J.L. supervised the entire project. J.C. and W.F. designed the experiments. W.F. and J.C. carried out most of the experiments. J.S., L.H., and L.W. performed some of the experiments. J.C. and J.L. wrote the manuscript.

Supplementary data

The following materials are available in the online version of this article.

Supplementary Figure S1. Mutant of *MIZ1* exhibited a reduced sensitivity to *trans*-zeatin.

Supplementary Figure S2. Mutant of *MIZ1* exhibited a reduced sensitivity to BAP.

Supplementary Figure S3. Cell division in the root meristematic region of *miz1-2* shown a reduced sensitivity to *trans*-zeatin.

Supplementary Figure S4. The response of type-A *ARRs*, excluding *ARR16* and *ARR17*, to *trans*-zeatin is not influenced by the mutation of *MIZ1*.

Supplementary Figure S5. The responses of type-A *ARRs*, excluding *ARR16* and *ARR17*, to *trans*-zeatin in the root tips of *miz1-2* are similar to those in Col-0.

Supplementary Figure S6. The detailed mutation alleles of *arr16-C* and *arr17-C*.

Supplementary Table S1. Primers used for genotyping in this research.

Supplementary Table S2. Primers used for DNA or cDNA cloning in our research.

Supplementary Table S3. Primers used for RT-qPCR in our research.

Supplementary Video S1. Mutant of *miz1-2* exhibited no hydrotropic response.

Supplementary Video S2. Mutant of *ahk2-5 ahk3-7 ahk4-2* exhibited a decreased hydrotropic response.

Supplementary Video S3. Mutant of *arr3 arr4 arr5 arr6 arr8 arr9 arr16-C arr17-C* exhibited a decreased hydrotropic response.

Supplementary Video Legends.

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Data availability

The data supporting the research are available in the article and its Supplementary materials.

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