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Are Histidine Kinases of Arbuscular Mycorrhizal Fungi Involved in the Response to Ethylene and Cytokinins?

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Signals are exchanged at all stages of the arbuscular mycorrhizal (AM) symbiosis between fungi and their host plants. Root-exuded strigolactones are well-known early symbiotic cues, but the role of other phytohormones as interkingdom signals has seldom been investigated. Here we focus on ethylene and cytokinins, for which candidate receptors have been identified in the genome of the AM fungus *Rhizophagus irregularis*. Ethylene is known from the literature to affect asymbiotic development of AM fungi, and in the present study, we found that three cytokinin forms could stimulate spore germination in *R. irregularis*. Heterologous complementation of a *Saccharomyces cerevisiae* mutant strain with the candidate ethylene receptor RiHHK6 suggested that this protein can sense and transduce an ethylene signal. Accordingly, its N-terminal domain expressed in *Pichia pastoris* displayed saturable binding to radiolabeled ethylene. Thus, RiHHK6 displays the expected characteristics of an ethylene receptor. In contrast, the candidate cytokinin receptor RiHHK7 did not complement the *S. cerevisiae* mutant strain or *Medicago truncatula* cytokinin receptor mutants and seemed unable to bind cytokinins, suggesting that another receptor is involved in the perception of these phytohormones. Taken together, our results support the hypothesis that AM fungi respond to a range of phytohormones and that these compounds bear multiple functions in the rhizosphere

beyond their known roles as internal plant developmental regulators. Our analysis of two phytohormone receptor candidates also sheds new light on the possible perception mechanisms in AM fungi.

Keywords: cytokinin, ethylene, mycorrhiza, receptor, *Rhizophagus*, symbiosis

Plants interact with myriad microorganisms, particularly in the soil compartment surrounding their roots. In this complex environment, both plants and microbes use chemical cues to mediate essential processes such as mutual recognition, nutrient exchange, or the development of compatible or incompatible interactions (Berlanga-Clavero et al. 2020). A wide array of chemical signals belonging to diverse biochemical categories are exchanged. Interestingly, in many cases, these signaling compounds were first associated with cell-to-cell communication within a multicellular organism, or within a species in the case of microbes. Only later were they discovered to mediate interkingdom communication (He et al. 2023).

Arbuscular mycorrhizal (AM) fungi from the order Glomeromycotina comprise the most common group of root-associated microbes. Their association with plants dates back to more than 400 million years ago, and concerns the vast majority of land plant species (Brachmann and Parniske 2006). In angiosperms, AM fungi penetrate root cortical cells to establish branched structures called arbuscules, where nutrient exchange takes place between the plant and fungus: AM fungi provide their host plants with water and minerals and receive carbon sources in the form of lipids and sugars (Lanfranco et al. 2018). The outstanding ecological success of AM symbiosis relies on a delicate balance between cost and benefit for each symbiont in a situation where multiple partners are available and nutrient constraints can vary greatly. It is therefore essential for the plant and fungus to identify suitable symbionts and adequately regulate the extent of root colonization, as well as the intensity of nutrient exchanges.

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A number of symbiotic signals regulating AM interactions have been identified. They include root-released phytohormones called strigolactones, *N*-acetyl-glucosamine derivatives of fungal or plant origin, fungal-secreted peptides, fungal volatile organic compounds, and the exchanged nutrients themselves (Lanfranco et al. 2018). Interestingly, strigolactones were identified as interkingdom signals in AM symbiosis through their effects on the morphology and metabolism of isolated AM fungi (Akiyama et al. 2005; Besserer et al. 2006, 2008), and their hormonal functions in plants were only discovered in 2008 (Gomez-Roldan et al. 2008; Umehara et al. 2008). In contrast, the effects of other phytohormones on AM symbiosis have mostly been interpreted in terms of impacts on the plant host, consistent with the fact that these compounds were essentially regarded as internal plant signals. While positive or negative impacts on the symbiosis have been reported depending on phytohormone class, plant species, and experimental conditions (Foo et al. 2013; Gutjahr 2014), experimental designs usually make it impossible to distinguish between impacts on the plant, the fungus, or both. There have been, however, a few direct observations of phytohormone effects on isolated AM fungi. Jasmonate was reported to stimulate hyphal elongation (Nagata et al. 2016). Ishii et al. (1996) reported the effects of ethylene on two species of AM fungi, *Gigaspora ramisporophora* and *Glomus mosseae*. Ethylene was mentioned to enhance spore germination in both species. Hyphal elongation responded positively to small concentrations of ethylene but was inhibited at the highest tested concentrations. More recently, growth conditions that allow the completion of a full developmental cycle of AM fungi in asymbiotic conditions have been defined. They have revealed the effect of abscisic acid, strigolactones, and jasmonate on asymbiotic sporulation (Liu et al. 2019; Tanaka et al. 2022).

Despite their classification as regulators of plant development, it is well known that phytohormones are produced and/or perceived by many microorganisms. A classic example is that of gibberellins, first discovered in a fungal rice pathogen (Kurosawa 1926). The release of phytohormones or analogous compounds is also associated with the plant-beneficial effects of many plant growth-promoting rhizobacteria (Egamberdieva et al. 2017). Phytohormone perception has been documented in the fungal pathogen *Ustilago maydis*, which possesses a sensor and signal transduction pathway to respond to salicylic acid (Rabe et al. 2016), and the bacterial pathogen *Xanthomonas campestris*, which enhances its virulence upon perception of host-released cytokinins (Wang et al. 2017). Likewise, a wide range of (micro)organisms including amoebae, bacteria, and cyanobacteria can sense ethylene to regulate their own development (Amagai 2011; Augimeri and Strap 2015; Lacey and Binder 2016) or possess ethylene receptor homologs (Bidon et al. 2020; Carlew et al. 2020; Papon and Binder 2019).

Because AM fungi have coevolved with plants as obligate biotrophs for hundreds of millions of years and clearly perceive and respond to strigolactones, it is conceivable that they use additional phytohormonal cues. In addition, AM-germinated spores were recently shown to contain or release four types of phytohormones: ethylene, the cytokinin *N*⁶-isopentenyladenosine, the auxin indoleacetic acid, and the gibberellin GA₄ (Pons et al. 2020). It is yet unknown whether AM fungi use these signals for their own sake or to modulate plant development. It is thus of interest to determine whether AM fungi possess a perception and signal transduction pathway for these hormones.

Receptors of ethylene and cytokinins in plants are histidine kinase (HK) proteins evolved from a bacterial two-component system (Capra and Laub 2012). Bacterial HKs sense various stimuli through their N-terminal domains. In the simplest cases, this perception triggers autophosphorylation of a conserved histidine residue in the C-terminal kinase domain, then the transfer

of the phosphate group to a response regulator protein. Typical plant HK receptors also comprise two main domains to achieve signal recognition and transduction. The N-terminal domain is required for ligand binding and, accordingly, differs between HK proteins depending on their respective ligands. For cytokinin receptors, a cyclase/histidine kinase-associated sensing extracellular (CHASE) domain surrounded by two transmembrane helices is required for ligand binding (Mougel and Zhulin 2001). For ethylene, the receptor characteristic feature is a combination of three transmembrane helices that contain seven conserved residues involved in ethylene binding (Hall et al. 2007).

The properties of the C-terminal domain and mechanisms underlying signal transduction differ between bacterial and plant HKs and for the plant receptors between cytokinin and ethylene sensors. Briefly, the cytokinin signaling pathways in plants differ from bacterial two-component systems in that they involve (i) an additional transfer of the phosphate group between domains of the HK protein and (ii) phosphotransfer proteins that relay the signal between HKs and response regulators (Keshishian and Rashotte 2015). The importance of kinase activity for signal transduction is less well established for plant ethylene receptors (Shakeel et al. 2015). Another difference between plant cytokinin and ethylene receptors is that ethylene receptors are active in the absence of their ligand to negatively regulate ethylene responses. They are inactivated upon ethylene binding, which releases the repression of ethylene responses (Binder 2020).

In a previous study, we explored the structural diversity of HK proteins in fungi (Hérivaux et al. 2017). Among other features, the presence of the characteristic N-terminal domains for ethylene or cytokinin binding was searched in fungal HK-encoding genes. This allowed the identification, in the genome of the model AM fungus *Rhizophagus irregularis*, of two genes that seem to harbor all the features required for perception and signal transduction of ethylene (*RiHHK6*) and cytokinin (*RiHHK7*). This led us to propose that AM fungi could perceive ethylene and cytokinins through these HK proteins.

The C-terminal kinase domain of HKs is highly conserved across eukaryotes (Loomis et al. 1997), which allows the functionality of HK proteins to be tested using heterologous complementation. In *Saccharomyces cerevisiae*, the osmosensor SLN1 is the only HK protein, and it is possible to complement *sln1* mutants with another HK receptor in the presence of its ligand. This type of experiment has allowed the functional characterization of various HKs, including the osmosensor AHK1 from *Arabidopsis thaliana* (Urao et al. 1999) and several plant cytokinin receptors (Daudu et al. 2017; Held et al. 2014; Héricourt et al. 2019; Murray et al. 2007). Such assays also allowed the sensitivity and specificity of candidate receptors for different cytokinin forms to be determined (Daudu et al. 2017).

The obligate biotrophy of AM fungi represents an obstacle to addressing the effects of compounds like phytohormones that also have an effect on the host plant. Nevertheless, the AM fungus *R. irregularis* can be grown in vitro from spores for about 2 weeks in the absence of a host plant (Taulera et al. 2020). This experimental system was used to examine the responses of *R. irregularis* to cytokinins without any interference from possible effects on the host plant. Then, candidate receptors for ethylene and cytokinins were characterized using complementation in heterologous systems such as yeast or *Medicago truncatula*. Finally, binding assays were carried out to estimate the ability of the candidate proteins to recognize their phytohormonal ligands.

Results

Characterization of the candidate ethylene receptor RiHHK6

The responses of AM fungi to ethylene mentioned in the introduction suggest that they possess a receptor for this gaseous

hormone. We therefore attempted to determine whether the previously identified receptor candidate RiHHK6 of *R. irregularis* (Hérivaux et al. 2017) could sense ethylene and transduce this signal.

Investigation of RiHHK6 functionality through heterologous expression in a yeast $\Delta sln1$ mutant strain. In an attempt to functionally characterize the candidate ethylene receptor RiHHK6 of *R. irregularis*, we expressed the corresponding cDNA in a $\Delta sln1$ mutant strain of *S. cerevisiae*. The $\Delta sln1$ mutant strain used in this study is deleted for its sole HK. It can grow when supplied with galactose as a carbon source, but not on glucose (Héricourt et al. 2016). Complementation of this strain with SLN1 or with a heterologous, active HK can restore growth on glucose (Héricourt et al. 2019). As known ethylene receptors possess an autoactive kinase domain that is turned off upon ethylene perception, one would expect such receptors to restore growth of the $\Delta sln1$ strain in the absence of ethylene and to lose this effect upon ethylene treatment.

Complementation assays were carried out using the *SLN1* gene as a positive control and an empty vector as a negative control. The strain carrying an empty vector was able to grow on galactose, but not on glucose. Both $\Delta sln1$ -*SLN1* and $\Delta sln1$ -*RiHHK6* strains were able to grow on glucose. The growth of $\Delta sln1$ -*RiHHK6* was severely impacted, in concentration-dependent fashion, when the ethylene donor ethephon was added to the medium at concentrations of 5 mM and above. It must be noted that the growth of the control strain $\Delta sln1$ -*SLN1* also decreased in the presence of ethephon, albeit to a much lesser extent than $\Delta sln1$ -*RiHHK6* (Fig. 1A and B). The areas under curves (AUCs) were determined to enable quantitative comparisons between strains. AUC values integrate several growth parameters (Ayhan et al. 2016) and are commonly used to monitor the effect of a given treatment on yeast growth (e.g., Coral-Medina et al. 2022; Groszmann et al. 2023). The effect of ethephon on yeast growth was significantly stronger with $\Delta sln1$ -*RiHHK6* than $\Delta sln1$ -*SLN1* (Fig. 1C).

Ethylene binding assays. Further experiments were carried out using radiolabeled ethylene to determine whether RiHHK6 can directly bind this ligand. The N-terminal part of RiHHK6, which is predicted to contain the ethylene-binding domain, was fused to glutathione *S*-transferase (GST) and expressed in the yeast *Pichia pastoris*. This resulted in high-affinity ethylene binding activity, which could be displaced by competition with a large excess of unlabeled ethylene (Fig. 2). The latter observation indicates saturability (Schaller and Bleecker 1995). Control experiments with intact yeast cells containing the empty vector showed no saturable ethylene binding activity. These results indicate that the N-terminal part of RiHHK6 directly binds ethylene.

Response of *R. irregularis* to cytokinins and characterization of the candidate receptor RiHHK7

Effect of cytokinins on *R. irregularis* grown in vitro. The effect of cytokinins on the germination of isolated *R. irregularis* spores grown in vitro was assessed by adding cytokinins directly to the culture medium. We first evaluated the effect of a mix of four cytokinin forms: *cis*- and *trans*-zeatin (cZ and tZ, respectively), *N*⁶-isopentenyladenine (iP), and *N*⁶-benzyl adenine (BA). Treatment with this mix stimulated spore germination as measured at 7 or 11 days over a concentration range of 10⁻⁷ to 10⁻⁵ M (Fig. 3A). To determine whether particular cytokinins were responsible for this effect, we tested each cytokinin form individually at a concentration of 10⁻⁷ M. While cZ, iP, and BA were active, tZ had no effect on spore germination (Fig. 3B). Active cytokinin forms increased spore germination rate approximately twofold compared with the mock condition, and this

effect was maintained throughout the course of the experiment (from 7 to 14 days).

Investigation of RiHHK7 functionality through heterologous expression in yeast and plant mutants and direct binding assays. The complementation of *S. cerevisiae* $\Delta sln1$ strain with RiHHK7 was attempted. The principle was as described earlier for ethylene, except that the expected results in the event of complementation were the opposite: the kinase domain of many known cytokinin receptors is inactive by default and is activated upon cytokinin binding (Hluska et al. 2021). One would thus expect, in the event of complementation, to observe restoration of growth on glucose in the presence of added cytokinins (Fig. 4). The following cytokinins were tested at two concentrations, 10⁻⁷M and 10⁻⁶M: tZ, cZ, iP, BA, dihydrozeatin, and the synthetic cytokinin thidiazuron. In these assays, the negative control strain $\Delta sln1$ transformed with an empty vector was unable to grow on glucose, and the positive control strain $\Delta sln1$ -*SLN1* was able to grow on glucose, regardless of treatment conditions. An

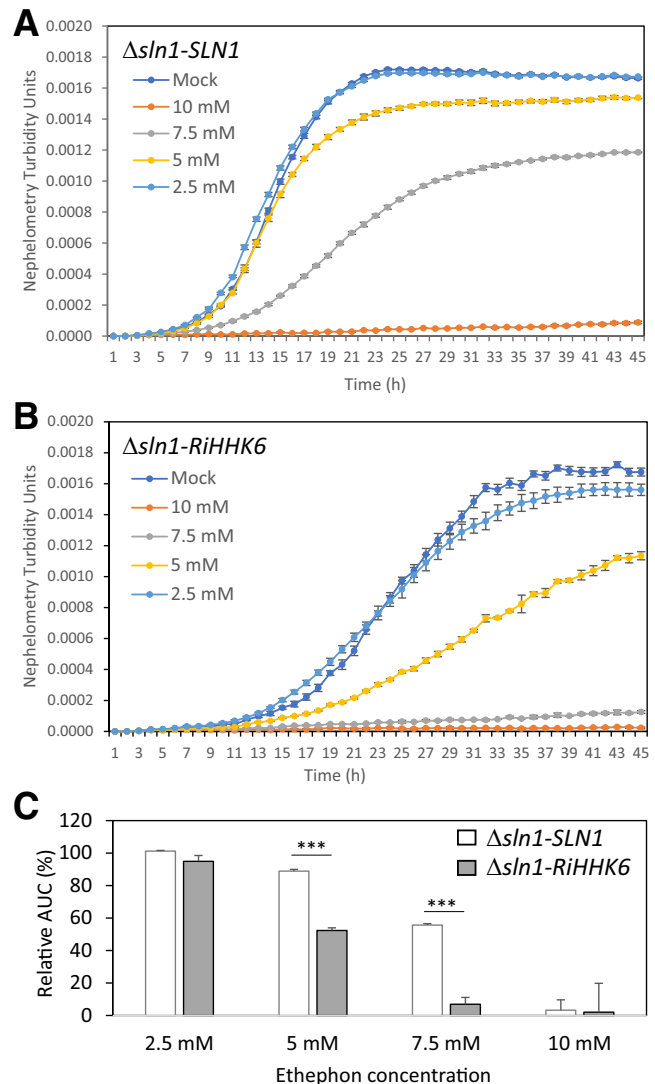


Fig. 1. Investigation of RiHHK6 functionality through heterologous expression in yeast $\Delta sln1$ mutants. **A and B,** The growth of strains $\Delta sln1$ -*SLN1* (A) and $\Delta sln1$ -*RiHHK6* (B) was monitored for 45 h by laser nephelometry in the absence or presence of ethephon at the indicated concentrations. Values are means \pm standard error of the mean (SEM) ($n = 3$). **C,** Curves expressing relative nephelometric units (ethephon-treated vs. mock) as a function of time were used to calculate the area under curve (AUC). The figure shows mean normalized AUC values \pm SEM ($n = 3$). Asterisks (***) indicate statistically significant differences according to Student's *t*-test ($P < 0.001$).

additional control strain was used, which expressed the poplar cytokinin receptor HK4a. As previously reported (Héricourt et al. 2019), the growth of this strain on glucose was restored when 10^{-6} M tZ was added. In addition, this strain was responsive to 10^{-6} M BA, and to a lesser extent to 10^{-6} M iP. The $\Delta sln1$ -*RiHhk7* strain was unable to grow on glucose, irrespective of cytokinin supply at any of the tested concentrations (Fig. 4).

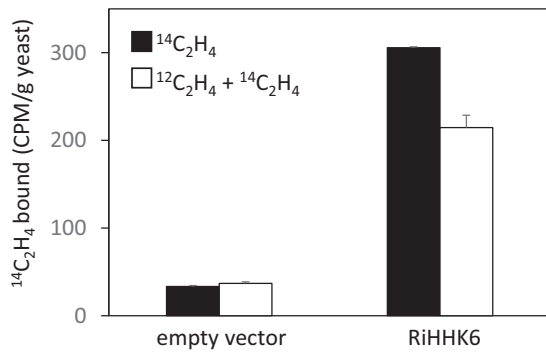


Fig. 2. Ethylene binding assays for RiHhk6. Ethylene binding to equal amounts of yeast carrying an empty vector or expressing the binding domain of RiHhk6 fused to glutathione *S*-transferase was compared between samples treated with $^{14}\text{C}_2\text{H}_4$ (0.1 $\mu\text{l/liter}$) and identical samples treated with $^{14}\text{C}_2\text{H}_4$ (0.1 $\mu\text{l/liter}$) plus $^{12}\text{C}_2\text{H}_4$ (1,000 $\mu\text{l/liter}$). The difference between these values shows saturable binding activity. Data show the mean counts per minute (CPM) + standard error of the mean ($n = 3$).

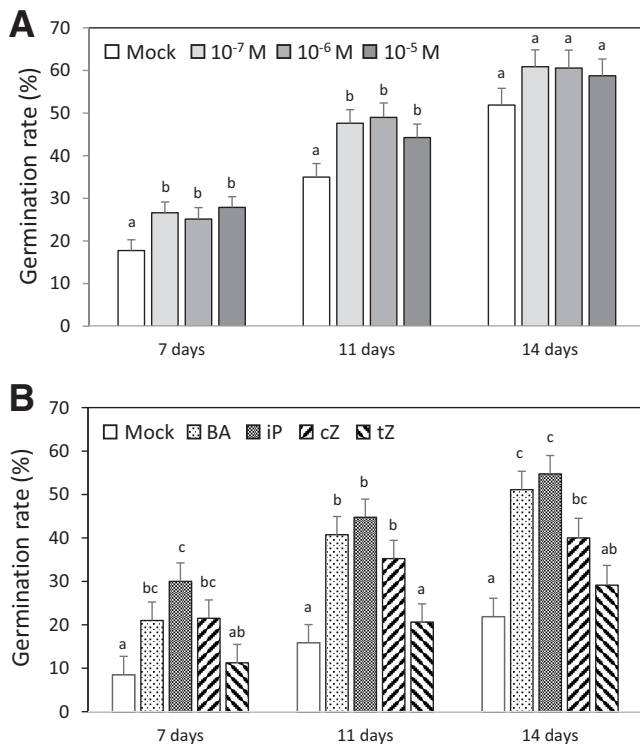


Fig. 3. Effect of cytokinins on *Rhizoglyphus irregularis* spore germination. Spores of *R. irregularis* were placed on solid medium and their germination was recorded over a period of 14 days. **A**, The medium was supplemented with a mix of N^6 -isopentenyladenine (iP), *cis*-zeatin (cZ), *trans*-zeatin (tZ), and N^6 -benzyl adenine (BA) at a final concentration of 10^{-7} M to 10^{-5} M each. Means topped by the same letter do not differ significantly by Duncan's multiple-range test ($P > 0.05$). **B**, The medium was supplemented with individual cytokinin forms at 10^{-7} M or the solvent alone for mock treatments. Conditions topped by the same letter do not differ significantly by Mann-Whitney test ($P > 0.05$). Bars represent the mean germination rate per plate + standard error of the mean. $n = 8$ to 9 plates per condition.

Cytokinin binding assays were then carried out using an *Escherichia coli* strain expressing the CHASE domain of RiHhk7 flanked by its two transmembrane domains and isotope-labeled tZ and iP. While a positive control strain expressing the cytokinin receptor *AtAHK4/AtCRE1* from *A. thaliana* was able to bind both tZ and iP, three independent strains expressing *RiHhk7* did not display any binding to either cytokinin (Fig. 5).

Finally, we attempted complementation of plant cytokinin-insensitive mutants. The legume species *M. truncatula* possesses four identified cytokinin receptors: MtCRE1/MtCHK1, MtCHK2, MtCHK3, and MtCHK4 (Boivin et al. 2016). Among the available mutant and knockdown lines, *cre1-1* mutants exhibit a strong phenotype of delayed nodulation. We attempted to complement this phenotype by genetic transformation with *RiHhk7*. To optimize our chances of success and because cytokinin signaling involves interactions of the receptor with other proteins, we built a chimeric construct comprising the N-terminal CHASE domain of *RiHhk7* surrounded by its two transmembrane domains, fused to the C-terminal kinase domain of *MtCRE1*, and hereafter designated as *RiHhk7(CHASE)-MtCRE1(kinase)*. Following root transformation with *Agrobacterium rhizogenes*, transformed roots were identified thanks to the presence of a gene encoding DsRed in the T-DNA, and untransformed roots were discarded. A full-length version of *MtCRE1* was used as a positive control, and a vector carrying DsRed alone as a negative control. Nodule numbers were scored 3 weeks postinoculation with *Sinorhizobium meliloti* (Fig. 6A). As expected, *cre1-1* plants displayed very few nodules in comparison with wild-type plants. While the *MtCRE1* full-length protein restored nodule numbers to wild-type or even higher levels, the *RiHhk7(CHASE)-MtCRE1(kinase)* fusion did not confer an improvement of nodulation ability compared with the empty vector control.

In addition to the nodulation assay, gene expression in the transformed *M. truncatula* plants was examined. The cytokinin-responsive gene *MtNIN* was used as a marker of cytokinin response (Plet et al. 2011). Its expression was not enhanced by cytokinin treatment in *cre1-1* mutant plants (Plet et al. 2011; Fig. 6B). Roots transformed with full-length *MtCRE1* responded to cytokinin treatment by overexpressing *MtNIN*, but roots transformed with the *RiHhk7(CHASE)-MtCRE1(kinase)* fusion did not (Fig. 6B).

Effect of cytokinins on the symbiotic ability of *R. irregularis*. We took advantage of *M. truncatula cre1-1* mutants to investigate whether cytokinins could affect the symbiotic ability of *R. irregularis*. The rationale was that *cre1-1* roots are largely insensitive to cytokinins (Plet et al. 2011), hence a putative effect of cytokinin treatment on the symbiosis would be indicative of an effect of cytokinins on the fungal partner. Plants inoculated with *R. irregularis* spores were treated with a mix of four cytokinin forms (iP, cZ, tZ, and BA) at regular intervals for 4 weeks, and the extent of root colonization was determined. Combined results of three independent experiments are shown in Figure 7. Root colonization rates were comparable between wild-type plants and *cre1-1* mutants. Treatment with the mix of cytokinins showed an overall tendency to reduce root colonization in both wild-type and mutant plants, but this effect was not statistically significant.

Discussion

Perception of ethylene by *R. irregularis*

The ethylene receptor candidate RiHhk6 was characterized using heterologous complementation in yeast. The *RiHhk6* cDNA introduced in a $\Delta sln1$ mutant strain of *S. cerevisiae* was able to restore its growth on glucose in the absence of added ethylene (Fig. 1), indicating that the RiHhk6 protein is correctly expressed and that its HK domain is able to interact with yeast

proteins to trigger a downstream response. The observation that the HK domain of RiHKK6 triggers the signaling cascade in the absence of ethylene points to similarities with plant ethylene receptors: In these proteins, the HK domain is active by default and inactivated upon ethylene binding (Voet-van-Vormizeele and Groth 2008). In addition, the growth of $\Delta sln1$ -RiHKK6 yeast was severely impaired upon addition of ethephon at 5 mM and above. The fact that the $\Delta sln1$ -SLN1 strain was also impacted (Fig. 1) suggests that ethephon and/or some of its degradation products could be toxic to yeast at the highest concentrations tested. Nonetheless, the higher sensitivity of $\Delta sln1$ -RiHKK6 to ethephon treatment indicates that the RiHKK6 protein responds differently from SLN1 and suggests an ability of RiHKK6 to sense ethylene.

Binding assays further support the ability of RiHKK6 to bind ethylene (Fig. 2). Combined results of heterologous complementation and binding assays strongly suggest that RiHKK6 is able to sense ethylene and triggers changes in the downstream signaling pathway in yeast in response. The observations therefore indicate that RiHKK6 displays the expected characteristics of an ethylene receptor.

The fact that RiHKK6 also displays some degree of nonspecific binding to ethylene (Fig. 2) can be related to similar observations on various fungal species (Wang et al. 2006).

The fact that high concentrations of ethephon are required to trigger a response in $\Delta sln1$ -RiHKK6 yeast can be explained in two ways. First, it is difficult to estimate the concentration of ethylene that yeast cells are actually exposed to. This is because the rate of decomposition of ethephon into ethylene is strongly dependent on chemical context, and in particular pH, which is difficult to monitor along the course of experiment (Zhang and Wen 2010). In addition, the solubility of ethylene in aqueous solutions is very low (Serra et al. 2011), thus most of the ethylene formed will accumulate in the headspace of the culture plates and will not be in contact with yeast cells. Second, it is possible that RiHKK6 senses ethylene with low affinity. From an evolutionary perspective, some ethylene receptors are thought to originate from copper-binding HK proteins whose primary role may not have been to sense ethylene (Wang et al. 2006). If such is the case for RiHKK6, this could explain its relatively weak response to ethylene.

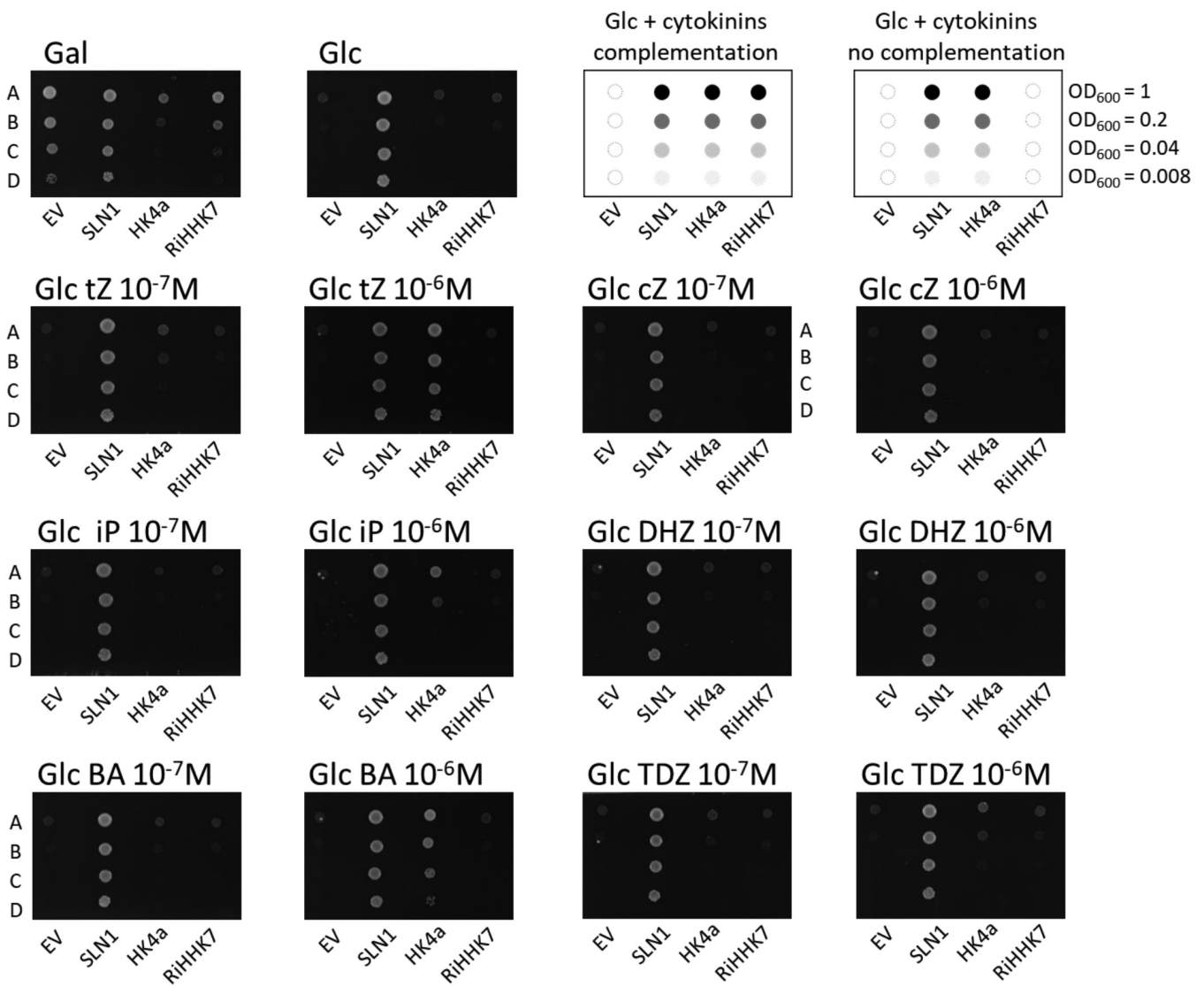


Fig. 4. Investigation of RiHKK7 functionality through heterologous expression in yeast $\Delta sln1$ mutants. Strains of $\Delta sln1$ *S. cerevisiae* carrying an empty vector (EV) or expressing the coding sequence of either *SLN1*, poplar *HK4a*, or *RiHKK7* were examined for their ability to grow on medium containing galactose (Gal, permissive condition, positive control) or glucose (Glc, restrictive condition) supplemented or not with different cytokinins. The two panels on the top right corner are a schematic representation of the assay and indicate the expected results in the respective events of complementation or not by RiHKK7 in the presence of cytokinins. Yeast suspensions were adjusted to an optical density at 600 nm = 1 (line A), 0.2 (line B), 0.04 (line C), or 0.008 (line D), and 2 μ l of each dilution was spotted on the medium. Growth was examined at 76 h postinoculation.

Responses of *R. irregularis* to cytokinins and heterologous complementation assays with RiHHK7

The germination of *R. irregularis* spores was significantly stimulated by exogenous treatment with either iP, BA, or cZ (Fig. 3B). Bioactivity was observed at concentrations as low as 10^{-7} M, which lie well in the range of typical bioactive concentrations of phytohormones. The lack of activity of tZ contrasted sharply with the activity of its isomer cZ. This observation likely reflects some degree of specificity in the fungal receptor(s) for these compounds, which could mirror the preference of some plant cytokinin receptors for particular cytokinin forms (Hluska et al. 2021). The biological relevance of such specificity in AM fungi, however, remains unclear. In contrast with strigolactones, which seem to be produced specifically by AM plant hosts, cytokinins can be released from multiple sources in the soil environment. These range from leaf litter to a diverse array of living organisms including, of course, plants, but also bacteria, fungi, cyanobacteria, nematodes, and even insects (Stirk and van Staden 2010). Mycorrhizal fungi are thus likely exposed to a complex cocktail of cytokinins. The fact that they also release the cytokinin iP-riboside themselves will make it very difficult to disentangle putative roles of cytokinins as endogenous fungal signals from their contribution to symbiotic or other interspecies signaling events.

Our mycorrhization experiments with *M. truncatula* did not reveal a clear effect of cytokinins on the symbiotic process (Fig. 7). Therefore, at least in this experimental system, increased spore germination did not translate into enhanced symbiotic capacity of the fungus. We can conclude that either this step is not limiting or events that occurred at later stages counterbalanced the positive effect of cytokinins on spore germination.

Regardless of its biological or ecological implications, the observed AM fungal response to low concentrations of cytokinins strongly suggests the existence of a corresponding receptor. While RiHHK7 was identified as a good candidate in a previous study (H erivaux et al. 2017), neither binding assays nor complementation experiments in yeast or *M. truncatula* supported the hypothesis that it could act as a cytokinin receptor (Figs. 4, 5, and 6). Several possible and not mutually exclusive interpretations can be put forward:

- (i) Complementation of the yeast $\Delta sln1$ mutant failed because the protein was not expressed correctly. Although its ethylene counterpart RiHHK6 appeared to be efficiently produced and active, we cannot exclude that despite codon

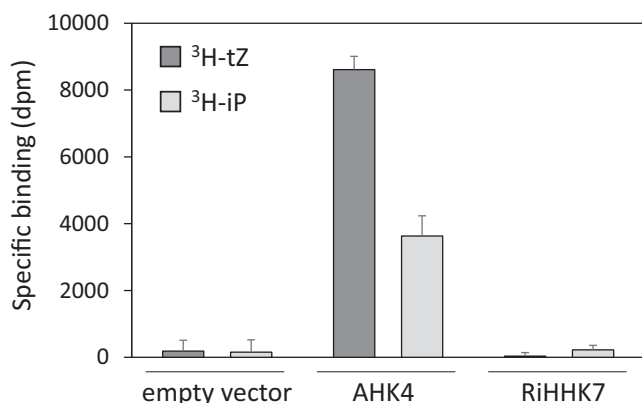


Fig. 5. Cytokinin binding assays for RiHHK7. Binding of $[2\text{-}^3\text{H}]\text{tZ}$ and $[2\text{-}^3\text{H}]\text{iP}$ in live-cell binding assays using *Escherichia coli* strains carrying an empty vector as a negative control, *AtAHK4* as positive control, or the cyclase/histidine kinase-associated sensing extracellular (CHASE) domain of *RiHHK7* flanked by its two transmembrane domains. The bars represent average specific binding + standard error of the mean ($n = 3$).

optimization, the RiHHK7 protein was for some reason poorly expressed or unstable in *S. cerevisiae*.

- (ii) Complementation of yeast $\Delta sln1$ mutant failed because RiHHK7 lacked protein interactants to trigger the signaling pathway that allows growth restoration.
- (iii) Complementation of *M. truncatula cre1-1* mutants failed because the chimeric RiHHK7-MtCRE1 protein was not correctly expressed. This explanation seems unlikely, because the full-length *MtCRE1* gene expressed in *cre1-1* mutants led to an increased nodulation compared with the empty vector transformants, indicating that this protein was expressed and active and that the chosen promoter was adequate to see an effect on nodulation.
- (iv) RiHHK7 does not act as a cytokinin receptor: It does not bind cytokinins and/or is unable to transduce the signal upon cytokinin binding. The fact that in vitro binding assays did not indicate binding of the N-terminal portion of RiHHK7 to several cytokinins supports the former hypothesis. If this is correct, then two questions arise: What is the function of

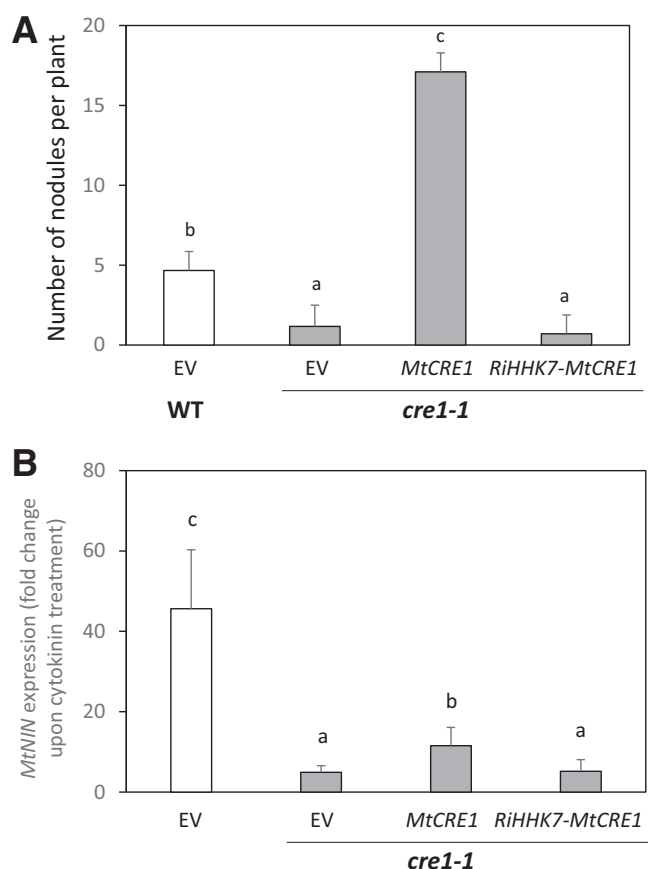


Fig. 6. Investigation of RiHHK7 functionality through heterologous expression in *Medicago truncatula cre1-1* mutants. Cytokinin-insensitive *cre1-1* mutants of *M. truncatula* were transformed with an empty vector (EV), the full-length *MtCRE1* gene, or an *RiHHK7*(CHASE)-*MtCRE1*(kinase) fusion (noted *RiHHK7-MtCRE1*). Wild-type plants were transformed with the empty vector. **A**, Transformed plants were inoculated with *Sinorhizobium meliloti*, and the number of nodules on transformed roots was scored 21 days postinoculation. Bars represent the mean number of nodules per plant; error bars show SEM. $n = 25$ to 30 plants per condition. **B**, Transformed plants were treated with a mix of N^6 -isopentenyladenine (iP), N^6 -benzyl adenine (BA), *cis*-zeatin (cZ), and *trans*-zeatin (tZ) at 10^{-7} M each for 1 h, and the expression of *MtNIN* was measured by RT-qPCR. Bars indicate the mean fold-change in expression in cytokinin-treated plants relative to mock-treated plants + standard error of the mean. $n = 6$ to 8 plants per condition. Conditions topped by the same letter do not differ significantly by Mann-Whitney test ($P > 0.05$).

RiHKK7, and what is the cytokinin receptor in *R. irregularis*?

An alternative function for RiHKK7 could be to perceive ligands other than the tested cytokinin forms. Indeed, CHASE domains are quite variable and are known to bind a number of ligands, including cytokinin-like compounds and unrelated molecules (Anantharaman and Aravind 2001). Cytokinin-like compounds are commonly found in soil (Pizzeghello et al. 2013) and can be released upon plant litter decomposition (Arthur et al. 2001). They can be perceived by some plant cytokinin receptors (Stirk and van Staden 2010). It is thus conceivable that RiHKK7 has a role in the perception of such compounds. Alternatively, and consistent with the hypothesis that RiHKK7 is thought to have evolved separately from plant cytokinin receptors (Hérivaux et al. 2017), this protein could bind compounds unrelated to cytokinins.

If RiHKK7 is not a cytokinin receptor, but *R. irregularis* responds to small concentrations of cytokinins, then there must be another protein acting as a cytokinin receptor. In support of this possibility, other organisms perceive cytokinins without any involvement of a CHASE domain-containing protein. For example, *Dictyostelium discoideum* responds to various cytokinins, and strains with mutations in its two CHASE-domain proteins retain this ability (Anjard and Loomis 2008). Similarly, the fungi *Botrytis cinerea* and *Sclerotium rolfsii* respond to cytokinins, but CHASE domain-encoding genes were not identified in their genomes (Anand et al. 2022). As alternative microbial cytokinin receptors have not been identified to date, it is difficult to speculate on the identity of the cytokinin receptor of *R. irregularis*.

Significance of phytohormone perception by AM fungi

Our observations for both ethylene and cytokinins lend support to the hypothesis that, like their host plants, AM fungi perceive these two signals. As they are also able to release these hormones (Pons et al. 2020), this perception could either contribute to the regulation of their own development and/or represent a response to plant-derived cues. Regardless of the answer to this question, we can add AM fungi to the long list of organisms responding to cytokinins or ethylene (Carlew et al. 2020; Kabbara et al. 2018). These compounds, despite their common designa-

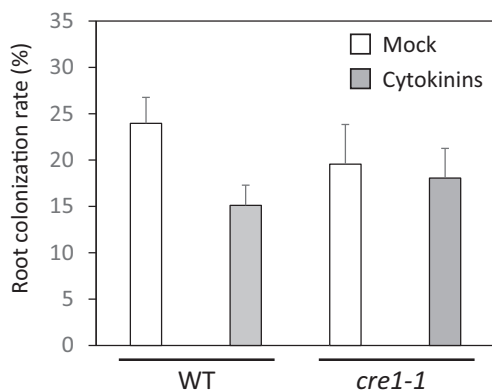


Fig. 7. Effect of cytokinins on AM symbiosis in *Medicago truncatula*. Wild-type (WT) and *cre1-1* plants of *M. truncatula* inoculated with *Rhizophagus irregularis* were treated weekly with a mix of cytokinins (*N*⁶-isopentenyladenine [iP], *N*⁶-benzyl adenine [BA], *cis*-zeatin [cZ], and *trans*-zeatin [tZ] at 10⁻⁷ M each in the nutrient solution) or with the nutrient solution alone for mock treatments. The extent of root colonization by *R. irregularis* was measured after 5 weeks as the percentage of root length showing fungal structures. Bars represent the mean root colonization rate per plant + standard error of the mean; *n* = 12 plants per condition. No significant differences were found according to Duncan's multiple-range test (*P* > 0.05).

tion as phytohormones, would be better described as common biological signals. Like several mammalian hormones or bacterial quorum-sensing signals, they seem to serve as interspecies or interkingdom mediators in a wide range of interactions (He et al. 2023; Kendall and Sperandio 2016). As ethylene and cytokinins are common in soil environments and of highly diverse origins, they seem unlikely to mediate recognition events with any degree of specificity. It would be interesting to investigate how they interplay with strigolactones, which are other phytohormones playing a decisive role in the first steps of AM interactions. Cross-talk between strigolactones and either ethylene or cytokinins has been documented on the plant side (Wu et al. 2022). While signaling pathways on the fungal side may be quite different, one could speculate that cross-talk could also take place in the fungal partner to integrate signals from different origins and at different stages of the symbiosis.

In this article, we used a candidate-based approach to identify AM fungal receptors for two classes of phytohormones. While RiHKK6 presents the expected characteristics of an ethylene receptor, our data do not support the identification of the RiHKK7 candidate as a bona fide cytokinin receptor. The use of mutants in AM fungi is precluded by the multinucleate nature of their cells (Kokkoris et al. 2020), and fungal biomass is only available in very limited quantities. It will thus be difficult to use forward genetics or biochemical approaches to identify a cytokinin receptor in these fungi. Likewise, as these organisms are not transformable, it is not possible to use reverse genetics to address the importance of putative phytohormone receptors. Similar obstacles will likely arise in the characterization of receptors of other phytohormones perceived by these fungi (e.g., jasmonate). There is thus a long path ahead to address to what extent, and how, these ancient microorganisms have learned to communicate with their long-standing partners.

Materials and Methods

Biological material

The AM fungus used in all experiments was *R. irregularis* strain DAOM197198. Sterile spores were purchased from Agronutrition. Before use, the storage solution was eliminated, and the spores were incubated in sterile water for 48 h at 4°C, then rinsed again with a large volume of sterile water.

Medicago truncatula cre1-1 mutants, obtained in the Jemalong A17 genotype, have been described by Plet et al. (2011).

The *S. meliloti* strain used for nodulation experiments was RCR2011 containing the pXLGD4 plasmid. Bacteria were grown in a modified tryptone-yeast medium with 20 mM CaCl₂ and 10 mg/liter tetracycline (Beringer 1974) for 48 h at 28°C, then pelleted and resuspended in sterile water.

The *S. cerevisiae* strain MH179 (*ura3 leu2 his3 sln1::LEU2 sho1::TRP1* + pGP22, with pGP22::GALp-PTP2/pRS413 [HIS3, CEN]) was used as recipient strain for complementation assays.

Analysis of AM fungal development in vitro

Experiments were carried out in plates containing M medium (Bécard and Fortin 1988) without sucrose, buffered with 100 mg/liter MES and solidified with 0.8% (wt/vol) agarose. Cytokinins (iP, tZ, cZ, and BA purchased from Olchemim) or the solvent alone for mock treatments were added to the medium after autoclaving. Spores of *R. irregularis* were suspended in sterile water and placed on the medium as 20 spots of one to three spores per plate. Plates were incubated horizontally at 30°C under 2% CO₂ for 7 to 14 days. The germination rate was determined following observation with a stereomicroscope (Leica MZ75).

Effect of cytokinin treatment on mycorrhization

Wild-type and *cre1-1* plants of *M. truncatula* were grown in 250-ml pots containing a zeolite substrate (0.5 to 2.5 mm) under a 16-h/8-h photoperiod at 22/20°C, and inoculated with 400 spores of *R. irregularis* per plant. The plants were watered once a week with a nutrient solution supplemented or not with a mix of cytokinins (iP, cZ, tZ, and BA at 10⁻⁷ M each). The nutrient solution was a modified Long Ashton solution (Hewitt 1966) containing 3.75 μM phosphate. Roots were harvested 5 to 6 weeks postinoculation. They were cleared in 10% KOH (wt/vol) for 3 days at room temperature and stained with Schaeffer black ink as described by Vierheilig et al. (1998) for 8 min at 95°C. Whole-root systems were examined under a stereomicroscope (Leica MZ75) to determine the percentage of roots showing AM fungal structures: arbuscules, vesicles, or intraradical hyphae.

Complementation of *S. cerevisiae* Δ*sln1* mutants

The full coding sequences of *RiHhk6* and *RiHhk7* (Hérivaux et al. 2017) were codon optimized and synthesized by GenScript. Each sequence was then cloned into the pYX212 linearized vector (*NcoI-XhoI* digested) to yield pYX212-*RiHhk6* and pYX212-*RiHhk7*. The pPD2133 plasmid expressing the *S. cerevisiae* SLN1 osmosensor was used as a positive control for kinase activity complementation, and the empty vector pYX212 as a negative control.

The *S. cerevisiae* strain MH179 was independently transformed with pYX212-*RiHhk6*, pYX212-*RiHhk7*, pPD2133, or pYX212 plasmids according to the lithium acetate method (Gietz and Schiestl 1995). Ura⁺ transformants were selected following 3 days of incubation at 30°C on galactose-containing medium lacking uracil (SG-U) (7 g/liter yeast nitrogen base, 0.8 g/liter Complete Supplement Mixture without Uracil, 20 g/liter galactose).

Kinase activity complementation and ethylene sensitivity tests were performed with pYX212-*RiHhk6* and the pYX212-SLN1 control strain in 96-well plates using a protocol adapted from Daudu et al. (2017). Briefly, Ura⁺ transformants were grown at 28°C in liquid SD-U broth (7 g/liter yeast nitrogen base, 0.8 g/liter Complete Supplement Mixture without Uracil, 20 g/liter glucose) supplemented or not with different concentrations of ethephon (Sigma-Aldrich). Plates were incubated in a laser-based nephelometer (BMG Labtech, Germany) for 45 h at 30°C. Nephelometry allows growth to be recorded as an increase in yeast suspension turbidity over time, as it measures light scattered by small particles. The intensity of scattered light is proportional to the number of particles over a wide dynamic range (Finger et al. 2012). During incubation, plates were shaken at 200 rpm for 5 min every 10 min, and growth was registered after each shaking. Growth curves expressing the relative nephelometric unit as a function of time were generated using GraphPad Prism 6.0, and AUCs were calculated.

Complementation in the presence of cytokinins was assessed by monitoring growth of pYX212-*RiHhk7* and control strains on solid SD-U medium. Pure standards of cytokinins were purchased from Olchemim, and added to the medium after autoclaving. Yeast suspensions were adjusted to an optical density at 600 nm (OD₆₀₀) = 1, then underwent serial fivefold dilution, and 2-μl samples were spotted on the medium. Plates were incubated at 28°C, and yeast growth was monitored at regular intervals by scanning the plates with a Chemidoc imaging system (Bio-Rad Laboratories).

Ethylene binding assays

A construct bearing the N-terminal 264 residues of *RiHhk6* fused to GST was ligated into the pPICZA plasmid and transformed into *P. pastoris*, using methods described in McDaniel and Binder (2012). The radioligand ethylene binding assay was

carried out on intact yeast expressing this construct or empty vector, according to previously published methods (Schaller and Bleecker 1995; Sisler 1979). Briefly, yeast cells grown in liquid medium were washed with water and collected on filter paper disks. These samples were incubated in sealed chambers with either 0.1 μl/liter [¹⁴C]ethylene alone or a mix of 0.1 μl/liter [¹⁴C]ethylene and 1,000 μl/liter [¹²C]ethylene. The amount of [¹⁴C]ethylene bound to yeast cells was determined using scintillation counting.

Cytokinin binding assays

Cytokinin binding assays in *E. coli* were carried out as previously described (Nisler et al. 2010; Romanov et al. 2006). Radiolabeled tZ ([2-³H]tZ) and iP ([2-³H]iP) were obtained from the Isotope Laboratory, Institute of Experimental Botany, The Czech Academy of Sciences. Live-cell cytokinin-binding assays were performed with an intact *E. coli* KMI001 strain carrying the pINIII vector. The TM-CHASE-TM coding sequence of *RiHhk7* was cloned into pINIII using *Bam*HI and *Spe*I restriction sites. *Escherichia coli* KMI001 strains harboring plasmid pINIII-*AtAHK4* (Suzuki et al. 2001) or the empty vector pINIII were used as positive control and negative controls, respectively. Bacterial cultures were grown in liquid M9 medium supplemented with 100 μg/ml ampicillin and 0.1% (wt/vol) casamino acids at 37°C with shaking (200 rpm) to reach an OD₆₀₀ of about 0.5, then 250 μM IPTG was added to induce expression of the constructs, and the cultures were further grown overnight at 20°C until they reached an OD₆₀₀ of about 0.7 and 0.9 for *AtAHK4*- and *RiHhk7*-expressing cultures, respectively. Bacterial samples were incubated with 3 nM [2-³H]tZ or 6 nM [2-³H]iP, in the presence or absence of a large excess (at least 3,000-fold) of unlabeled cytokinins. A control experiment was performed with the solvent alone (0.1% [vol/vol] dimethyl sulfoxide) instead of cytokinins. After at least 30 min incubation at 4°C, the sample was centrifuged (6,000 × g, 6 min, 4°C), the supernatant was carefully removed, and the bacterial pellet was resuspended in 1 ml of scintillation cocktail (Beckman) in an ultrasonic bath. Radioactivity was measured using a Hidex 300 SL scintillation counter (Hidex). Assays with only labeled cytokinins measured total binding, while competition assays with an excess of unlabeled cytokinins measured nonspecific binding. The difference between total and nonspecific binding was taken as a measure of specific binding.

Complementation of *cre1-1 M. truncatula* mutants

Constructs for plant genetic transformation were obtained through Golden Gate cloning (Engler and Marillonnet 2014). The N-terminal 1,965 bp of *RiHhk7* were cloned upstream of the C-terminal 1,869 bp of *MtCRE1*, and the fusion was placed under the control of a double 35S promoter. The full-length wild-type sequence of *MtCRE1* was also cloned under the control of the same promoter as a positive control. Constructs were first cloned into the Level-1 vector EC47811 and were then combined in the Level-2 binary vector EC50507 with a pNOS:Kan^R cassette and a *pAtUbi:DsRed* cassette to allow selection of transformed roots. The “empty vector” negative control contains the kanamycin resistance and DsRed cassettes. *Agrobacterium rhizogenes* strain K599 was transformed with the binary vectors by thermal shock (Höfgen and Willmitzer 1988).

Composite plants with transformed hairy roots were obtained as described in Boisson-Dernier et al. (2001). After 3 weeks in culture, roots were examined for DsRed expression. Non-transformed roots were removed, and plants were transferred to pots containing a zeolite substrate (0.5 to 2.5 mm). They were grown for 1 week under a 16-h-day and 8-h-night photoperiod at 22/20°C, and watered with a modified Long Ashton solution (Hewitt 1966) containing 5 μM nitrogen and 1 mM phosphate.

For nodulation tests, each plant was inoculated with 5 ml of an *S. meliloti* suspension ($OD_{600} = 0.02$). Roots were harvested 3 weeks postinoculation and observed under a fluorescence microscope (AxiozoomV16, Zeiss) to count the number of nodules on transformed roots.

For gene expression analysis, composite plants were treated with nutrient solution containing a mixture of iP, cZ, tZ, and BA at 10^{-7} M each, or an equivalent amount of solvent for the mock treatment. Roots were harvested 1 h after treatment and immediately frozen in liquid nitrogen.

RT-qPCR analysis

Total RNA was extracted from *M. truncatula* roots with the NucleoSpin RNA Plus kit (Macherey-Nagel) following the manufacturer's instructions. RNA samples were treated with RQ1 DNase (Promega), and 0.5 µg of RNA was reverse transcribed using M-MLV RT (H-) (Promega) according to the supplier's protocols, using an oligo-dT primer. Primers for qPCR were designed using Primer3 software. *PDF2* was used as a reference gene. Primer sequences were as follows. *NIN-F*: 5'-gtgcttcagagtgaaggcaactt and *NIN-R*: 5'-aatccccacattgcttgca (Moreau et al. 2011); *PDF2-F*: 5'-gtgtttgctccgcggtt and *PDF2-R*: 5'-ccaatctgtcctcatctg. The efficiency of the PCR reaction was estimated using a cDNA dilution series, and was greater than 98% for both primer pairs. Quantitative PCR was carried out in 10-µl reactions using LightCycler 480 SYBR Green I Master Mix (Roche). Each reaction contained 4 µl of a twofold dilution of the cDNA sample. Reactions were carried out in a CFX Opus 384 Real-Time PCR thermocycler (Bio-Rad Laboratories).

Statistical analyses

Data were analyzed using Statgraphics Centurion software (SigmaPlus). Duncan's multiple-range test was used when normality or homoscedasticity criteria were met. In other cases, data sets were analyzed using the Kruskal-Wallis test, followed by pairwise comparisons using the Mann-Whitney test.

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