the plant journal

SEB Society for Experimental Biology

The Plant Journal (2017) **92,** 211–228 doi: 10.1111/tpj.13647

Divergent expression of cytokinin biosynthesis, signaling and catabolism genes underlying differences in feeding sites induced by cyst and root-knot nematodes

Carola D. Dowd[1], Demosthenis Chronis[1], Zoran S. Radakovic[2], Shahid Siddique[2], Thomas Schmülling[3], Tomáš Werner[3,4], Tatsuo Kakimoto[5], Florian M. W. Grundler[2] and Melissa G. Mitchum[1,*]

[1]*Division of Plant Sciences and Bond Life Sciences Center, University of Missouri, Columbia, MO 65211, USA,*
[2]*Rheinische Friedrich-Wilhelms-University of Bonn, INRES – Molecular Phytomedicine, Karlrobert-Kreiten-Straße 13, D-53115 Bonn, Germany,*
[3]*Institute of Biology/Applied Genetics, Dahlem Centre of Plant Sciences, Freie Universität Berlin, D-14195 Berlin, Germany,*
[4]*Institute of Plant Sciences, Department of Plant Physiology, University of Graz, Schubertstraße 51, 8010 Graz, Austria, and*
[5]*Department of Biology, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan*

Received 1 June 2017; revised 15 July 2017; accepted 21 July 2017; published online 26 July 2017.
*For correspondence (e-mail goellnerm@missouri.edu).

SUMMARY

Cyst and root-knot nematodes are obligate parasites of economic importance with a remarkable ability to reprogram root cells into unique metabolically active feeding sites. Previous studies have suggested a role for cytokinin in feeding site formation induced by these two types of nematodes, but the mechanistic details have not yet been described. Using Arabidopsis as a host plant species, we conducted a comparative analysis of cytokinin genes in response to the beet cyst nematode (BCN), *Heterodera schachtii*, and the root-knot nematode (RKN), *Meloidogyne incognita*. We identified distinct differences in the expression of cytokinin biosynthesis, catabolism and signaling genes in response to infection by BCN and RKN, suggesting differential manipulation of the cytokinin pathway by these two nematode species. Furthermore, we evaluated Arabidopsis histidine kinase receptor mutant lines *ahk2/3*, *ahk2/4* and *ahk3/4* in response to RKN infection. Similar to our previous studies with BCN, these lines were significantly less susceptible to RKN without compromising nematode penetration, suggesting a requirement of cytokinin signaling in RKN feeding site formation. Moreover, an analysis of *ahk* double mutants using *CycB1;1:GUS*/*ahk* introgressed lines revealed contrasting differences in the cytokinin receptors mediating cell cycle activation in feeding sites induced by BCN and RKN.

Keywords: Heterodera, Meloidogyne, beet cyst nematode, root-know nematode, nematode, cytokinin, Arabidopsis histidine kinase, isopentenyltransferase, cytokinin oxidases/dehydrogenases, Arabidopsis response regulators.

INTRODUCTION

Cyst nematodes (CN) and root-knot nematodes (RKN) are the two most economically important plant-parasitic nematodes worldwide, causing more than 100 billion dollars in crop losses annually (Sasser, 1977; Abad *et al.*, 2003; Jones *et al.*, 2013). After molting into a second-stage juvenile (J2), these nematodes hatch from eggs in the soil and migrate towards plant roots. Once at the root surface, juveniles use a hollow mouth spear (stylet) for mechanical and enzymatic disruption of cell walls, moving either intra- (cyst) or inter (root-knot)-cellularly through root tissues. Once they reach the vascular cylinder, the nematode selects a single cell (CN) or group of cells (RKN) to initiate feeding and becomes sessile. At this point, a complex reprogramming of the chosen cell(s) leads to the formation of a hypermetabolic 'feeding site', which nurtures the nematode until the completion of its life cycle. The CN feeding site (syncytium) is composed of hundreds of cells fused together as a consequence of cell wall dissolution, while the RKN feeding site is made up of several individual hypertrophied giant-cells embedded within a gall (Grundler and Böckenhoff, 1997; Gheysen and Fenoll, 2002; Mitchum *et al.*, 2012). Although the feeding sites induced by these two types of nematodes function in a similar capacity as nutrient sinks, the molecular events leading up to their

formation differ with respect to cell cycle activation and the extent of cell division. Nuclei of cells within syncytia do not divide, but enlarge through repeated rounds of endoreduplication. In contrast, giant-cell nuclei undergo extensive endoreduplication and repeatedly divide (de Almeida Engler *et al.*, 1999; de Almeida Engler and Gheysen, 2012). In addition, the degree of cell division occurring in cells surrounding the feeding sites is dramatically different between these species. In the case of CN, division of cells surrounding the developing syncytium is limited, whereas cells surrounding giant-cells induced by RKN divide extensively giving rise to characteristic 'root-knots' or galls on the roots.

A role for cytokinin in plant–nematode interactions has long been suspected given the importance of this hormone in cell cycle, cell division and nutrient mobilization, all of which represent key cellular processes altered for feeding site formation. Plant cytokinins (CKs) are N^6 adenine derivatives commonly associated with meristematic cell division in the shoot apical meristem, the root apical meristem (RAM) and the vascular cambium. Whether or not meristematic cells proliferate or differentiate is mediated by CK signaling pathways (Zürcher and Müller, 2016). In *Arabidopsis thaliana*, CKs are synthesized by two distinct gene families encoding seven AMP/ADP/ATP isopentenyltransferases (IPT) and two tRNA-isopentenyltransferases (tRNA-IPT), which utilize cytoplasmic dimethylallyl diphosphate and AMP/ADP/ATP or tRNA as precursors, respectively (Kakimoto, 2001; Takei *et al.*, 2001). Previous studies have shown that *IPT* genes are involved in the biosynthesis of isopentenyladenines (iP), which are subsequently interconverted into *trans*-zeatins (tZ) by cytoplasmic P450 monooxygenase CYP735A (Takei *et al.*, 2004). In contrast, tRNA-IPTs are known to produce *cis*-zeatins (cZ). CK homeostasis is maintained by the action of cytokinin oxidases/dehydrogenases (CKX), which catalyze the irreversible degradation of CK molecules into an adenine/adenosine and an isoprenoid chain (Schmulling *et al.*, 2003), while CK perception in higher plants is based on a two-component phosphorelay system (TCS; Hwang and Sheen, 2001; Hwang *et al.*, 2002; Heyl and Schmülling, 2003). In *A. thaliana*, this system includes three histidine kinase membrane-bound receptors [Arabidopsis histidine kinase (AHK)2, AHK3, AHK4/CRE1], five histidine phosphotransfer proteins (AHP1–AHP5), and 10 type-A and 11 type-B response regulators (Inoue *et al.*, 2001; Suzuki *et al.*, 2001; Ueguchi *et al.*, 2001a,b; Mason *et al.*, 2004; To *et al.*, 2004; Hutchison *et al.*, 2006). CK recognition causes autophosphorylation of the receptors and a subsequent mobilization of a phosphoryl group into the nucleus via phosphorelay. Arabidopsis histidine phosphoproteins (AHPs) serve as 'molecular shuttles' between the cytoplasm and the nucleus, where they physically interact with type-B Arabidopsis response regulators (ARRs), which act as transcriptional activators of CK-responsive genes (Heyl and Schmülling, 2003). Finally, CK signaling is modulated by an additional class of ARRs, called type-A ARRs, which act as negative regulators of the CK pathway (To *et al.*, 2004).

We recently demonstrated the required early activation of cytokinin signaling during syncytium formation by the beet cyst nematode (BCN) *Heterodera schachtii* in Arabidopsis. This signaling, mediated predominantly by AHK3 and AHK4, was shown to affect the expression of *Cyclin B1;1* to promote cell cycle reactivation within the developing syncytium and neighboring cells, and is required for feeding site formation (Siddique *et al.*, 2015). Much less is known in plant interactions with RKN. However, cytokinin likely plays an important role in these interactions because fewer nematode-induced root galls were observed on *Lotus japonicas* hairy root lines expressing cytokinin oxidase, an enzyme that degrades cytokinin (Lohar *et al.*, 2004). Although increasing evidence supports a positive role for cytokinin in plant interactions with both CN and RKN (Lohar *et al.*, 2004; Siddique *et al.*, 2015), potential differences in the expression of host plant cytokinin biosynthesis, signaling and catabolism genes between these two nematode species have not been investigated. In the present study, we established an important role for cytokinin signaling in RKN parasitism, and identified key differences in the expression of cytokinin biosynthesis, signaling and catabolism genes that may underlie differences in feeding site formation by CN and RKN. Understanding the different mechanisms of nematode parasitism and the essential plant factors required for completion of the nematode life cycle may lead to more effective control strategies.

RESULTS

Reduced levels of endogenous cytokinin decrease susceptibility of Arabidopsis to RKN

A prior study reported a reduction in RKN-induced gall formation on *L. japonicus* hairy roots overexpressing cytokinin oxidases *AtCKX3* and *ZmCKX1* compared with a vector control (Lohar *et al.*, 2004). To determine whether cytokinin contributes to RKN infection in Arabidopsis, we evaluated a cytokinin oxidase overexpressing line (*35S:CKX2*) using either solid agar medium or a sand–soil mix substrate. When grown in either agar medium or soil, *35S:CKX2* plants were significantly less susceptible compared with the wild-type control, Col-0 (Figure 1), showing that decreasing cytokinin content in Arabidopsis, similarly to Lotus, causes reduced nematode infection. These findings also indicate that RKN, like BCN, requires normal levels of cytokinin to successfully infect Arabidopsis and that cytokinin plays a positive role in both interactions.

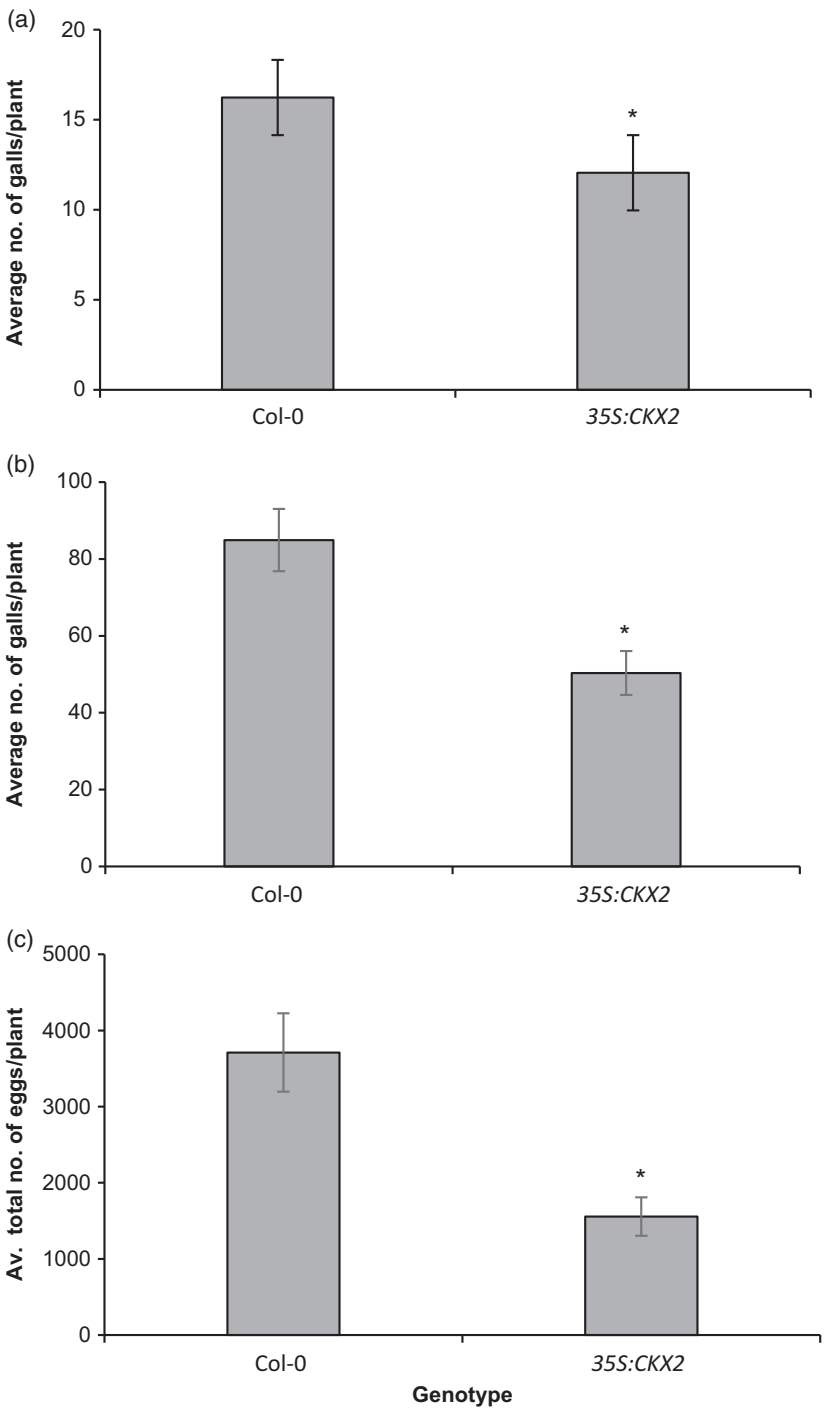

Figure 1. Overexpression of cytokinin oxidase 2 (*35S:CKX2*) reduces root-knot nematode (RKN) *Meloidogyne incognita* infection in Arabidopsis.
(a) Average number of galls per plant that developed at 40 days post-inoculation (dpi) in Knop's media infection assays, plant number (*n*) per line were $n = 30$ for Col-0, $n = 36$ for *35S:CKX2*. Data represent means ± SE of three independent biological replicates.
(b) Average number of galls per plant at 7 weeks post-inoculation (wpi) in soil infection assays. Data represent mean ± SE, $n = 14$ for Col-0, $n = 12$ for *35S:CKX2*. Data are representative of at least two independent biological replicates.
(c) Total eggs per plant were harvested and counted at 7 wpi in soil assays. Data represent mean ± SE, $n = 14$ for Col-0, $n = 10$ for *35S:CKX2*. Data are representative of at least two independent biological replicates. Asterisk indicates statistically significant differences compared with Col-0 using a two-tailed Student's *t*-test ($P < 0.05$).

Cytokinin biosynthesis (*IPT*) gene expression is differentially expressed in response to BCN and RKN

Next, we wanted to assess the status of cytokinin biosynthesis in response to nematode infection, and determine whether BCN and RKN might be altering plant cytokinin biosynthesis in different ways. Our prior results indicated that cytokinins involved in the expansion of BCN feeding sites are partially produced by the host plant (Siddique *et al.*, 2015), but the identities of the cytokinin biosynthesis genes expressed in the syncytium were unknown. Seven *IPT:GUS* reporter lines, including five adenylate *IPT* biosynthesis genes, *IPT1, IPT3, IPT5*, *IPT7*, *IPT8*, and two tRNA-IPT biosynthesis genes, *IPT2* and *IPT9*, were inoculated either with BCN or RKN infective juveniles. Infected plants were harvested at different time points representing the main stages of nematode development. A histochemical

β-glucuronidase (GUS) assay was conducted on infected plants. In the absence of nematode infection, *IPT1:GUS* is normally only expressed in root tips (Miyawaki *et al.*, 2004). However, as a consequence of BCN infection, GUS expression was visible in developing feeding sites (Figure 2a,b). In contrast to BCN infection, *IPT1:GUS* expression was not detected in galls at any time point evaluated (Figure 2d–f). *IPT3*, on the other hand, had a different response and is highly expressed in the root vasculature of uninfected plants (Miyawaki *et al.*, 2004). However, when *IPT3:GUS* was challenged with either BCN or RKN, we observed downregulation in both syncytia and galls at all developmental stages (Figure 2g–l). No changes in expression were found for *IPT5:GUS*, *IPT7:GUS* and *IPT8:GUS* in response to either BCN or RKN infection. The upregulation of *IPT1:GUS* in response to BCN, but not RKN, led us to

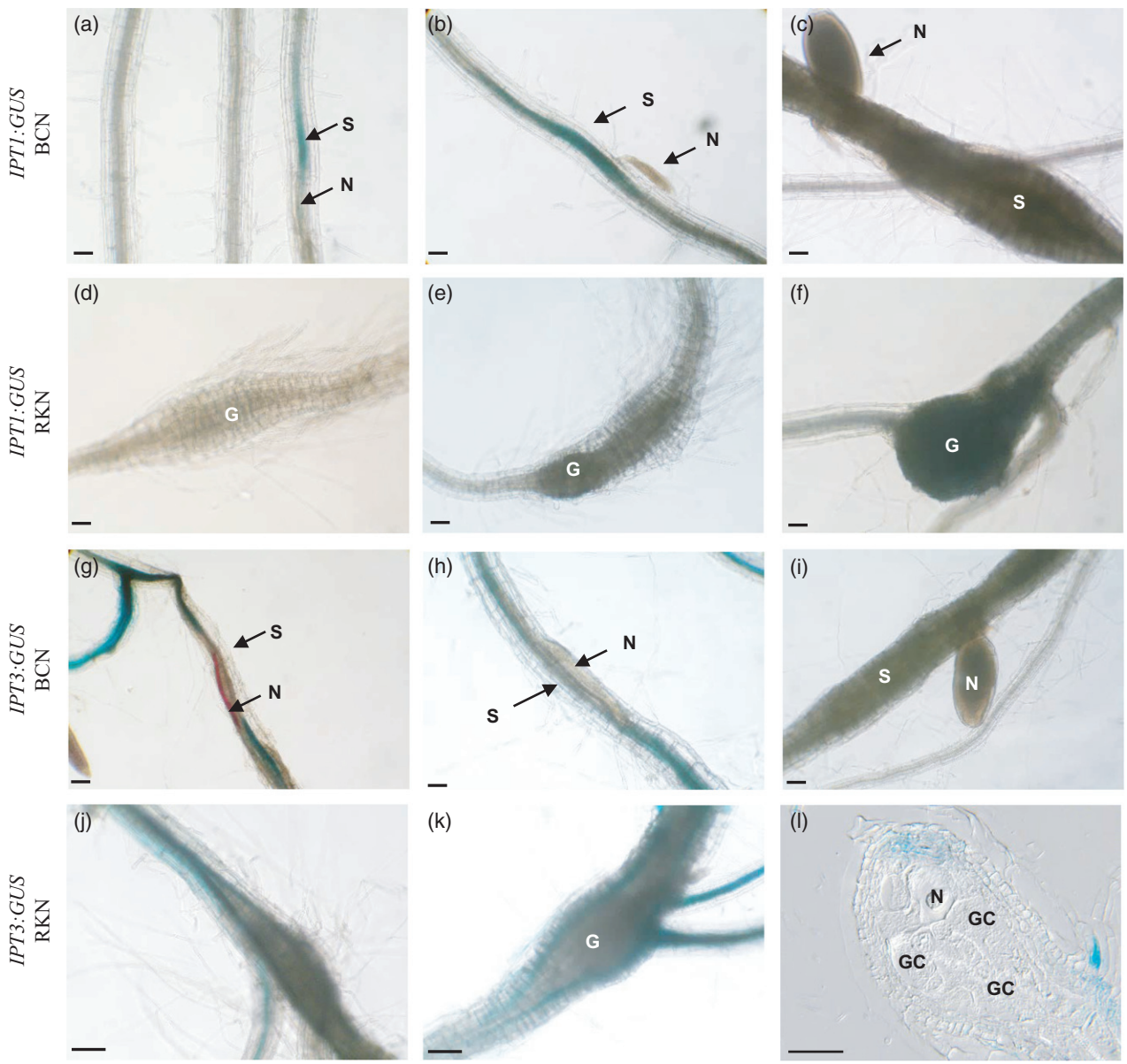

Figure 2. Cytokinin biosynthesis isopentenyltransferases *IPT1* and *IPT3* expression in response to the beet cyst nematode (BCN) *Heterodera schachtii* and the root-knot nematode (RKN) *Meloidogyne incognita*.
(a–c) *IPT1:GUS* and (g–i) *IPT3:GUS* expression in BCN-infected Arabidopsis roots during early and later stages of parasitism: (a,g) second-stage juveniles (J2), (b,h) third-stage juveniles (J3) and (c,i) fourth-stage juveniles (J4).
(d–f) *IPT1:GUS* and (j–l) *IPT3:GUS* expression in RKN-infected Arabidopsis roots during early and later stages of parasitism: (d,j) 3, (e,k) 6 and (f,l) 11 days post-inoculation (dpi). (l) Longitudinal section of a gall showing reduced *IPT3:GUS* expression in GC. Abbreviations: N, nematode; S, syncytium; G, gall; GC, giant-cell. Scale bar: 100 μm, 50 μm (l).

evaluate *ipt1-1* in response to BCN infection; however, no phenotypic changes were observed, possibly due to functional redundancy in this gene family (Figure S1). Changes in *IPT1* and *IPT3* gene expression were confirmed by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) of BCN- and RKN-infected plants compared with mock-inoculated controls (Figure S2). Overall, these results suggest a specific regulation of certain members of the cytokinin biosynthesis gene family in response to BCN and RKN infection, with only one adenylate *IPT* gene (*IPT1*) being upregulated in response to BCN and no major upregulation of adenylate *IPT* genes in response to RKN.

Beet cyst nematodes and RKN were also able to modulate tRNA-*IPT* gene expression. In the absence of nematode infection, *IPT2:GUS* is expressed in root tips and lateral root primordia (Miyawaki *et al.*, 2004). However, after nematode infection, *IPT2:GUS* expression was observed within developing syncytia and galls of nematode-infected roots at multiple developmental stages before declining (Figure 3a–e). Cross-sections of galls revealed expression of this gene specifically within giant-cells (Figure 3f). *IPT9:GUS* expression, on the contrary, was downregulated in syncytia and galls (Figure 3g–l), despite strong expression in the root vasculature of uninfected and infected roots.

The upregulation of *IPT2* in both syncytia and giant-cells led us to evaluate the Arabidopsis mutant line *ipt2-1* in response to infection by these two nematode species. The *ipt2-1* plants did not show any visible growth phenotype compared with wild-type, as previously described (Miyawaki *et al.*, 2006). Interestingly, when *ipt2-1* was challenged with BCN, we observed a significantly higher number of females than on the wild-type Col-0 (Figure S3a). *ipt2-1* RKN-infected plants also showed a trend of higher average gall number compared with Col-0; however, the lack of significance may be the result of the difficulty in detecting the small effects given the variability in gall numbers among samples (Figure S3b). These results suggest IPT2 has a divergent role in plant–nematode interactions that is not associated with the reduced susceptibility we observed for the cytokinin biosynthesis and receptor mutants described in our previous study (Siddique *et al.*, 2015), and was therefore not evaluated further here.

Cytokinin catabolism (*CKX*) gene expression is differentially expressed in response to BCN and RKN

Cytokinin levels are modulated by a family of seven cytokinin oxidases (CKX1– CKX7) involved in the degradation of cytokinin. We tested six *CKX:GUS* lines, including *CKX1:GUS*, *CKX2:GUS*, *CKX4:GUS*, *CKX5:GUS*, *CKX6:GUS* and *CKX7:GUS* in infection assays with BCN and RKN. *CKX1:GUS* and *CKX2:GUS* are expressed in aerial plant parts with limited or no expression in roots (Werner *et al.*, 2003), and no expression in response to nematode parasitism. *CKX4:GUS* is expressed in root tips of uninfected plants (Werner *et al.*, 2003), but was not expressed in syncytia or galls of nematode-infected plants. *CKX5:GUS* expression in uninfected roots is limited to the RAM (Werner *et al.*, 2003). However, *CKX5:GUS* was upregulated during early stages of BCN infection and declined as the nematode molted into an adult stage (Figure 4a–c). Interestingly, *CKX5:GUS* expression was not observed in galls at any time point evaluated (Figure 4d–f). In contrast, *CKX6:GUS*, which is expressed in the root vasculature (Werner *et al.*, 2003), showed upregulation during early stages of syncytium development, and during early and late stages of gall development (Figure 4g–l). *CKX7:GUS* also showed upregulation during early stages of syncytium formation and a restricted pattern of expression within galls that quickly declined (Figure 4m–r). Expression of a subset of *CKX* genes was confirmed by qRT-PCR of BCN- and RKN-infected plants compared with mock-inoculated controls (Figure S2). In an attempt to confirm a biological role for *CKX* gene family members in nematode infection, we tested CKX mutant lines *ckx5-1*, *ckx6-2* and *ckx5-1 ckx6-2* in response to BCN, and *ckx6-2* and *ckx5-1 ckx6-2* in response to RKN infection. Interestingly, we observed a significant reduction in the number of BCN females and RKN galls on the *ckx5-1 ckx6-2* mutant compared with Col-0, suggesting the importance of CKX enzymes and proper cytokinin balance for BCN and RKN infection (Figure S4).

Cytokinin signaling is activated at BCN and RKN feeding sites

To further elucidate cytokinin signaling status in feeding sites induced by BCN and RKN, we evaluated a novel version of TCS:GFP, *TCSn:GFP*, which has higher sensitivity and stronger expression across multiple plant tissues (Zürcher *et al.*, 2013). For this, we carried out *in vivo* imaging of gall and syncytium formation in *TCSn:GFP* infected lines starting 18 h after infection, monitoring each infection site daily until 10 days post-inoculation (dpi). Consistent with our prior report (Siddique *et al.*, 2015), we observed green fluorescent protein (GFP) in developing syncytia as early as 2 dpi, with a decline at about 10 dpi when the nematodes molted into an adult stage (Figure 5a–c). Similarly, galls induced by RKN showed early upregulation of *TCSn:GFP* (Figure S5), with expression increasing over time until the nematode reached maturity (Figure 5d–f). Altogether, these findings suggest cytokinin signaling is not only activated in response to BCN, but also in response to RKN infection.

Differential expression of cytokinin receptors in response to RKN

To determine if cytokinin receptor genes are differentially expressed during the interaction with RKN, we analyzed *AHK* expression in galls using *AHK2:GUS*, *AHK3:GUS* and *AHK4:GUS* reporter lines. GUS expression was observed in

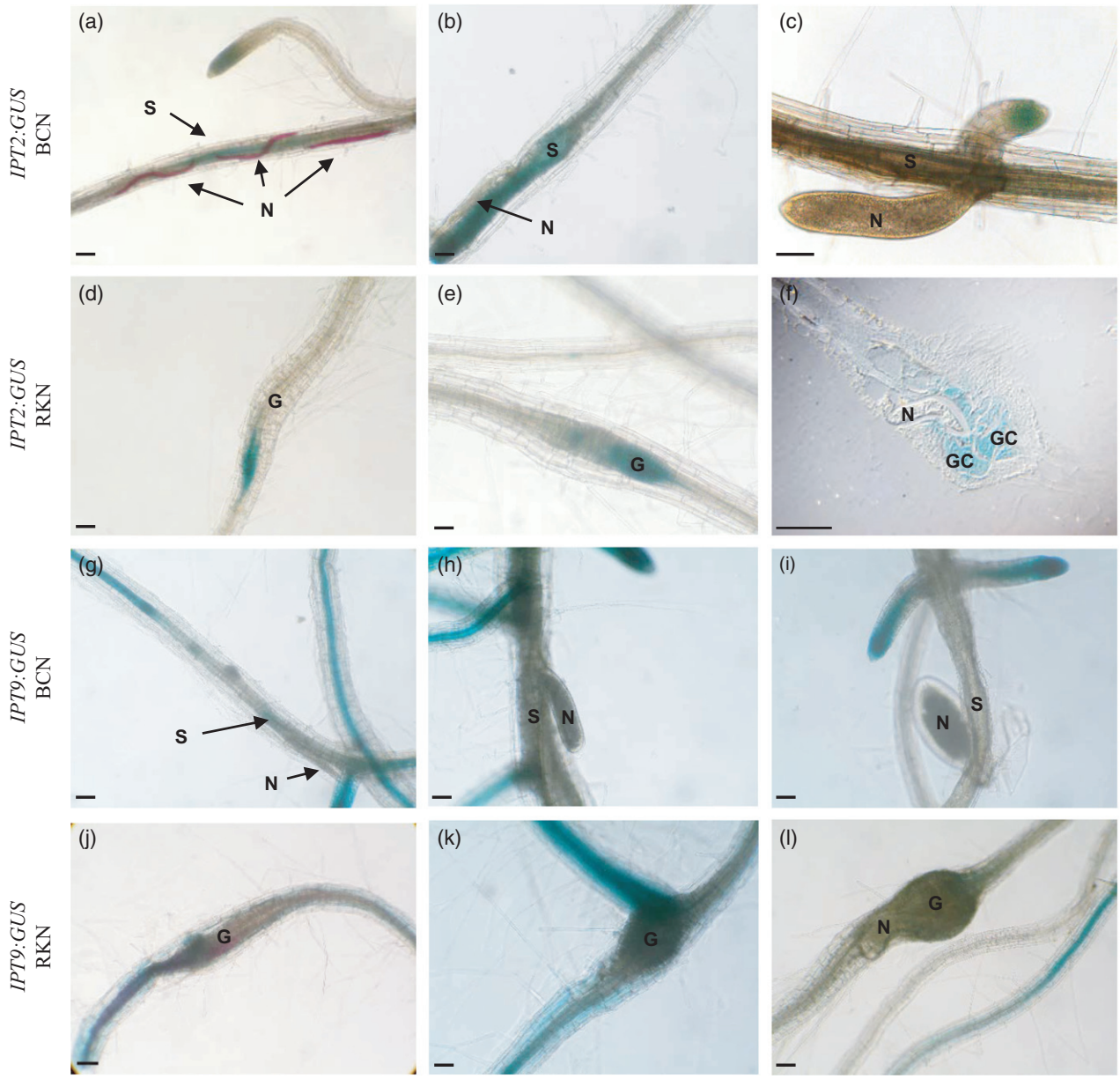

Figure 3. Cytokinin biosynthesis trans-isopentenyltransferases *IPT2* and *IPT9* expression in response to the beet cyst nematode (BCN) *Heterodera schachtii* and the root-knot nematode (RKN) *Meloidogyne incognita*.
(a–c) *IPT2:GUS* and (g–i) *IPT9:GUS* expression in BCN-infected Arabidopsis roots during early and later stages of parasitism: (a,g) second-stage juveniles (J2), (b,h) third-stage juveniles (J3) and (c,i) fourth-stage juveniles (J4).
(d–f) *IPT2:GUS* and (j–l) *IPT9:GUS* expression in RKN-infected Arabidopsis roots during early and later stages of parasitism: (d,j) 3, (e,k) 6 and (f,l) 11 days post-inoculation (dpi). (f) Longitudinal section of a gall showing *IPT2:GUS* expression in GC. Abbreviations: N, nematode; S, syncytium; G, gall; GC, giant-cell. Scale bar: 100 μm, 50 μm (f).

roots of all uninfected *AHK:GUS* lines as previously described (Higuchi *et al.*, 2004). However, we observed differential expression of the *AHK* receptor–reporter gene fusions compared with BCN. In response to BCN, *AHK2:GUS*, *AHK3:GUS* and *AHK4:GUS* are all expressed in developing syncytia (Siddique *et al.*, 2015). In contrast, *AHK4:GUS* was strongly downregulated in developing galls (Figure 6g–i), while *AHK2:GUS* and *AHK3:GUS* remained expressed (Figure 6a,b,d and e). Cross-sections through galls showed expression of *AHK2:GUS* and *AHK3:GUS* in giant-cells and surrounding gall tissues (Figure 6c and f). To further corroborate *AHK4:GUS* downregulation, we analyzed ARR15, a type-A ARR downstream component of the cytokinin signaling pathway whose expression is dependent on AHK4 (Kiba *et al.*, 2002). *ARR15:GUS* plants were evaluated in response to BCN and RKN infection. In

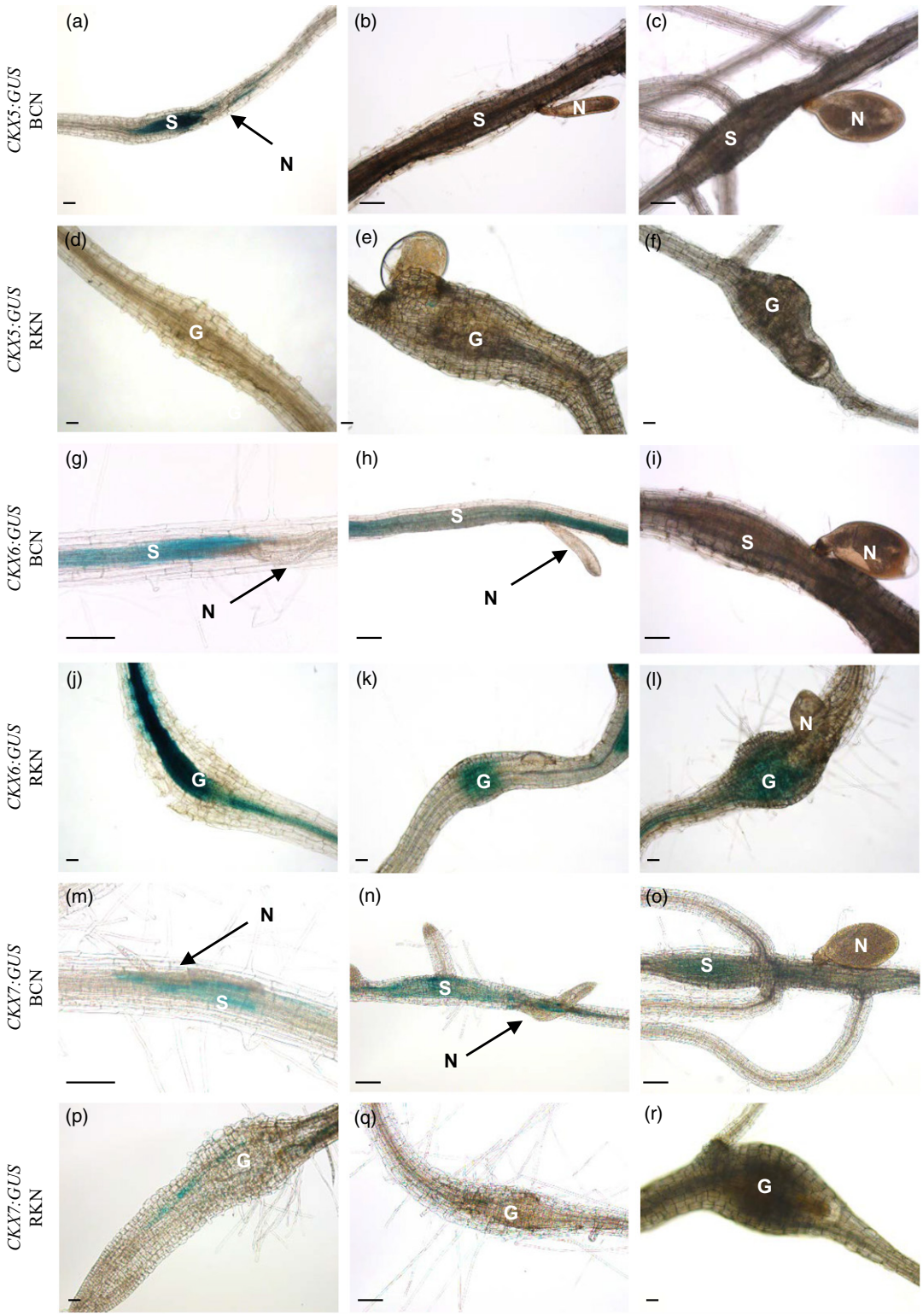

Figure 4. Cytokinin oxidases *CKX5*, *CKX6* and *CKX7* expression in response to the beet cyst nematode (BCN) *Heterodera schachtii* and the root-knot nematode (RKN) *Meloidogyne incognita*.
(a–c) *CKX5:GUS*, (g–i) *CKX6:GUS*, (m–o) *CKX7:GUS* expression in BCN-infected Arabidopsis roots during early and later stages of parasitism: (a,g,m) second-stage juveniles (J2), (b,h,n) third-stage juveniles (J3) and (c,i,o) fourth-stage juveniles (J4).
(d–f) *CKX5:GUS*, (j–l) *CKX6:GUS*, (p–r) *CKX7:GUS* expression in RKN-infected Arabidopsis roots during early and later stages of parasitism: (d,j,p) 3, (e,k,q) 6 and (f,l,r) 11 days post-inoculation (dpi). Abbreviations: N, nematode; S, syncytium; G, gall. Scale bar: 100 μm.

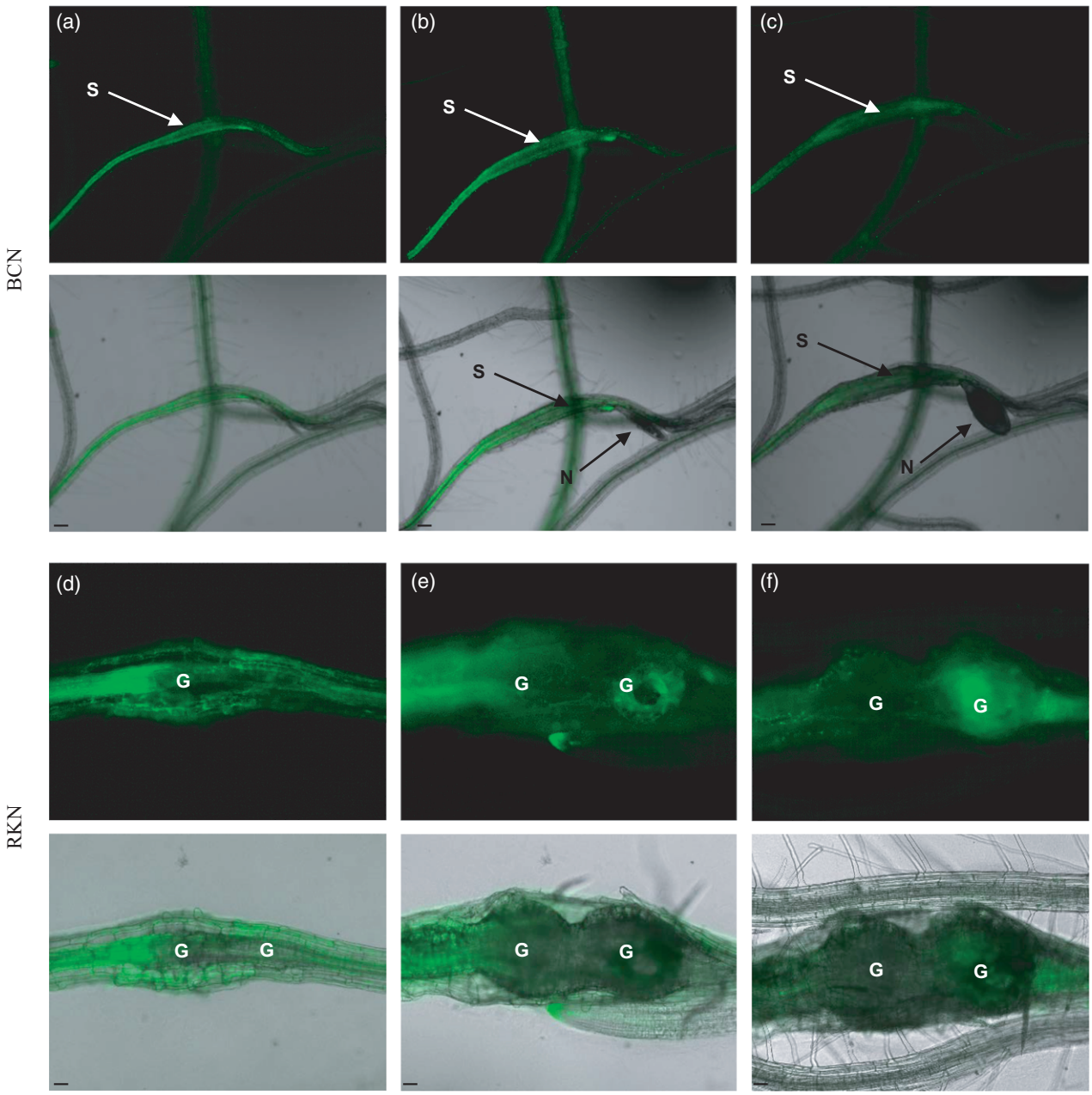

Figure 5. Expression of *TCSn:GFP* in response to the beet cyst nematode (BCN) *Heterodera schachtii* and the root-knot nematode (RKN) *Meloidogyne incognita*.
(a–c) Green fluorescent protein (GFP) expression in BCN-infected Arabidopsis roots during early and later stages of parasitism: (a) second-stage juveniles (J2), (b) third-stage juveniles (J3) and (c) fourth-stage juveniles (J4).
(d–f) GFP expression in RKN-infected Arabidopsis roots showing two galls developing during early and later stages of parasitism: (d) 3, (e) 8 and (f) 13 days post-inoculation (dpi). Abbreviations: N, nematode; S, syncytium; G, gall. Scale bar: 100 μm.

uninfected roots, *ARR15:GUS* showed a low level of expression in the root vasculature. Upon BCN infection, *ARR15:GUS* showed increased expression in developing syncytia during J2 and J3 developmental stages, with a decline in expression when nematodes reached the J4 developmental stage (Figure 7a–c). Opposite to BCN, no GUS expression was detected in galls or giant-cells (Figure 7d–f), resembling the results observed for *AHK4:GUS*. *AHK4* and *ARR15* downregulation was confirmed using qRT-PCR with specific primers (Figure S2). An additional type-A ARR gene, *ARR5*, was also evaluated. *ARR5:GUS* was expressed in the root vasculature and root tips, and showed early upregulation in response to both BCN and RKN further suggesting differential expression of type-A *ARR* genes in response to each nematode species (Figure 7g–l).

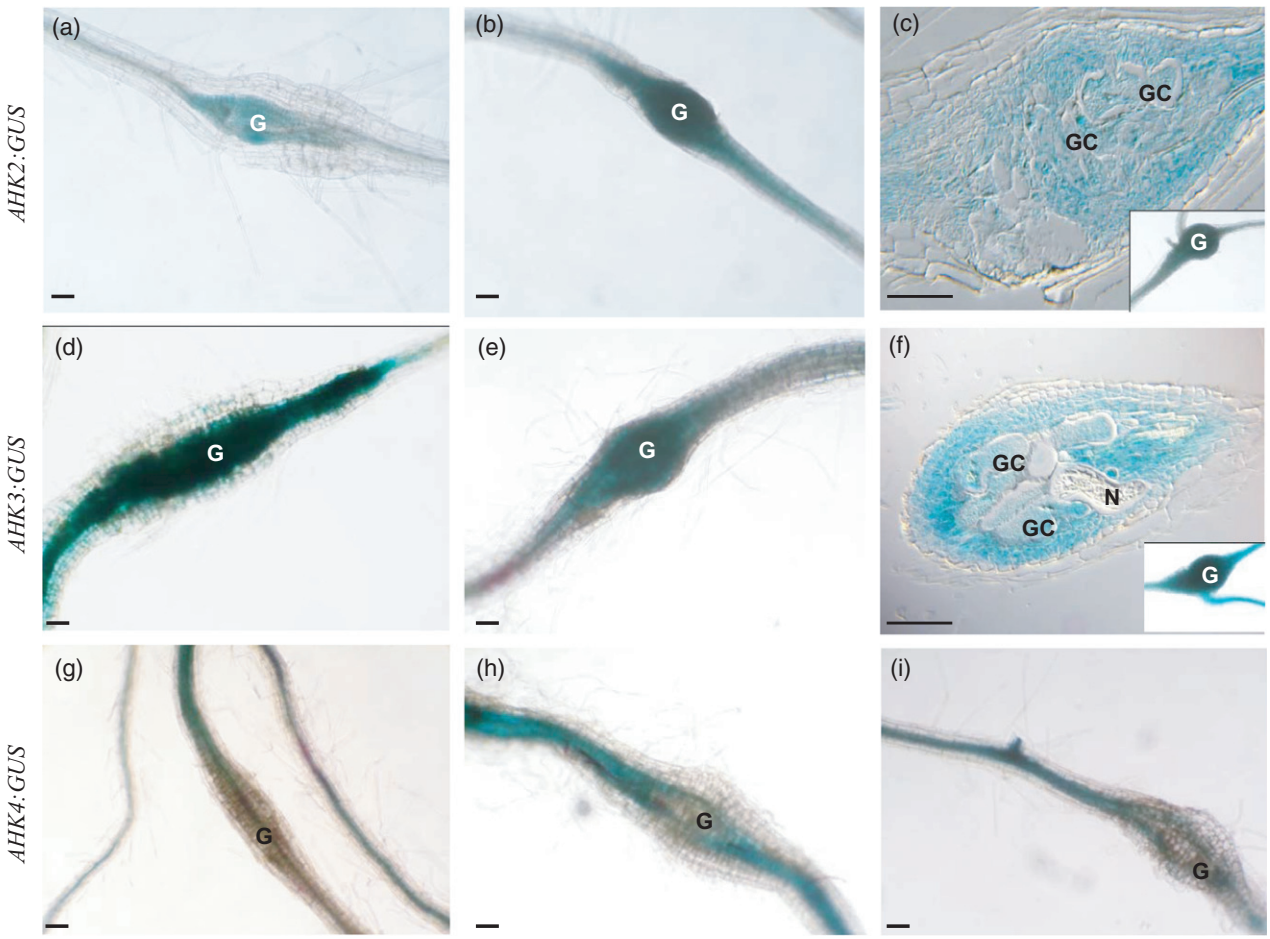

Figure 6. Histidine kinase receptor *AHK2*, *AHK3* and *AHK4* expression in response to the root-knot nematode (RKN) *Meloidogyne incognita*.
(a–c) *AHK2:GUS*; (d–f) *AHK3:GUS*; (g–i) *AHK4:GUS* during early and later stages of parasitism: (a,d,g) early and (b,e,h,i) later. (c,f) Longitudinal sections of a gall showing expression in GC and surrounding gall tissues (c) *AHK2:GUS* and (f) *AHK3:GUS* expression in GC. Abbreviations: N, nematode; G, gall; GC, giant-cell. Scale bar: 100 μm, 50 μm (c,f).

Cytokinin signaling is required for RKN parasitism

To determine the functional significance of the AHK receptors in RKN parasitism, we tested AHK receptor mutant lines. Single, *ahk2-2*, *ahk3-3*, *ahk4/cre1-12*, and double, *ahk2-2 ahk3-3* (*ahk2/3*), *ahk2-2 cre1-12* (*ahk2/4*) and *ahk3-3 cre1-12* (*ahk3/4*), mutant lines were infected with RKN following standard procedures, and gall numbers and size were measured. Triple mutants are dwarf, with limited shoot and root growth, and therefore not suitable for nematode infection assays (Riefler *et al.*, 2006). We did not observe any significant differences in the single receptor mutants (Figure S6); however, double receptor mutants showed significantly fewer galls, smaller galls and delayed nematode development (Figure 8a–c). The *ahk2/3* double mutant showed the greatest decrease in the number of galls of all the double mutant combinations, and was significantly different from *ahk2/4* and *ahk3/4*. These results are consistent with the high level of expression observed for *AHK2* and *AHK3* compared with *AHK4* throughout gall development (Figure 6). To rule out whether the reduced infection observed in the double cytokinin receptor mutants was caused by reduced nematode attraction or penetration, we counted the number of juveniles that entered the roots at 3 dpi. No significant differences were found among genotypes (Figure 8d). Altogether, these results suggest that cytokinin signaling is not required during nematode penetration, but rather is required for successful gall formation.

Cell cycle activation in RKN galls is mediated by cytokinin receptors

Our previous studies showed cytokinin is a trigger for cell cycle reactivation in syncytia, and that this is mainly mediated through cytokinin receptors AHK3 and AHK4 (Siddique *et al.*, 2015). To determine if cell cycle reactivation is dependent on cytokinin signaling in forming galls and

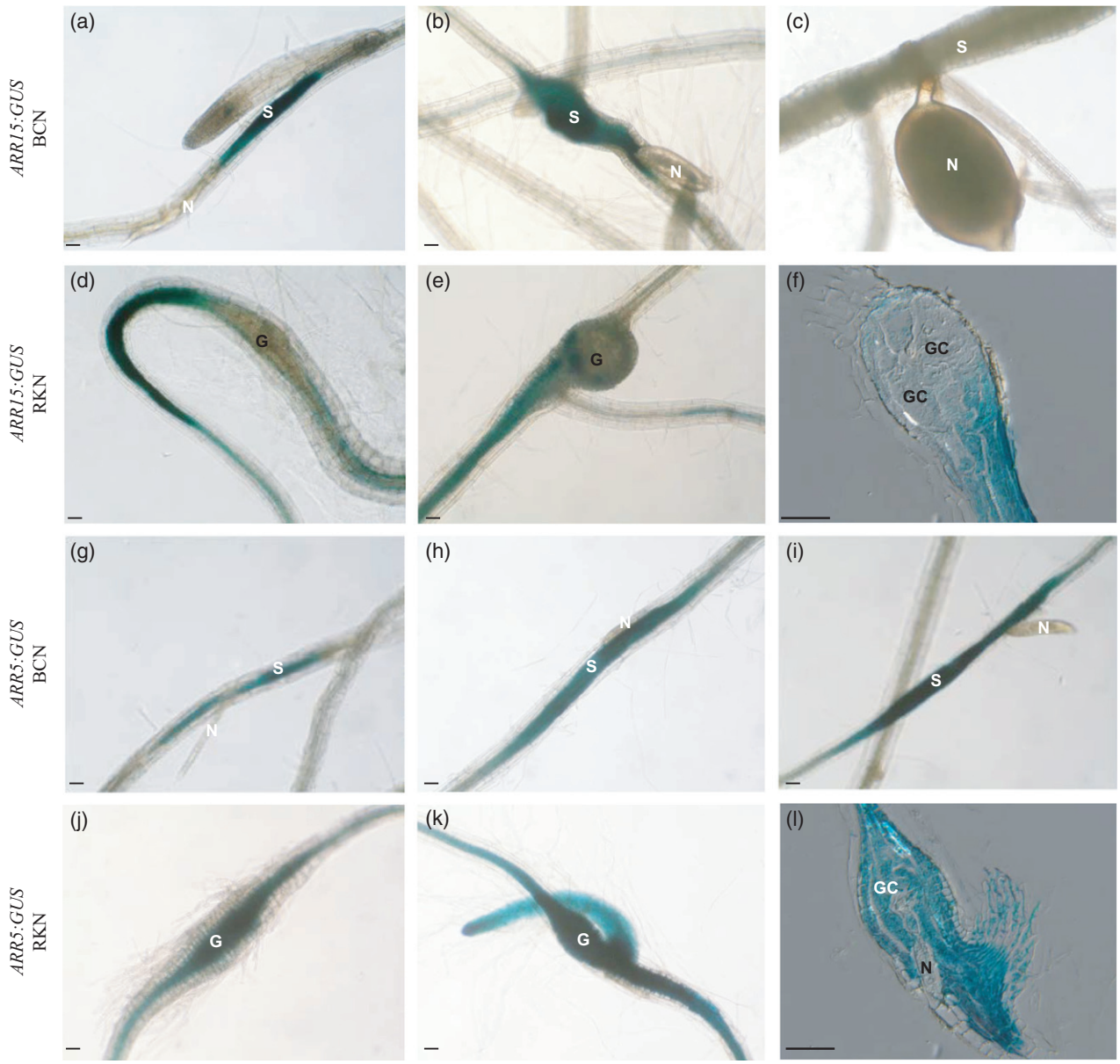

Figure 7. Cytokinin type-A Arabidopsis response regulators (ARR) *ARR15* and *ARR5* expression in response to the beet cyst nematode (BCN) *Heterodera schachtii* and the root-knot nematode (RKN) *Meloidogyne incognita*.
(a–c) *ARR15:GUS* and (g–i) *ARR5:GUS* expression in BCN-infected Arabidopsis roots during early and later stages of parasitism: (a,g) early and (b,h,c,i) later stages.
(d–f) *ARR15:GUS* and (j–l) *ARR5:GUS* expression in RKN-infected Arabidopsis roots during (d,j) early and (e,k) later stages. (f) Longitudinal section of a gall showing reduced *ARR15:GUS* expression in GC. (l) Longitudinal section of a gall showing expression of *ARR5:GUS* in GC. Abbreviations: N, nematode; S, syncytium; G, gall; GC, giant-cell. Scale bar: 100 μm, 50 μm (f, l).

whether receptor specificity varies between syncytia and galls, we evaluated expression of the *Cyclin B1;1:GUS* gene (*CycB1;1*) in *ahk2/3*, *ahk2/4* and *ahk3/4* double mutants in response to RKN infection. *CycB1;1* is highly expressed during the G2-to-M phase transition (Niebel *et al.*, 1996; Shaul *et al.*, 1996), and therefore was used here as a mitotic marker. Evaluation of *CycB1;1:GUS* transformed Col-0 roots showed strong expression in early developing galls (4 dpi), and expression was sustained till 11 dpi (Figure 9a–c). *CycB1;1:GUS* expression was also detected in receptor mutant combinations *ahk2/4* and *ahk3/4* (Figure 9g–l), but was frequently absent in *ahk2/3* (Figure 9d–f). To determine if significant differences existed between Col-0 and the receptor mutants, we quantified the number of galls in each mutant background expressing *CycB1;1:GUS*. We observed significant

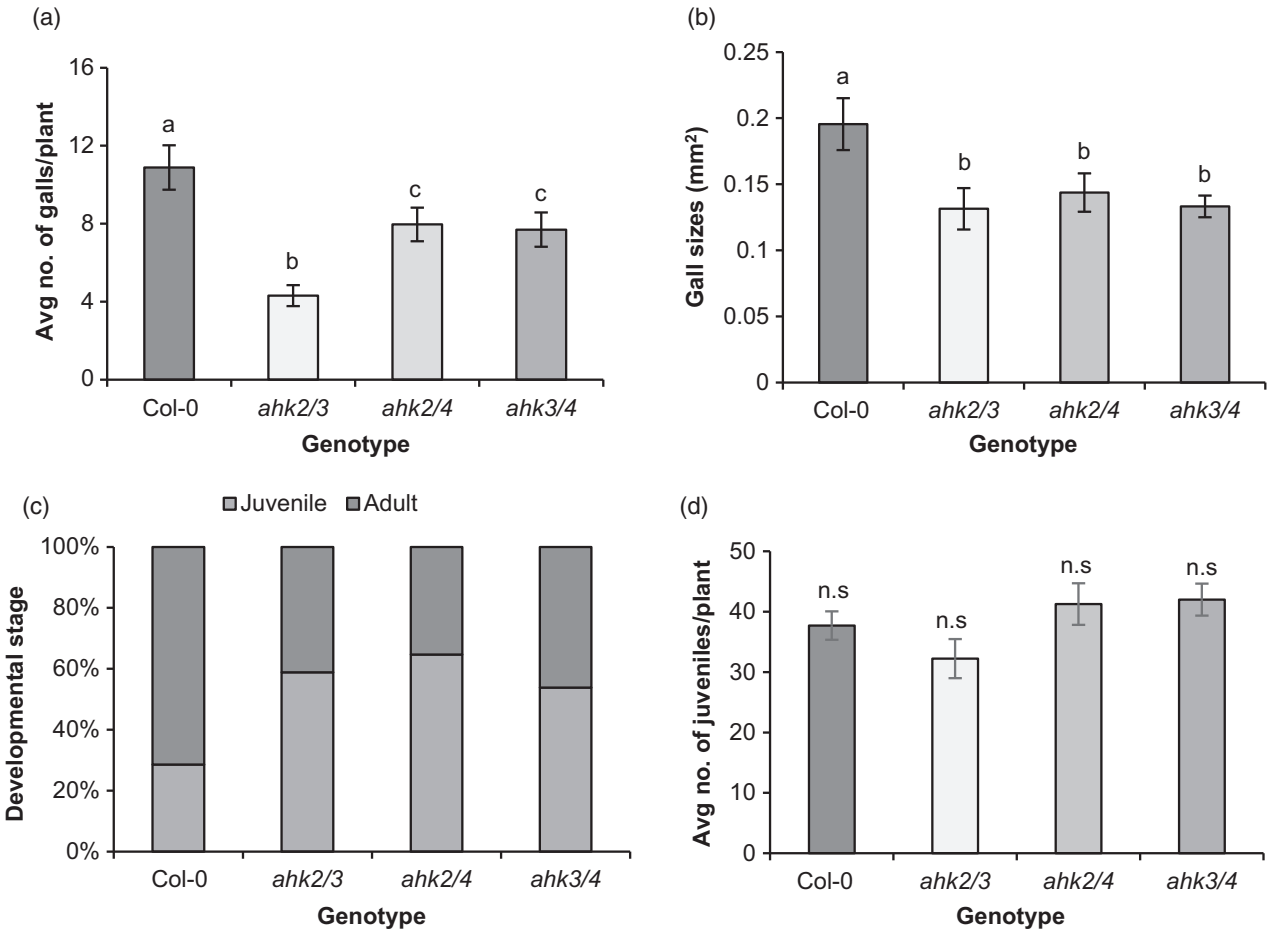

Figure 8. Evaluation of cytokinin double receptor mutants in response to the root-knot nematode (RKN) *Meloidogyne incognita* infection.
(a) Average number of galls per plant that developed in *ahk2-2/ahk3-3* (*ahk2/3*); *ahk2-2/cre1-2* (*ahk2/4*); *ahk3-3/cre1-12* (*ahk3/4*) at 40 days post-inoculation (dpi), plant numbers (*n*) per line were $n = 26$ for Col-0, $n = 29$ for *ahk2/3*, $n = 26$ for *ahk2/4*, $n = 23$ for *ahk3/4*.
(b) Average gall sizes that developed per plant after 20 dpi. Gall numbers per line were 25 for Col-0; 10 for *ahk2/3*; 34 for *ahk2/4*; 28 for *ahk3/4*.
(c) Percentage of galls that remained juvenile or became adult in each line at 11 dpi, developing galls were marked at 4 dpi and followed during course of infection. Gall numbers per line were 7 for Col-0; 17 for *ahk2/3*; 17 for *ahk2/4*; 13 for *ahk3/4*.
(d) Average number of juveniles that penetrated per plant in each line; $n = 10$ for Col-0, $n = 9$ for *ahk2/3*, $n = 12$ for *ahk2/4*, $n = 10$ for *ahk3/4*. Data are representative means ± SE of at least three independent biological replicates. Different letters above columns indicate statistically significant differences using a two-tailed Student's *t*-test ($P < 0.05$).

reductions across all lines at 5 and 7 dpi, with the greatest decrease observed for *ahk2/3* (Figure 9m and n), suggesting AHK2 and AHK3 are the major receptors for cell cycle activation in developing galls.

DISCUSSION

In this study, through a comparative analysis using a single host plant species we demonstrate that cytokinin signaling is a requirement of both BCN and RKN interactions, but these two nematode species differentially regulate genes involved in cytokinin biosynthesis, catabolism, signaling and response for the establishment of feeding sites. We first analyzed Arabidopsis GUS reporter lines of cytokinin biosynthesis genes in response to BCN and RKN infection and found commonalities, but also gene-specific differences between both nematode species. *IPT1* and *IPT3* are two of the five ATP/ADP IPT genes in Arabidopsis, and are involved in the production of iP and tZ, the most abundant forms of cytokinins in Arabidopsis plants (Miyawaki *et al.*, 2004). In this study, we found *IPT1* to be upregulated during early stages of BCN feeding site formation. *IPT1* expression was observed in infection zones developing near the vasculature of main roots, suggesting BCN can overrule repression of this gene in mature tissue and reactivate its expression presumably to help in the growth of the feeding site. *IPT1* expression within the syncytium confirms the unique nature of the feeding site, which is partially unrelated to other organs in the plant. In contrast to BCN infection, RKN infection did not stimulate *IPT1* expression, unveiling what may be a key difference in the modulation of cytokinin biosynthesis between these two nematode species. A second member of the ATP/ADP IPT

family, *IPT3*, displayed an expression pattern opposite to *IPT1* as it was downregulated by both nematode species at all developmental stages of feeding site growth. Determining whether or not *IPT3* downregulation is required for proper feeding site formation is beyond the reach of these studies; however, the fact that *IPT3* is downregulated by nutrient deficiencies such as low phosphorus and low nitrogen in roots (Ramireddy *et al.*, 2014) suggests that *IPT3* gene expression might be responding to internal nutrient content changes as a result of continuous nematode feeding. Similar to ATP/ADP *IPT* gene expression, tRNA-*IPT* genes *IPT2* and *IPT9* also express differently in response to BCN and RKN, further suggesting a differential and targeted manipulation of gene expression exerted by these two nematode species.

Proper function of cytokinin in root and shoot meristems depends heavily on a stringent regulation of cytokinin catabolism (Werner *et al.*, 2003; Bartrina *et al.*, 2011). To identify and compare cytokinin catabolism genes involved in BCN and RKN feeding site formation, we tested GUS reporter lines of *CKX* genes in response to nematode infection. Among all genes evaluated, *CKX6* was upregulated by both species, and *CKX5* and *CKX7* were upregulated by BCN, but not by RKN. Interestingly, *ckx5-1 ckx6-2* but not *ckx6-2* showed a significant reduction in BCN and RKN infection compared with Col-0, suggesting CKX5 could possibly complement CKX6 function in the *ckx6-1* mutant. Considering that CKX enzymes have distinct substrate specificities, these results may suggest a difference in the cytokinin pool accumulating in syncytia versus galls. Our previous studies showed an increase in cytokinin content in BCN-infected roots with a higher percent of iP and tZ cytokinins. Biochemical characterization of AtCKXs expressed in transgenic tobacco showed CKX5, CKX6 and CKX7 have mild activities against these types of cytokinins compared with other CKXs, such as CKX2 and CKX4 (Galuszka *et al.*, 2007). Potentially, upregulation of *CKX5*, *CKX6* and *CKX7*, but not *CKX2* and *CKX4*, coupled with the local upregulation of biosynthesis genes *IPT1* and *IPT2* and secretion of nematode-derived cytokinins may allow for a steady increase in iP and tZ cytokinin accumulation in syncytia explaining the high levels previously observed. CKX7 activity has also been correlated with a strong activity in cZ degradation (Kollmer *et al.*, 2014), which supports the lower levels for this type of cytokinin found in BCN-infected roots (Siddique *et al.*, 2015).

The overall differential expression of biosynthesis and catabolism genes by BCN and RKN suggest divergent initial cues delivered by these two nematode species into the initial feeding cell(s). It is well established that CN and RKN differ in their repertoire of effector molecules and host targets (Mitchum *et al.*, 2012). Thus, each nematode species may be utilizing a distinct set of effectors to directly or indirectly target specific genes within the cytokinin pathway. Previous studies have shown CN are able to manipulate auxin transport and signaling pathways through the use of effector molecules (Lee *et al.*, 2011; Hewezi *et al.*, 2015). As more effectors are identified and characterized from CN and RKN, additional effectors targeting hormonal pathways are likely to be identified. An alternative (but not exclusive) explanation to our findings relies on the existence of cytokinin molecules of nematode origin. Our previous studies have shown that CN have an *IPT* cytokinin biosynthesis gene homologue and are able to secrete cytokinins (Siddique *et al.*, 2015). Degradation of nematode *IPT* transcripts by dsRNA gene silencing resulted in deficient feeding sites and smaller cysts. Additionally, we showed that BCN-derived cytokinins were necessary for the activation of cell cycle in initial syncytial cells (Siddique *et al.*, 2015). Considering BCN mainly secretes isopentenyladenosine and benzyladenine, while RKN mainly secretes zeatin and benzyladenine (De Meutter *et al.*, 2003), it is possible that the differences we observed in cytokinin biosynthesis and catabolism gene expression derive from differences in the type of cytokinins secreted by CN and RKN, and will be interesting to explore further.

To determine whether cytokinin signaling was necessary for RKN parasitism, we evaluated single and double AHK receptor mutants against *Meloidogyne incognita* infection. No significant differences were found among single receptors mutants, likely due to functional redundancy among these receptors; however, the *ahk2/3* double mutant showed significantly fewer galls compared with the wild-type control, Col-0, followed by *ahk2/4* and *ahk3/4*. The observed reductions in gall number and nematode development compared with Col-0 were not explained by a decrease in nematode penetration efficiency, suggesting cytokinin signaling is necessary for feeding site formation rather than for initial infection of the host plant. No significant differences were found across double mutants in terms of nematode development or gall size, suggesting that even though fewer galls are formed in the *ahk2/3* mutant compared with *ahk2/4* and *ahk3/4*, the nematodes that are able to infect *ahk2/3* can still develop and form galls of comparable size to the nematodes growing in *ahk2/4* and *ahk3/4*. This suggests that receptors AHK2 and AHK3 play a role in early rather than late gall developmental stages. Consistent with this, *AHK2* and *AHK3* were expressed in galls over consecutive developmental stages from early stages until galls reached full maturity, whereas *AHK4/CRE1* was downregulated in galls following the initial stages of gall formation. The expression of the type-A *ARR* gene *ARR15*, and the cytokinin oxidase *CKX7* gene depend on AHK4/CRE1, both of which showed downregulation in expanding galls suggesting a reduced function of the AHK4/CRE1 receptor in response to RKN. These results support the stronger phenotypes observed for the *ahk2/ahk3* double mutant compared with *ahk2/ahk4* and *ahk3/*

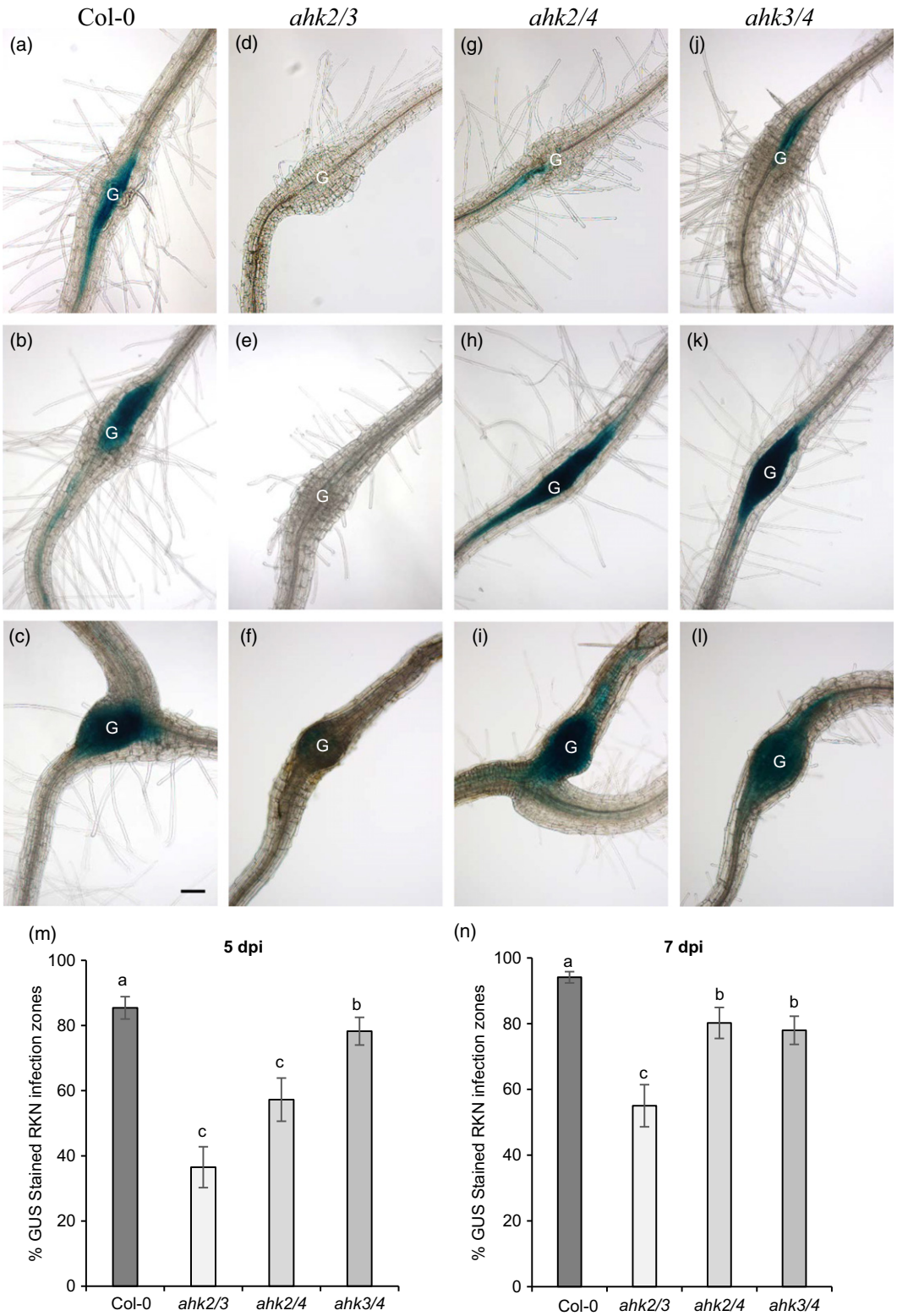

Figure 9. *CyclinB1;1* (*CycB1;1*) GUS expression in root-knot nematode (RKN) *Meloidogyne incognita* induced galls developing in Arabidopsis wild-type Col-0 (a–c) and cytokinin receptor mutants *ahk2/3* (d–f), *ahk2/4* (g–i), *ahk3/4* (j–l) at 4, 7 and 11 days post-inoculation (dpi) (top-bottom). Abbreviations: G, gall. Scale bar: 100 μm. Quantification of RKN galls expressing *CycB1,1:GUS* at 5 and 7 dpi. Data are representative means ± SE of two independent biological replicates with $n = 28$ for Col-0, $n = 29$ for *ahk2/3*, $n = 28$ for *ahk2/4* and $n = 29$ for *ahk3/4*. Different letters above columns indicate significant differences by a two-tailed Student's *t*-test ($P < 0.05$). Scale bar: 100 μm.

ahk4. However, we cannot exclude a role for AHK4/CRE1 during the initial stages of gall formation due to the expression of *AHK4/CRE1* in early galls and only partial reduction in gall formation in the double mutants. To further analyze the role of AHK receptors in early gall formation, we evaluated the function of cytokinin receptors in gall cell cycle activation. We tested *CycB1;1:GUS*, introgressed into Col-0, *ahk2/3*, *ahk2/4* and *ahk3/4* lines, and measured the number of infection sites expressing GUS. CycB1;1 is a cell cycle marker highly active in dividing cells expressed in galls (de Almeida Engler *et al.*, 1999). Evaluation of *CycB1;1:GUS* transformed Col-0 plants, showed *CycB1;1:GUS* expression in the majority of galls at 5 dpi and 7 dpi. In contrast, cytokinin receptor mutants had a significantly lower number of expressing sites compared with Col-0. The *ahk2/ahk3* mutant showed the greatest reduction in expression among all mutant combinations. These results strongly suggest cytokinin signaling is required for cell cycle progression in developing galls, and that *ahk2* and *ahk3* have a stronger effect on *CycB1;1:GUS* expression in galls than *ahk4*. Lack of cell cycle *CycB1;1:GUS* expression in galls could explain the fewer galls formed in the *ahk2/ahk3* mutant, further supporting the importance of AHK2 and AHK3 in gall formation. This is in stark contrast to our previous studies showing that AHK3 and AHK4/CRE1 act as the main receptors involved in the interaction with BCN (Siddique *et al.*, 2015).

The differences observed in cytokinin signaling between CN and RKN (Figure 10) may explain the underlying cellular changes induced by these two pathogens to form quite distinct feeding sites. Whereas CN induce limited cell division prior to incorporation of cells into the syncytium and require a normal activation of cytokinin signaling, RKN induce an abnormal increase in cell division giving rise to the characteristic root galls. Whether RKN achieves this by hyperactivation of cytokinin signaling is not yet understood, but presumably the observed differential expression of AHK receptors in the same tissue could be involved. In roots, AHK4 is the main receptor involved in cytokinin perception as *ahk4* mutants have a clear delay in cytokinin responsiveness to exogenous applications, and exhibit longer roots compared with *ahk2* and *ahk3* mutants, which behave similarly to the wild-type (Higuchi *et al.*, 2004; Nishimura *et al.*, 2004). AHK4 has also been associated with downregulation of cytokinin signaling under environmental stresses (Franco-Zorrilla *et al.*, 2002) and the direct control of negative regulators of cytokinin signaling (Kiba *et al.*, 2002). In this study, we observed a predominant role

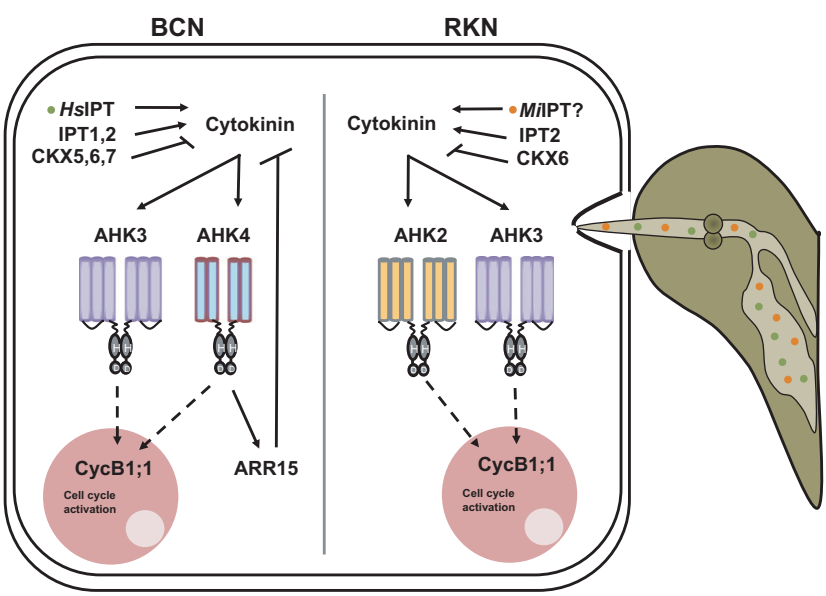

Figure 10. Model depicting divergent expression of cytokinin metabolism and signaling genes in response to the beet cyst nematode (BCN) *Heterodera schachtii* and the root-knot nematode (RKN) *Meloidogyne incognita*.
Cytokinin is synthesized by the host or the nematode, and recognized by receptors AHK3 and AHK4 in the case of syncytium formation (left) and AHK2 and AHK3 in the case of gall formation (right). Expression of *AHK4* and the negative regulator *ARR15* is upregulated during syncytium formation, but absent during gall formation. Upon cytokinin binding, autophosphorylation of cytokinin receptors initiates a phosphorelay resulting in upregulation of *CycB1;1* expression via an unknown pathway, which in turn activates cell cycle of infected cells. Host cytokinin levels in feeding sites are modulated by isopentenyltransferase genes *IPT1* and *IPT2* in the case of syncytia and cis-zeatin (cZ)-type cytokinin biosynthesis gene *IPT2* in the case of galls and cytokinin oxidase genes *CKX5, CKX6* and *CKX7* (syncytia) and *CKX6* (galls). BCN have an *IPT* cytokinin biosynthesis gene homologue and are able to secrete cytokinins (Siddique *et al.*, 2015). *MilPT* cytokinin biosynthesis gene/s remain to be identified. Abbreviations: *Hs*IPT: *Heterodera schachtii* isopentenyltransferase; *Mil*PT: *Meloidogyne incognita* isopentenyltransferase; IPT: *Arabidopsis thaliana* isopentenyltransferase; CKX: *A. thaliana* cytokinin oxidase/dehydrogenase, ARR: Arabidopsis response regulator; CycB1;1: Cyclin B1;1; AHK: Arabidopsis histidine kinase receptor; H: histidine residue; D: aspartic acid residue. Dotted lines represent unknown signaling pathways. The green and orange dots depict putative nematode stylet-secreted cytokinin.

for AHK2 and AHK3, but not AHK4 in gall formation. RKN may be downregulating AHK4, the main receptor involved in cytokinin sensitivity in roots in order to avoid a negative feedback, which would have naturally occurred over time as a result of an extensive exposure to cytokinin. This would result in partial cytokinin insensitivity and the abnormal growth observed in gall tissue, which is not observed in CN feeding sites. Future studies focusing on the identification of RKN cytokinin biosynthesis genes and the downstream components mediated by each AHK cytokinin receptor may uncover the role of differential cytokinin signaling in syncytium and gall formation.

EXPERIMENTAL PROCEDURES

Nematode cultures

Cultures of BCN *Heterodera schachtii* were maintained on greenhouse-grown sugarbeet plants (*Beta vulgaris* cv. Monohi). Cultures of RKN *Meloidogyne incognita* were maintained on tomato (*Lycopersicon esculentum* cv. Tiny Tim) and eggplant (*Solanum melongena* cv. Black Beauty).

Plant material

IPT:GUS and *AHK:GUS* reporter lines were described by Miyawaki *et al.* (2004). *CKX:GUS* (*CKX1:GUS* – *CKX6:GUS*) reporter lines were described by Werner *et al.* (2003). *CKX7:GUS* was reported in Kollmer *et al.* (2014). The Arabidopsis cytokinin biosynthesis mutant lines *ipt1-1* and *ipt2-1* used in this study were described by Miyawaki *et al.* (2006). Lines *ckx5-1*, *ckx6-2* and *ckx5-1/ckx6-2* were described by Bartrina *et al.* (2011). *TCSn::GFP* line was described by Zürcher *et al.* (2013). *CycB1;1* introgressed lines were described by Siddique *et al.* (2015). The Arabidopsis cytokinin receptor mutant lines used in this study included *ahk2-2*, *ahk3-3*, *cre1-12* (*ahk4*), *ahk2-2 ahk3-3*, *ahk2-2 cre1-12*, *ahk3-3 cre1-12*, and were described by Higuchi *et al.* (2004). Seeds were surface-sterilized with chlorine gas as previously described (Wang *et al.*, 2011) and cold-stratified for 3 days at 4°C. Seeds were plated on 0.5% Murashige Skoog (MS) medium (MS Basal Salts; Caisson Laboratories, North Logan, UT, USA), 2% sucrose and 0.8% Type A Agar (Sigma, St Louis, MO, USA) and placed in a growth chamber set at 22°C under constant light for 7 days. Seedlings were transplanted from the medium using forceps to trays of commercially available potting mix (SunGro) containing 50–60% horticulture grade vermiculite, Canadian sphagnum peat moss, horticulture grade perlite and dolomitic limestone. Trays of seedlings were covered with a plastic dome and placed in a walk-in growth chamber set at 22°C, 70% RH and 14 h photoperiod for 6 weeks. The plastic dome was removed 4 days after transplanting. Plants were bottom-watered with tap water every 3 or 4 days, and fertilized with a commercially available soluble fertilizer, Miracle-Gro (N-P-K: 24-8-16) every 2 weeks.

Inoculation of Arabidopsis reporter lines

For inoculations with BCN, surface-sterilized seeds were plated in square plates on Knop's medium prepared with 0.8% Daishin agar (Brunschwig Chemie, http://www.brunschwig-ch.com/; Sijmons, 1991). Plates were positioned vertically in a growth chamber set at 24°C, 70% RH with a 12 h photoperiod with a light intensity of 100–150 μmol m^{-2} sec^{-1}. Two days before inoculation, BCN eggs were isolated from pot cultures and J2s hatched at 28°C as previously described (Mitchum *et al.*, 2004). J2s were surface-sterilized in a solution containing 0.004% mercuric chloride, 0.004% sodium azide and 0.002% Triton X-100 for 8 min, washed with sterile water five times and suspended in 0.1% agarose. Nematodes were pelleted at 2000 rpm per 30 sec between washes. Roots of 10-day-old seedlings were inoculated with 50 surface-sterilized J2s. Plates were sealed with parafilm and placed back in the growth chamber until harvesting.

For inoculations with RKN, surface-sterilized seeds were plated one seed per well in six-well plates (GUS reporter lines) or glass-bottom microwell dishes (MatTek, Ashland, MA, USA) (*TCSn:GFP*) containing Knop's medium prepared with 0.8% Daishin agar. Plates were wrapped twice with parafilm to avoid dehydration and placed in a growth chamber set to the conditions above. Four days before inoculation, RKN eggs were harvested from pot cultures by rinsing soil from the roots and releasing eggs from egg masses by agitating roots in 10% sodium hypochlorite for 4 min. The egg suspension and plant debris was poured over a stack of sieves: 850 μm (No. 20); 250 μm (No. 60); 75 μm (No. 200); and 25 μm (No. 500). Eggs collected on the No. 500 sieve were rinsed under tap water for 5 min and set up to hatch as previously described (Mitchum *et al.*, 2004). J2s were surface-sterilized according to the above, except the incubation step in sterilization solution was for 5 min. Fourteen-day-old (GUS reporter lines) or 10-day-old (*TCSn:GFP*) seedlings were inoculated with 1000 surface-sterilized J2s per plant. Plates were resealed with parafilm and placed back in the growth chamber until harvesting.

Histochemical GUS assay

Nematode-inoculated and mock-inoculated seedlings were transferred from six-well plates into plates containing GUS solution [100 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS), 1 mM 5-bromo-4-chloro-3-indolyl glucuronide, pH 7.0, 50 mM NaCl, 0.06% Triton X-100, 2 mM potassium ferricyanide]. Plates were vacuum-infiltrated twice for 10 min and incubated at 37°C overnight. Seedlings were washed three times with 70% ethanol at intervals of 30 min, and incubated in 70% ethanol at 4°C overnight to remove all remaining chlorophyll prior to evaluation under an upright stereoscope. Representative samples were excised using micro-scissors and forceps (DUMOXEL #4), and placed in 12-well plates containing 70% ethanol. At least 12 seedlings were analyzed per line per time point.

Fixation, embedding and sectioning of GUS-stained Arabidopsis roots

Root segments containing syncytia or galls were embedded in 1.5% low-melting agarose by carefully dragging each root with forceps through a 250-μl drop of agarose placed inside a Petri dish. Agarose blocks containing root sections were cut and transferred to 12-well plates containing 4% paraformaldehyde (PFA) in a 1 × phosphate-buffered saline (PBS) solution (11.9 mM phosphate buffer containing KH_2PO_4 and Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4). Tissues were vacuum-infiltrated three times for 5 min. Following the last infiltration, PFA was replaced with new solution, and tissues were incubated for 17 h at room temperature and then for 24 h at 4°C. After incubation, tissue was gradually dehydrated starting with 1 × PBS solution for 15 min twice, and replacing the solution with 30, 40, 50, 60 and 70% ethanol for 30 min each. Agarose blocks were placed between foam pads of a biopsy cassette, submerged in 70% ethanol, and sent to the MU Histology Core for paraffin embedding and sectioning at 10 μm. Mounted sections were placed on a slide warmer at 40°C overnight for adhesion. Slides were vertically submerged in

xylene for 10 min, and then laid flat to dry before mounting sections in Shandon consul mount and coverslipped for imaging.

Brightfield microscopy

Root pieces containing syncytia or galls were placed on a plain microscope slide (Fisher Scientific, Hampton, NH, USA) in a drop of sterile distilled water and gently covered with a microscope cover glass (Fisher Scientific, Hampton, NH, USA). Photos of whole roots and root sections containing feeding sites were taken with a digital Nikon COOLPIX5000 camera manually attached to a Nikon Eclipse TS100 (Melville, NY, USA) inverted microscope (*IPT:GUS*, *AHK2:GUS*, *AHK3:GUS*). Additional photos were taken with an Olympus Vanox AHBT3 microscope (*CKX:GUS*) or a Leica DM5500B upright microscope (*AHK4:GUS*) equipped with a Leica DFC295 color digital camera at the MU Cytology Core.

Widefield fluorescence microscopy

TCSn:GFP seedlings were grown in 35-mm glass-bottom microwell dishes (MatTek, Ashland, MA, USA) containing 2 ml of Knop's medium for 7 days and inoculated as described above. *TCSn:GFP* lines infected with BCN or RKN were monitored daily for 14 days starting hours after inoculation. Syncytia and galls forming in *TCSn:GFP* infected lines were marked and labeled in the bottom of the plate. Photos were taken using an Olympus IX70 inverted microscope with Orca ER digital camera at the MU LSC Cytology Core.

Infection assays

Knop's medium. Sterilized seeds were plated one seed per well in sterile 12-well plates (BD Biosciences, http://www.bdbiosciences.com/) containing modified Knop's medium and 0.8% Daishin agar following a randomized block design. Plates were sealed with parafilm to avoid dessication and kept in a growth chamber at 24°C, 65% RH in a 12 h photoperiod with 100–150 μmol m^{-2} sec^{-1} average of light intensity. Fourteen-day-old seedlings were inoculated with 1000 surface-sterilized J2 resuspended in 25 μl of 0.01% sterile agarose and returned to the chamber. Galls were counted at 43 dpi. Thirty-six plants were evaluated per genotype per experiment, and each experiment was independently repeated three times.

Sand–soil-based substrate. Surface-sterilized seeds were imbibed in sterile water and placed at 4°C for 48 h under constant rotation. Seeds were plated on " MS, 2% sucrose medium in Petri plates, and kept at 22°C in a Percival chamber set to constant light. Seven-day-old seedlings were transferred to individual pots filled with riversand:Fafard Growing mix (3:1) and placed in trays. Pots were bottom watered for about 2 min until the soil surface looked wet. Trays were completely covered and placed in a growth chamber for 5 days. Covers were then cracked for 2 days. On day 7 after transplanting, the cover was completely removed. Fourteen-day-old seedlings were inoculated with 1500 RKN eggs. A 200-μl pipette tip was used to punch four holes around each plant. Each plant was inoculated with 1 ml (1500 eggs per ml) of egg inoculum suspended in sterile water. Tube was agitated by pipetting up and down before inoculating each plant and strongly agitated between trays. Plants were fertilized once a week with Miracle-Gro (N-P-K: 24-8-16). At 42 dpi, roots were carefully removed from the pots and gently rinsed in a bucket of water. Galls were counted under a stereomicroscope. Each root system was then cut into small pieces with scissors and placed in a 50-ml tube with 10% bleach and agitated for 4 min. The root slurry was poured over a nested stack of sieves (no. 20/60/200/500) while rinsing with a hand sprayer to release egg masses. Eggs were rinsed from the no. 500 sieve into a round-bottom tube or small beaker, and the volume was adjusted to 25 ml with water. Two milliliters of acid fuchsin was added, and the sample was microwaved for 45 sec and then cooled to RT. One milliliter of solution was placed into a Huxley slide and the eggs were counted.

Penetration assay

Arabidopsis seedlings were grown in six-well plates and inoculated with nematodes according to the protocol described above. Three days after inoculation, plants were carefully removed and placed into 1% bleach for 4 min. Plants were briefly rinsed to remove excess bleach, placed into 1/25 dilution of acid fuschin solution (250 ml lactic acid, 750 ml water, 0.35 g acid fuchsin) and microwaved for 3 min and 30 sec. After cooling down inside the fume hood, seedlings were stored in 12-well plates containing 95% ethanol until further analysis. Each seedling was placed on a microscope slide and the roots were carefully spread out for counting. Stained nematodes were counted using a Nikon Eclipse TS100 inverted microscope. Twelve to 24 plants were used per genotype per experiment. Each experiment was repeated three times.

Nematode development assessment

Experiments were conducted following the infection assay protocol and conditions above described. Developing galls were marked in the bottom of each well plate 3 days after inoculation. Photos of marked galls were taken at 11 dpi using Leica M205 FA stereoscope with a color digital camera at the MU Cytology Core. Pictures of each gall were evaluated for the presence of juvenile or an adult nematode. The numbers of adults and juveniles were counted, and the adult/juvenile ratio was calculated per genotype. This experiment was independently replicated three times using eight to 38 galls per genotype per experiment.

RNA isolation and qRT-PCR

One-hundred wild-type Col-0 seedlings were grown on Knop's medium and inoculated with BCN- or RKN-infective J2. Root segments containing syncytium or galls were harvested at 6 dpi and immediately placed in RNA*later* stabilization solution (Qiagen, Hilden, Germany) following manufacturer's instructions. Tissue was stored at −80°C until further processing. Each sample was homogenized in liquid nitrogen using a mortar and pestle, and total RNA was isolated with NucleoSpin RNA Plant Kit (Macherel-Nagel, Düren, Germany) following user manual instructions. cDNA was synthesized using First Strand cDNA Synthesis Kit following manufacturer's guidelines (Roche Diagnostics, Mannheim, Germany). cDNA was used in subsequent qRT-PCR reactions. qPCR reactions were prepared with template and a non-template control containing 15 μl of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 5 μl of 0.2 μM primers and 5 μl of 1:10 diluted cDNA in 96-well plates. qPCR was carried out with the following conditions: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min using an Applied Biosystems 7500 Real Time PCR system. A dissociation curve analysis was conducted for each primer set. Four technical replicates per each of two biological replicates were used. Average expression levels relative to mock were calculated using the ΔΔCt calculation method and *AtActin* as endogenous control. *AtActin* gene (AT1G49240) was amplified using forward primer 5′ AGTGGTCGTACAACCGGTATTGT and reverse primer 5′ GAGG

ATAGCATGTGGAAGTGAGAA. Gene-specific primer sequences used in this study are listed in Table S1.

ACKNOWLEDGEMENTS

The authors thank Robert Heinz, Amanda Howland and Clinton Meinhardt for nematode population maintenance, Aleksandr Jurkevic and Frank Baker for assistance with microscopy, and Bruno Müller for providing the *TCSn:GFP* seed. This work was supported by a grant from the National Science Foundation (NSF) (Grant IOS-1456047 to M.G.M). C.D.D. was funded in part by a Daniel F. Millikan graduate research assistantship. The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

C.D.D., D.C. and Z.S.R. performed experiments, analyzed and interpreted the data; T.S., T.W. and T.K. provided materials and assisted with data interpretation; M.G.M, F.M.W.G. and S.S. supervised the experimental work and assisted with data analysis and interpretation; M.G.M. co-wrote the article with C.D.D. All authors reviewed and commented on the manuscript.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Evaluation of cytokinin biosynthesis mutant *ipt1-1* in response to beet cyst nematode (BCN) *Heterodera schachtii*.

Figure S2. Quantification of cytokinin gene expression levels in beet cyst nematode (BCN) *Heterodera schachtii*- and the root-knot nematode (RKN) *Meloidogyne incognita*-infected roots at 6 dpi.

Figure S3. Evaluation of cytokinin biosynthesis mutant *ipt2-1* in response to beet cyst nematode (BCN) *Heterodera schachtii* and the root-knot nematode (RKN) *Meloidogyne incognita*.

Figure S4. Evaluation of cytokinin oxidase mutants in response to the beet cyst nematode (BCN) *Heterodera schachtii* and the root-knot nematode (RKN) *Meloidogyne incognita* infection.

Figure S5. *TCSn:GFP* expression in developing galls formed by the root-knot nematode (RKN) *Meloidogyne incognita*.

Figure S6. Evaluation of AHK receptor mutants *ahk2-2*, *ahk3-3* and *cre1-12* (*ahk4*) in response to the root-knot nematode (RKN) *Meloidogyne incognita*.

Table S1. Gene-specific primer sequences used in this study.

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