Molecular Plant Pathology

WILEY

Molecular Plant Pathology

SHORT COMMUNICATION OPEN ACCESS

Cytokinin Plays a Multifaceted Role in *Ralstonia solanacearum*-Triggered Plant Disease Development

Xiang Wang[1] | Qichang Gong[1] | Shengyang Cheng[1] | Ning Qin[1] | Tao Cao[1] | Yue Chen[1] | Dongdong Wang[1] | Marc Valls[2,3] | Núria S. Coll[2,4] | Qin Chen[5] | Cuizhu Zhao[1] | Haibin Lu[1]

[1]State Key Laboratory of Crop Stress Resistance and High-Efficiency Production, College of Agronomy, Northwest A&F University, Yangling, China | [2]Centre for Research in Agricultural Genomics (CRAG), CSIC-IRTA-UAB-UB, Campus UAB, Bellaterra, Barcelona, Spain | [3]Department of Genetics, University of Barcelona, Barcelona, Spain | [4]Consejo Superior de Investigaciones Científicas (CSIC), Barcelona, Spain | [5]State Key Laboratory of Crop Stress Resistance and High-Efficiency Production, College of Food Science and Engineering, Northwest A&F University, Yangling, China

Correspondence: Haibin Lu (luhaibin011@hotmail.com) | Cuizhu Zhao (zhaocuizhu2002@163.com)

Received: 9 October 2024 | **Revised:** 21 November 2024 | **Accepted:** 2 December 2024

Funding: The work was supported by the Nature Science Basic Research Plan of Shaanxi Province (2024JC-YBMS-144) for Haibin Lu, the Fundamental Research Fund of Northwest A&F University Seeds Industry Innovation (Z1090322151) for Haibin Lu, the Special Research Project from Shaanxi province (F2020221001) for Haibin Lu, the Program of Introducing Talents of Innovative Discipline to Universities (Project 111) from the State Administration of Foreign Experts Affairs (B18042) for Haibin Lu and the Natural Science Foundation of Shaanxi Province, China (2024JC-YBMS-180) for Cuizhu Zhao.

Keywords: bacterial wilt | cytokinin | plant immunity | *Ralstonia solanacearum* | root disease symptoms

ABSTRACT

Cytokinin signalling plays both positive and negative roles in plant resistance to pathogens. It is not clear whether the role of cytokinin changes at the different stages of pathogen infection. *Arabidopsis thaliana* sequentially exhibits distinct root morphological symptoms during *Ralstonia solanacearum* infection, which offers a good system to investigate function of cytokinin in the whole pathogen infection process. Using this system, we found increase of cytokinin signalling by *Lonely Guy 2* (*LOG2*) overexpression or depletion of type-A *Arabidopsis Response Regulators* (*ARRs*), negative regulators of cytokinin signalling pathway, promoted cell death, wilting symptom and bacterial growth, but attenuated primary root growth inhibition and lateral root formation. The decrease of cytokinin signalling by mutation on *Isopentenyl Transferases* (*IPTs*) inhibited root hair formation, cell death, wilting symptom and bacterial colonisation. Application of different concentration of exogenesis 6-benzylaminopurine (6-BA) showed first promoted, then decreased root hair formation. Moreover, application of 6-BA accelerated cell death but suppressed lateral root formation and primary root growth inhibition. The diverse roles of cytokinin in these different root disease phenotypes suggested function of cytokinin during plant responses to *R. solanacearum* is cell type-specific, which provides new insights on roles of cytokinin signalling in regulation on plant–pathogen interactions.

To survive in the natural environment, plants have acquired a sophisticated immune system to perceive pathogens and fend them off. The plant immune system consists of two branches: pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl 2006). The activation of PTI and ETI alters the homeostasis of phytohormones, directly influencing the outcome of plant immunity (Zhou and Zhang 2020). It is well known that the phytohormone salicylic acid (SA) plays a critical role in plant resistance to hemibiotrophic pathogens, while jasmonic acid (JA) and ethylene are responsible for plant resistance to necrotrophic pathogens and insects (Burger and Chory 2019; Nakano, Omae, and Tsuda 2022). Moreover, auxin and abscisic acid (ABA) have been demonstrated to suppress plant resistance to

Xiang Wang, Qichang Gong, Shengyang Cheng and Ning Qin contributed equally to this work.

hemibiotrophic pathogens (Burger and Chory 2019; Nakano, Omae, and Tsuda 2022). In contrast, the function of cytokinin in plant defence is still not clear.

Many bacteria (*Agrobacterium tumefaciens*, *Rhodococcus fascians*, *Streptomyces turgidiscabies*), oomycetes (*Plasmodiophora brassciae*), fungi (*Magnaporthe oryzae*) and nematodes (cyst and root-knot nematodes) can directly synthesise cytokinins (Bacete et al. 2020; Chanclud et al. 2016; Joshi and Loria 2007; Shanks et al. 2016; Siddique et al. 2015; Siemens et al. 2006; Spallek et al. 2018). Abolishment of cytokinin biosynthesis in those pathogens suppresses their growth on plants and prevents disease symptom development (Bacete et al. 2020; Chanclud et al. 2016; Shanks et al. 2016; Siddique et al. 2015; Siemens et al. 2006). However, it has also been reported that cytokinin enhances plant resistance to *Pseudomonas syringae* (bacterium), *Verticillium longisporum*, *Botrytis cinerea* and *Plectosphaerella cucumerina* (fungi), *Hyaloperonospora arabidopsidis* (oomycete) and viruses (Alonso-Diaz et al. 2021; Argueso et al. 2012; Bacete et al. 2020; Choi et al. 2010; Grosskinsky et al. 2011; Gupta et al. 2020; Naseem et al. 2012; Naseem, Wolfling, and Dandekar 2014; Reusche et al. 2013). Together, these data seem to indicate that the negative or positive regulation of cytokinin on plant resistance is pathogen species-specific. However, HopQ1, an effector from *P. syringae*, activates cytokinin signalling and interferes with plant immunity (Hann et al. 2014), which conflicts with the positive role of cytokinin in plant immunity to *P. syringae* (Choi et al. 2010; Grosskinsky et al. 2011; Wang et al. 2017). Furthermore, cytokinin is essential for the pathogenicity of *M. oryzae* in rice, but it is also required for rice blast-triggered defence gene expression (Chanclud et al. 2016; Jiang et al. 2013). Similar conflicting roles for cytokinin in plant defence also exist in the interaction between *R. solanacearum* and plants. Lack of cytokinin receptors *Arabidopsis* Histidine Kinases (AHK2, AHK3 and AHK4) in *Arabidopsis* accelerates bacterial wilt symptom development (Alonso-Diaz et al. 2021). Mutation of the negative regulator of cytokinin signalling *ARR6* increases *Arabidopsis* susceptibility to *R. solanacearum* (Bacete et al. 2020). These studies imply that the role of cytokinin in plant immunity is context-dependent. Investigation on roles of cytokinin at different stages of pathogen infection will help to elucidate the context-dependent function of cytokinin in the interaction between plants and microbes.

Ralstonia solanacearum, a soilborne vascular pathogen, invades many plant species including *Arabidopsis* and causes bacterial wilt disease, resulting in tremendous loss on crop production (Mansfield et al. 2012). Previously, we have demonstrated that *Arabidopsis* root architecture displayed dynamic changes as infection progresses and divided root infection processes into four stages as root hair formation (at 24 h post-inoculation [hpi]), cell death on root tip and inhibition of primary root growth (at 48 hpi) and lateral root formation (at 72 hpi) (Figure S1) (Lu et al. 2018; Zhao et al. 2019). These scenarios during *R. solanacearum* infection provide a very good system to explore the context-dependent role of cytokinin in the interaction between plants and pathogens. Here, taking advantage of these well-described root disease symptoms and late wilting symptoms, we characterised the function of cytokinin in plant responses to *R. solanacearum* at different infection stages. Our data indicate that cytokinin plays differential roles in different stages of the pathogen infection. These findings provide new insights on understanding the complex and important role of cytokinin in plant resistance to pathogens.

To ascertain the role of cytokinin in plant defence to *R. solanacearum*, we re-analysed global transcriptome data of *Arabidopsis* roots in response to the pathogen (Zhao et al. 2019). The genes involved in cytokinin biosynthesis (*LOGs* and CYP735A2 [Cytochrome P450 enzyme 735A2]), degradation (CKXs [Cytokinin Oxidases]) and signalling (type-A *ARRs* and *KMDs* [Kiss Me Deads]) exhibited dynamic changes in response to *R. solanacearum* infection (Figure S2, Table S1). *KMDs*, a subfamily of F-box proteins targeting type-B ARR proteins for degradation (Kim et al. 2013), started going down at 6 hpi and maintained very low expression after 24 hpi, in comparison to its expression at 0 hpi (Figure S2), suggesting type-B ARRs become stable and prepare for upstream cytokinin signals after 6 hpi. The expression of metabolic genes (*CYP735A2*, *LOG2*, *LOG6*, *CKX2*, *CKX3* and *CKX5*) and signalling genes (*ARR3*, *ARR4*, *ARR5*, *ARR7* and *ARR16*) of cytokinin significantly went up at 12 hpi in comparison to their expression at 0 hpi and reached a peak at 24–48 hpi, suggesting cytokinin is synthesised and cytokinin signalling is activated at this stage. The activation of biosynthesis genes (*LOG2* and *LOG6*), degradation genes (*CKX3* and *CKX5*) and signalling genes (*ARR3*, *ARR4*, *ARR5* and *ARR7*) at 24 hpi was further confirmed by reverse transcription-quantitative PCR (RT-qPCR) (Figure S2). These data indicated that the cytokinin signalling pathway was activated at the root hair formation stage (24 hpi) and cell death appearance and primary root growth inhibition stage (48 hpi), implying cytokinin may affect root disease symptom development. In comparison to 48 hpi, the expression of some cytokinin-related genes (*LOG2*, *ARR16*, *ARR4*, *ARR5*, *CKX2* and *CYP735A2*) quickly went down at 72 hpi. Interestingly, others (*LOG6*, *ARR3*, *ARR7* and *CKX5*) maintained very high expression after 48 hpi (Figure S2). This suggests cytokinin signalling is still activated, but changes at these stages. Altogether, these differential expression patterns of cytokinin-related genes at different infection stages might be related to diverse disease symptoms, which hints that cytokinin signalling is fine-tuned during the whole *R. solanacearum* infection.

The biosynthesis gene *LOG2* was upregulated at 24–48 hpi (Figure S2). To determine whether *LOG2* expression would have an impact on the plant responses to *R. solanacearum*, we investigated root disease symptoms and wilting symptoms on *log2* mutant plants (Kuroha et al. 2009, Figure S3). We found that the *log2* mutant plants displayed similar phenotypes of root hair formation, cell death on root tip, lateral root formation, inhibition on primary root growth and wilting symptoms as wild-type plants (Figure 1a–f). Moreover, the bacterial loads in *log2* plants were similar to that in wild-type plants at 4 days post-inoculation (dpi) (Figure 1g). These data indicate that mutation of *LOG2* does not affect disease development and resistance to *R. solanacearum*. Considering that the expression of *LOG6* is also significantly activated after *R. solanacearum* infection (Figure S2) and seven *LOGs*, including *LOG2*, show overlapping expression patterns and functional redundancy in *Arabidopsis* (Kuroha et al. 2009; Tokunaga et al. 2012), it is possible that LOG6 or other LOGs functionally compensate

for LOG2. Hence, we generated *Arabidopsis* transgenic plants overexpressing *LOG2-FLAG* under the control of a CaMV 35S constitutive promoter (Figure S3) and investigated the disease development at different stages of infection. *LOG2* overexpressors displayed longer root hairs and earlier cell death in response to *R. solanacearum* in comparison to wild-type plants

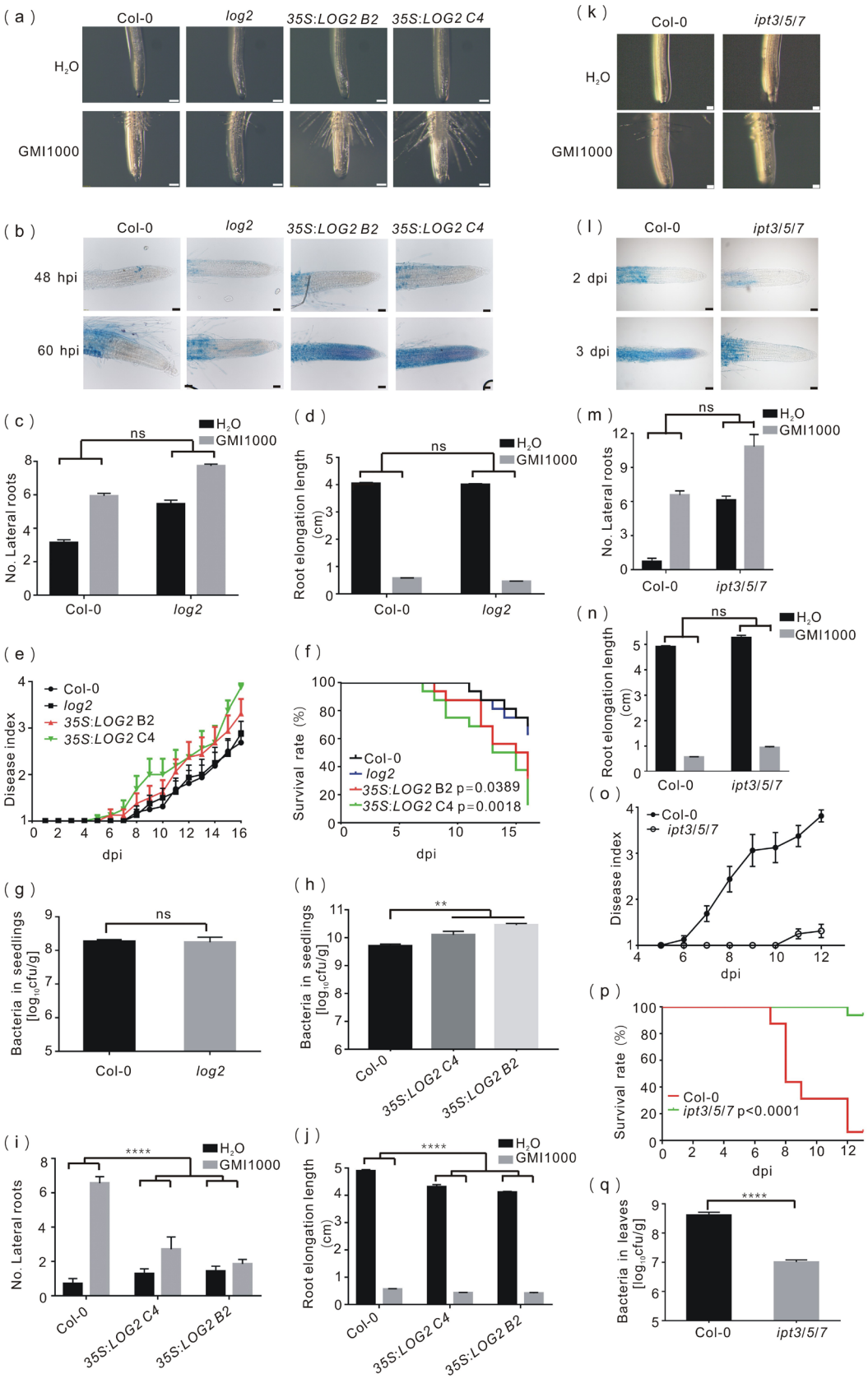

FIGURE 1 | Legend on next page.

FIGURE 1 | Ectopic expression of cytokinin biosynthesis-related genes alters *Ralstonia solanacearum* disease symptom development on plants. (a) Overexpression of *LOG2* promoted *R. solanacearum*-induced root hair formation. (b) Overexpression of *LOG2* accelerated *R. solanacearum*-induced cell death. (c, d) Lack of *LOG2* did not affect lateral root formation and inhibition on primary root growth caused by *R. solanacearum*. (e, f) Overexpression of *LOG2* accelerated bacterial wilt symptom development. (g) Lack of *LOG2* did not change plant sensitivity to *R. solanacearum*. (h) Overexpression of *LOG2* increased plant susceptibility to *R. solanacearum*. (i) Overexpression of *LOG2* decreased *R. solanacearum*-caused lateral root formation. (j) Overexpression of *LOG2* inhibited *R. solanacearum*-caused primary root growth inhibition. (k) Triple mutant *ipt3/5/7* displayed shorter root hair. (l) Triple mutant *ipt3/5/7* showed delayed cell death. (m, n) Loss of function of *IPT3/5/7* had minor effect on lateral roots and primary root growth inhibition. (o, p) *ipt3/5/7* plants displayed delayed wilting symptom. (q) Bacterial colonisation was restricted in *ipt3/5/7* plants. Seven-day-old seedlings grown in vitro were infected with *R. solanacearum* GMI1000. Root hairs were photographed at 24 h post-inoculation (hpi) with an Olympus microscope (a, k; bar = 100 μm; $n = 6$–7). Cell death on infected seedlings was visualised with Evans blue staining and photographed with an Olympus BX53 microscope at the indicated time (b, l; bar = 50 μm; $n = 6$). The number of lateral roots was counted at 4–5 days post-inoculation (dpi) (c, i and m; $n = 10$). Root elongation from the moment of *R. solanacearum* infection was measured with a ruler at 4–5 dpi (d, j and n; $n = 10$). Bacterial population in whole seedlings was measured at 4 dpi (g, h; $n = 6$). Roots of 5-week-old plants were cut with a razor blade and soil-drenched with a *R. solanacearum* solution ($OD_{600} = 0.1$). Wilting disease development was recorded and given a disease index score (1–4) by day (e and o; $n = 16$). For survival rate, disease index score ≤ 2 was regarded as '0' while disease index score ≥ 3 was regarded as '1' (f, p; $n = 16$). Bacterial population in *ipt3/5/7* leaves was determined at 13 dpi (q; $n = 6$). Statistical analysis was done by Student's *t* test (c,d, g, m, n and q; ns, no significance, $^{****}p < 0.0001$), one-way analysis of variance Dunnett's (h–j; $^{**}p < 0.01$; $^{****}p < 0.0001$) or log-rank (Mantel–Cox) test (f, p; *p* value is indicated in the picture). All experiments were repeated at least three times with similar results. Values in figures represent mean ± *SE*.

(Figure 1a,b). *LOG2* overexpression also resulted in quicker wilting symptoms and a lower survival rate (Figure 1e,f). Consistent with these disease phenotypes, *LOG2* overexpressing plants showed more bacterial colonisation than wild-type plants (Figure 1h), indicating that cytokinin accelerates disease development (root hair formation and cell death) and enhances plant susceptibility to *R. solanacearum*. However, lateral root formation and primary root growth inhibition caused by *R. solanacearum* were decreased on *LOG2*-overexpressing plants (Figure 1i,j), indicating the increase of cytokinin content in *Arabidopsis* inhibits *R. solanacearum*-triggered lateral root formation and primary root growth inhibition. These data suggest cytokinin acts in different roles in different *R. solanacearum*-triggered root disease symptoms.

To further investigate the effects of the decrease in endogenous cytokinin content on *R. solanacearum*-triggered root disease symptoms, we focused on IPTs, a family of proteins catalysing the first step of cytokinin biosynthesis (Figure S2), because multiple *LOG* mutants containing *log2* exhibit dwarf and very short primary roots, precluding any meaningful analysis (Tokunaga et al. 2012). *IPT3*, *IPT5* and *IPT7* (*IPT3/5/7*), but not other *IPTs,* are highly expressed and are mainly involved in cytokinin biosynthesis in vegetative plants (Miyawaki, Matsumoto-Kitano, and Kakimoto 2004; Miyawaki et al. 2006). The *ipt3/5/7* triple mutant displays lower endogenous levels of cytokinin than wild-type plants and weak growth defect in vegetative growth (Miyawaki et al. 2006). To ascertain the effect of low endogenous cytokinin on disease development, we further investigated disease symptoms of *ipt3/5/7* triple mutant plants after *R. solanacearum* infection. Root hair formation and cell death were reduced on *ipt3/5/7* triple mutants (Figure 1k,l). It has been reported that IPT3/5/7 inhibits primary root growth and lateral root formation (Miyawaki et al. 2006), but mutation of *IPT3/5/7* had little effect on primary root growth inhibition and lateral root formation triggered by *R. solanacearum* (Figure 1m,n). It is possible that cytokinin plays a minor role in *R. solanacearum*-triggered lateral root formation and primary root growth inhibition. Consistent with weakened root hair formation and cell death on *ipt3/5/7* triple mutants, mutation of *IPT3/5/7* inhibited bacterial wilting symptom and *R. solanacearum* colonisation (Figure 1o–q). Altogether, our data indicate cytokinin plays a positive role in disease symptom development such as root hair formation, cell death, bacterial wilt and plant susceptibility to *R. solanacearum*, but a negative role in lateral root and primary root inhibition, suggesting the role of cytokinin in disease development is disease symptom-specific.

Type-A ARRs, hallmarks of activation of cytokinin signalling, act as negative-feedback regulators of cytokinin signalling via suppression on key cytokinin signalling regulators type-B ARRs activation (Kieber and Schaller 2018; Svolacchia and Sabatini 2023). Induction of type-A *ARRs* expression by *R. solanacearum* suggests that cytokinin signalling might be important for disease development (Figure S2). Therefore, we investigated root disease development on type-A *ARR* single mutants *arr5*, *arr6* and *arr7* after pathogen infection. *arr5*, but not *arr6* nor *arr7* displayed shorter root hairs and fewer lateral roots after *R. solanacearum* inoculation (Figure 2a,b). However, inhibition of primary root growth, cell death, and bacterial growth were not different between wild-type plants and these individual *ARR* mutants (Figure 2c–e). Considering that the *Arabidopsis* genome contains 10 type-A *ARRs* (To et al. 2004), the minor effect of individual *ARRs* mutation on root disease symptom might be related to functional redundancy of this family of proteins. To at least partially overcome this problem, we analysed root disease symptoms on type-A *ARR* quadruple mutants *arr3/4/5/6*, *arr3/4/8/9* and *arr5/6/8/9* after *R. solanacearum* infection (To et al. 2004). Root hair and lateral root formation were attenuated on these three quadruple mutants (Figures 2f,g and S4). With the exception of *arr5/6/8/9*, these quadruple mutants exhibited weaker primary root growth inhibition (Figure 2h). Unexpectedly, cell death was accelerated in the quadruple mutants (Figure 2i). Regarding wilting disease development, *arr3/4/8/9* and *arr5/6/8/9* showed earlier wilting and lower survival rate than wild-type plants (Figure 2j,k). Consistent with bacterial wilt symptoms, the mutant plants supported more bacterial growth than wild-type plants (Figure 2l). All these data indicate that type-A ARRs negatively regulate

cell death, wilting symptoms and plant sensitivity to *R. solanacearum*, but positively regulate root hair formation, lateral root formation and primary root growth inhibition, which further confirms the notion indicated by *LOG2* overexpression that the function of cytokinin in disease development is root disease symptom-dependent.

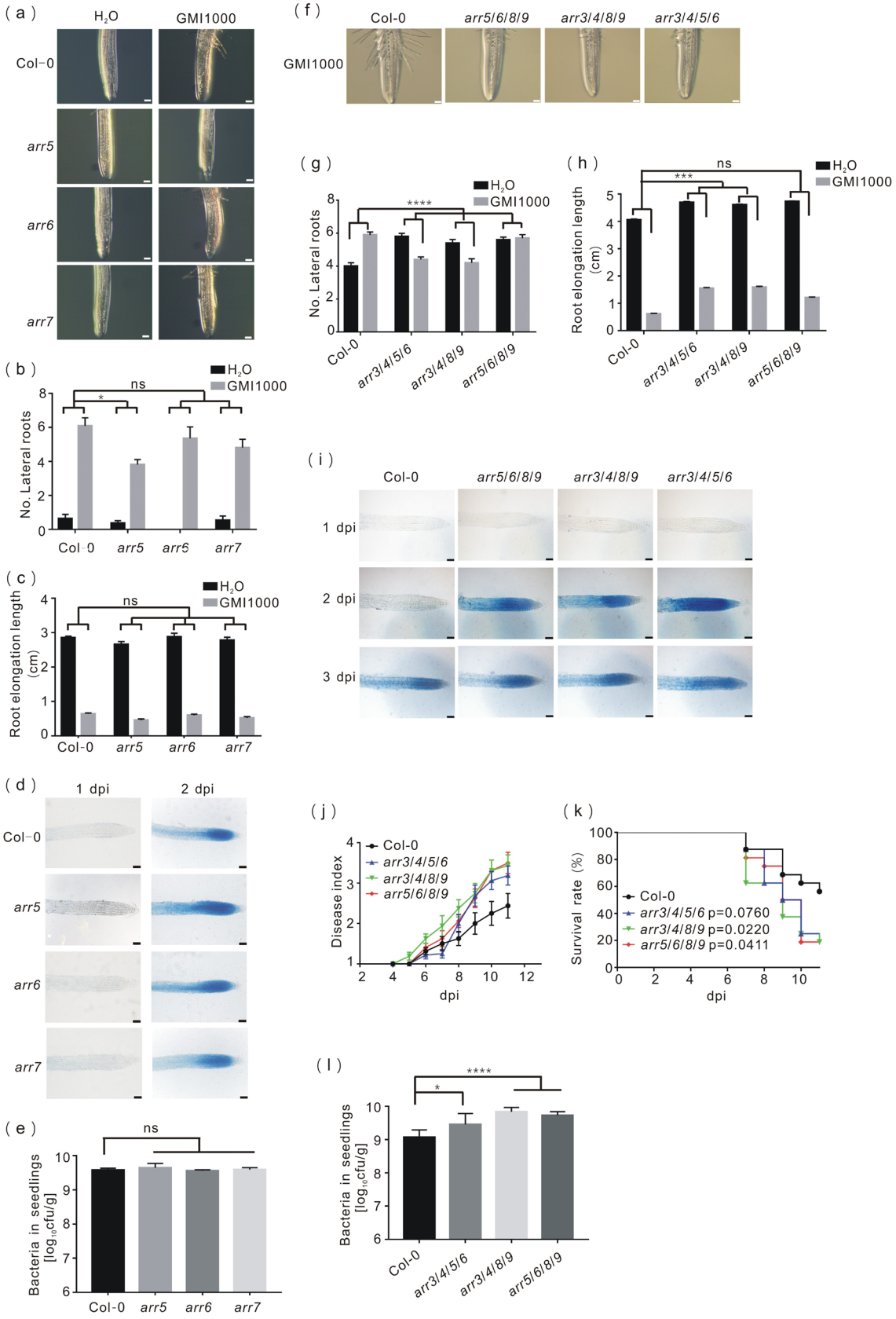

FIGURE 2 | Legend on next page.

FIGURE 2 | Loss of function of multiple type-A *ARRs* affects *Ralstonia solanacearum*-induced plant disease symptoms and plant susceptibility to *R. solanacearum*. (a) *arr5* mutant displayed shorter root hairs. (b) *arr5* mutant produced fewer lateral roots than wild-type plants. (c) The *arr* single mutants did not affect inhibition on primary root growth. (d) The *arr* single mutants did not affect cell death. (e) Single *arr* mutants exhibited similar plant sensitivity to *R. solanacearum*. (f) Simultaneous mutations of multiple type-A *ARRs* blocked root hair formation. (g, h) Simultaneous mutations of multiple type-A *ARRs* lowered lateral root formation and primary root growth inhibition. (i) Simultaneous mutations of multiple type-A *ARRs* resulted in earlier cell death on root tip. (j, k) Type-A *ARRs* quadruple mutants displayed quicker and stronger wilting symptom than wild-type plants. (l) Simultaneous mutations of multiple type-A *ARRs* increased plant susceptibility to *R. solanacearum*. Seven-day-old seedlings grown in vitro were infected with *R. solanacearum* GMI1000. Root hairs were photographed at 24 h post-inoculation (hpi) with Olympus microscope (a, f; bar = 100 μm; $n = 6–7$). The number of lateral roots was counted at 4–5 days post-inoculation (dpi) (b, g; $n = 10$). Root elongation length from the moment of *R. solanacearum* infection was measured with a ruler at 4–5 dpi (c, h; $n = 10$). Cell death on infected seedlings was observed by staining with Evans blue solution and photographed by an Olympus BX53 at the indicated time (d, i; bar = 50 μm; $n = 6$). Bacterial population in whole seedlings was measured at 4 dpi (e, l; $n = 6$). Roots of 5-week-old plants were cut with a razor blade and soil-drenched with *R. solanacearum*. Wilting disease development was recorded and given a disease index score (1–4) by day (j; $n = 16$). For survival rate, disease index score ≤ 2 was regarded as '0' while disease index score ≥ 3 was regarded as '1' (k; $n = 16$). Statistical analysis was done by one-way analysis of variance Dunnett's (b, c, e, g, h and l; ns, no significance, $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$) or log-rank (Mantel–Cox) test (k; p value is indicated in the picture). All experiments were repeated at least three times with similar results. Values in figures represent mean ± *SE*.

Interestingly, loss of function of multiple type-A *ARRs* and *LOG2* overexpression showed differential root hair formation phenotype (Figures 1a and 2a), although both of them boost cytokinin signalling by removing brake of cytokinin signalling or increasing supply of endogenous cytokinin (Kieber and Schaller 2018; Miyawaki et al. 2006; To et al. 2004; Tokunaga et al. 2012), which might be related to amplitude of cytokinin signalling. To answer this question, we investigated the effect of a series of concentrations of the synthetic cytokinin 6-benzylaminopurine (6-BA) on *R. solanacearum*-triggered root hair formation and other disease symptoms. Low concentrations of 6-BA (≤ 200 nM) promoted root hair formation after infection. When the concentration increased to 1000 nM, root hair formation was completely blocked by 6-BA (Figure 3a). These data indicated the differential amplitude of cytokinin signalling leads to distinct effects on *R. solanacearum*-triggered root hair formation, which might explain the distinct effects on root hair formation that appeared on multiple type-A *ARR* mutants and overexpressing *LOG2* plants (Figures 1a and 2a). Adding a low concentration of 6-BA (25 nM) significantly accelerated cell death at root tips after *R. solanacearum* infection (Figure 3b). In contrast to root hair formation, higher concentrations of 6-BA did not attenuate *R. solanacearum*-triggered cell death (Figure 3b). Additionally, with increasing concentrations of 6-BA, *R. solanacearum*-induced lateral root formation and primary root growth inhibition were decreased (Figure 3c,d). This is consistent with our findings that overexpressing *LOG2* or loss of function of type-A *ARRs* decreases lateral root formation and primary root inhibition (Figures 1i,j and 2b,c), and further confirming our prior observations showing that cytokinin negatively modulates *R. solanacearum*-mediated lateral root formation and primary root growth inhibition. The concentration of 6-BA displayed three different effects on root hairs, cell death, lateral root formation and primary root growth inhibition; we wondered how the amplitude of cytokinin signalling affects *Arabidopsis* sensitivity to *R. solanacearum*. In vitro bacterial growth assays showed that a rise of 6-BA concentration first promoted, then inhibited the pathogen growth in plants (Figure 3e). Altogether, our data suggest the fine-tuned cytokinin signalling in different infection stages determines the root responses to *R. solanacearum* infection.

Using sequential disease symptoms exhibited by plants infected with *R. solanacearum* (Lu et al. 2018; Zhao et al. 2019), we demonstrated that cytokinin plays diverse roles in different root disease symptoms: (1) cytokinin significantly enhanced *R. solanacearum*-triggered cell death but increasing cytokinin concentration did not further promote cell death appearance compared to 25 nM cytokinin. (2) Cytokinin inhibited *R. solanacearum*-triggered lateral root formation; the inhibitory effect became stronger as the concentration of cytokinin increased. (3) Primary root growth inhibition was also alleviated in the presence of cytokinin. Increasing cytokinin concentration strengthened the inhibitory effect of cytokinin on *R. solanacearum*-triggered primary root growth inhibition. Considering that *LOG2* overexpression and application of 6-BA both inhibited primary root growth regardless of infection (Figures 1j and 3d), we could not rule out that the weakened primary root growth inhibition is caused by the direct strong inhibitory effect of cytokinin on root growth (Kieber and Schaller 2018). (4) Cytokinin displayed a parabolic effect on *R. solanacearum*-triggered root hair formation and bacterial colonisation. Root hair formation and bacterial growth were enhanced at low concentrations of cytokinin but reduced by higher concentrations of cytokinin. Taken together, our data indicates the function of cytokinin in the plant–pathogen interaction varies as the pathogen infection progresses. The diverse roles of cytokinin in these root disease symptoms might be related to level of cytokinin content in roots during *R. solanacearum* infection. Our RNA-seq data indicated the expressions of genes involved in cytokinin biosynthesis and signalling were activated and dynamically changed as infection progressed. In addition, *TCS(n)::GFP*, a reporter for cytokinin signalling pathway, is activated in *Arabidopsis* after *R. solanacearum* infection (Alonso-Diaz et al. 2021). These data suggest that cytokinin content increases in infected roots, which is supported by the fact that endogenous cytokinin contents in roots of adult plants increase over the infection time (Alonso-Diaz et al. 2021). In future, time-resolved quantification of cytokinin contents in different root cell types of *Arabidopsis* seedlings during *R. solanacearum* infection will greatly help to elucidate the diverse roles of cytokinin in these root disease symptoms in different root cell types.

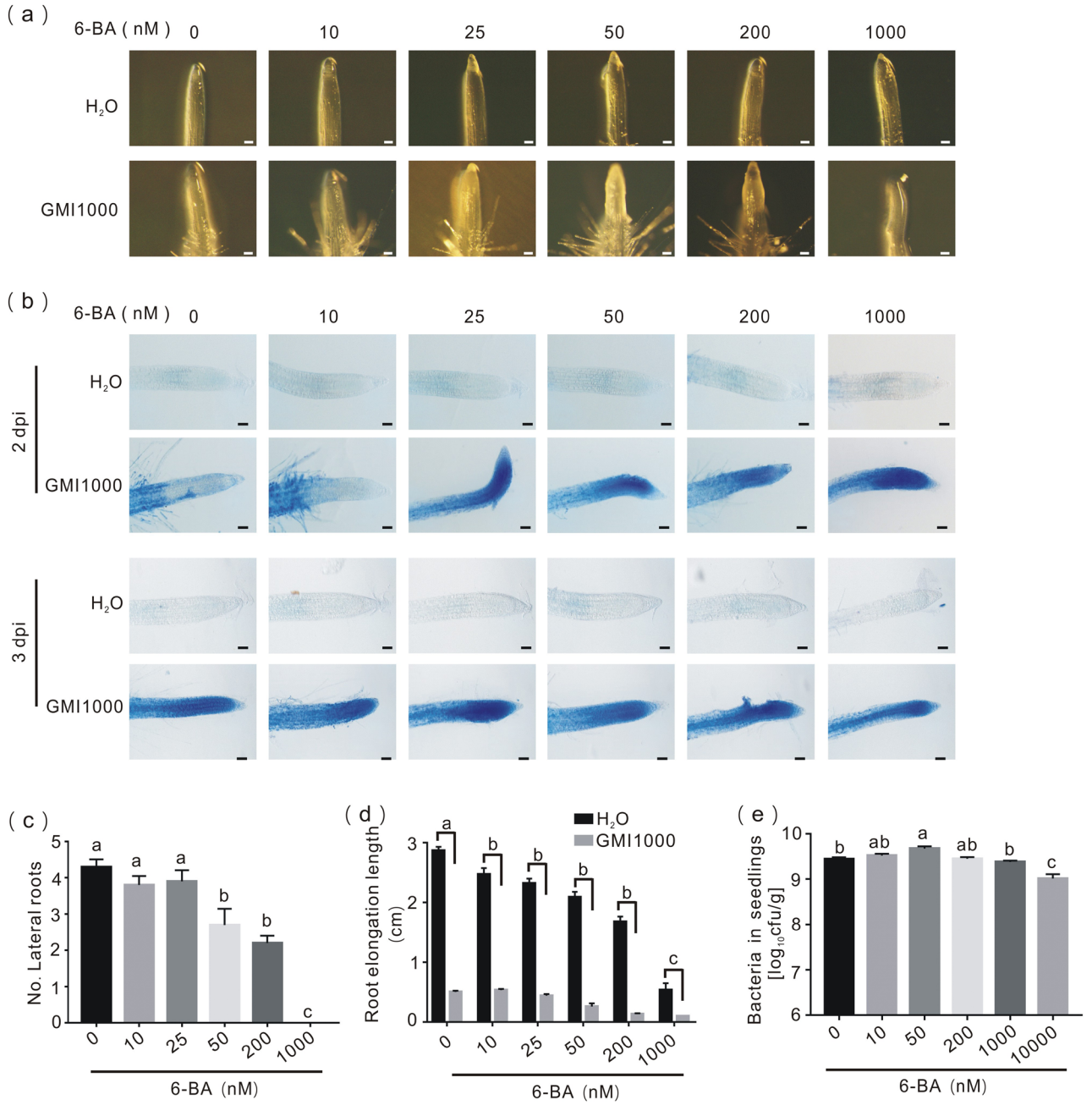

FIGURE 3 | Influence of 6-benzylaminopurine (6-BA) on different *Ralstonia solanacearum*-triggered disease symptoms and plant resistance. (a) Effect of exogeneous 6-BA on root hair formation. (b) Application of 6-BA promoted cell death. (c, d) Application of 6-BA reduced lateral root formation and inhibition on primary root growth caused by *R. solanacearum*. (e) Effect of 6-BA on plant susceptibility is dependent on its concentration. Six-day-old seedlings grown in vitro were transferred to Murashige and SkoogS- or agar plates containing various concentrations of 6-BA for 24 h, then infected with *R. solanacearum* GMI1000. Root hairs was photographed at 24 h post-inoculation (hpi) with an Olympus microscope (a; bar = 100 μm; $n = 6–7$). Cell death on infected seedlings was observed by staining with Evans blue solution and photographed by an Olympus BX53 microscope at the indicated time (b; bar = 50 μm; $n = 6$). Number of lateral roots was counted at 4–5 days post-inoculation (dpi) (c; $n = 10$). Root elongation length from the moment of *R. solanacearum* infection was measured with a ruler at 4–5 dpi (d; $n = 10$). Bacterial population in whole seedlings was measured at 4 dpi (e; $n = 6$). Statistical analysis was done by one-way analysis of variance Turkey's test (c–e; letters on columns indicate statistically significant differences). All experiments were repeated at least three times with similar results. Values in figures represent mean ± SE.

Diverse *R. solanacearum*-mediated root disease symptoms sequentially appear at different infection stages (Lu et al. 2018; Zhao et al. 2019). The negative or positive regulation of cytokinin on those root disease symptoms suggests the function of cytokinin is infection stage (temporal)-dependent during *R. solanacearum* infection. Moreover, root hairs are derived from root epidermis cells (Vissenberg et al. 2020), lateral roots originate from pericycle cells (Torres-Martinez, Napsucialy-Mendivil, and Dubrovsky 2022) and cell death happens to many different cell types in the root meristem zone (Lu et al. 2018; Zhao et al. 2019). All of these cues indicate that the role of cytokinin in plant defence to the pathogen is also spatially dependent.

The differential roles of cytokinin in different spatially arranged cells have been reported in the interaction between plants and rhizobacteria, in which cytokinin limits rhizobacterial invasion of the root epidermis and promotes rhizobacterial colonisation of the root cortex during nodule formation (Miri et al. 2016). Therefore, future investigation on molecular events in different cell types over pathogen infection time is necessary for explicitly elucidating the molecular mechanism in the interaction between plant and pathogen. Newly developed single-nuclei/cell transcriptome and spatial transcriptome techniques have been used to investigate new and rare cell types, gene expression heterogeneity across cells and trajectories of cell fate choices during plant development and provide new insights for complex mechanisms of plant development (Giacomello 2021; Yin, Xia, and Xu 2023; Yu, Liu, and Sun 2023). The time-resolved single-cell and spatial gene regulatory atlas of plant under pathogen attack will help to decipher the multifaceted function of cytokinin in the interaction between plants and pathogens and advance our understanding about cell type-specific immune responses during plant disease development or plant resistance, which will greatly help molecular breeding in the near future.

EXPERIMENTAL PROCEDURES

The details of materials, methods and primers used in this study were described in Methods S1 and Table S2.

Acknowledgements

The authors thank Dr. Shuhua Yang for sharing *arr5*, *arr6* and *arr7* seeds; Dr. Jamie Winshell for sharing *arr3/4/5/6*, *arr3/4/8/9* and *arr5/6/8/9* seeds; and Xigang Liu for sharing *ipt3/5/7* seeds. We also thank Jianchu Zhu and Huixia Li (the Crop Biology Innovation Center at the College of Agronomy, Northwest A&F University, China), Xiaona Zhou and Hua Zhao (the Public Platform in the State Key Laboratory of Crop Stress Biology for Arid Areas, Northwest A&F University, China) for help with ROS measurement and microscopy. The work was supported by the Nature Science Basic Research Plan of Shaanxi Province (2024JC-YBMS-144) for Haibin Lu, the Fundamental Research Fund of Northwest A&F University Seeds Industry Innovation (Z1090322151) for Haibin Lu, the Special Research Project from Shaanxi province (F2020221001) for Haibin Lu, the Program of Introducing Talents of Innovative Discipline to Universities (Project 111) from the State Administration of Foreign Experts Affairs (B18042) for Haibin Lu and the Natural Science Foundation of Shaanxi Province, China (2024JC-YBMS-180) for Cuizhu Zhao.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All the experimental data related to this work were included in the manuscript, figures or Supporting Information.

References

Alonso-Diaz, A., S. B. Satbhai, R. de Pedro-Jove, et al. 2021. "A Genome-Wide Association Study Unravels Cytokinin as a Major Component in the Root Defense Responses Against *Ralstonia solanacearum*." *Journal of Experimental Botany* 72: 2727–2740.

Argueso, C. T., F. J. Ferreira, P. Epple, et al. 2012. "Two-Component Elements Mediate Interactions Between Cytokinin and Salicylic Acid in Plant Immunity." *PLoS Genetics* 8: e1002448.

Bacete, L., H. Melida, G. Lopez, et al. 2020. "*Arabidopsis* Response Regulator 6 (ARR6) Modulates Plant Cell-Wall Composition and Disease Resistance." *Molecular Plant–Microbe Interactions* 33: 767–780.

Burger, M., and J. Chory. 2019. "Stressed out About Hormones: How Plants Orchestrate Immunity." *Cell Host & Microbe* 26: 163–172.

Chanclud, E., A. Kisiala, N. R. Emery, et al. 2016. "Cytokinin Production by the Rice Blast Fungus Is a Pivotal Requirement for Full Virulence." *PLoS Pathogens* 12: e1005457.

Choi, J., S. U. Huh, M. Kojima, H. Sakakibara, K. H. Paek, and I. Hwang. 2010. "The Cytokinin-Activated Transcription Factor ARR2 Promotes Plant Immunity via TGA3/NPR1-Dependent Salicylic Acid Signaling in *Arabidopsis*." *Developmental Cell* 19: 284–295.

Giacomello, S. 2021. "A New Era for Plant Science: Spatial Single-Cell Transcriptomics." *Current Opinion in Plant Biology* 60: 102041.

Grosskinsky, D. K., M. Naseem, U. R. Abdelmohsen, et al. 2011. "Cytokinins Mediate Resistance Against *Pseudomonas syringae* in Tobacco Through Increased Antimicrobial Phytoalexin Synthesis Independent of Salicylic Acid Signaling." *Plant Physiology* 157: 815–830.

Gupta, R., L. Pizarro, M. Leibman-Markus, I. Marash, and M. Bar. 2020. "Cytokinin Response Induces Immunity and Fungal Pathogen Resistance, and Modulates Trafficking of the PRR LeEIX2 in Tomato." *Molecular Plant Pathology* 21: 1287–1306.

Hann, D. R., A. Dominguez-Ferreras, V. Motyka, et al. 2014. "The *Pseudomonas* Type III Effector HopQ1 Activates Cytokinin Signaling and Interferes With Plant Innate Immunity." *New Phytologist* 201: 585–598.

Jiang, C. J., M. Shimono, S. Sugano, et al. 2013. "Cytokinins Act Synergistically With Salicylic Acid to Activate Defense Gene Expression in Rice." *Molecular Plant–Microbe Interactions* 26: 287–296.

Jones, J. D., and J. L. Dangl. 2006. "The plant immune system." *Nature* 444: 323–329.

Joshi, M. V., and R. Loria. 2007. "*Streptomyces turgidiscabies* Possesses a Functional Cytokinin Biosynthetic Pathway and Produces Leafy Galls." *Molecular Plant–Microbe Interactions* 20: 751–758.

Kieber, J. J., and G. E. Schaller. 2018. "Cytokinin Signaling in Plant Development." *Development* 145: dev149344.

Kim, H. J., Y. H. Chiang, J. J. Kieber, and G. E. Schaller. 2013. "SCF(KMD) Controls Cytokinin Signaling by Regulating the Degradation of Type-B Response Regulators." *Proceedings of the National Academy of Sciences of the United States of America* 110: 10028–10033.

Kuroha, T., H. Tokunaga, M. Kojima, et al. 2009. "Functional Analyses of LONELY GUY Cytokinin-Activating Enzymes Reveal the Importance of the Direct Activation Pathway in *Arabidopsis*." *Plant Cell* 21: 3152–3169.

Lu, H., A. S. Lema, M. Planas-Marques, A. Alonso-Diaz, M. Valls, and N. S. Coll. 2018. "Type III Secretion-Dependent and -Independent Phenotypes Caused by *Ralstonia solanacearum* in *Arabidopsis* Roots." *Molecular Plant–Microbe Interactions* 31: 175–184.

Mansfield, J., S. Genin, S. Magori, et al. 2012. "Top 10 Plant Pathogenic Bacteria in Molecular Plant Pathology." *Molecular Plant Pathology* 13: 614–629.

Miri, M., P. Janakirama, M. Held, L. Ross, and K. Szczyglowski. 2016. "Into the Root: How Cytokinin Controls Rhizobial Infection." *Trends in Plant Science* 21: 178–186.

Miyawaki, K., M. Matsumoto-Kitano, and T. Kakimoto. 2004. "Expression of Cytokinin Biosynthetic Isopentenyltransferase Genes in *Arabidopsis*: Tissue Specificity and Regulation by Auxin, Cytokinin, and Nitrate." *Plant Journal* 37: 128–138.

Miyawaki, K., P. Tarkowski, M. Matsumoto-Kitano, et al. 2006. "Roles of *Arabidopsis* ATP/ADP Isopentenyltransferases and tRNA Isopentenyltransferases in Cytokinin Biosynthesis." *Proceedings of the National Academy of Sciences of the United States of America* 103: 16598–16603.

Nakano, M., N. Omae, and K. Tsuda. 2022. "Inter-Organismal Phytohormone Networks in Plant–Microbe Interactions." *Current Opinion in Plant Biology* 68: 102258.

Naseem, M., N. Philippi, A. Hussain, G. Wangorsch, N. Ahmed, and T. Dandekar. 2012. "Integrated Systems View on Networking by Hormones in *Arabidopsis* Immunity Reveals Multiple Crosstalk for Cytokinin." *Plant Cell* 24: 1793–1814.

Naseem, M., M. Wolfling, and T. Dandekar. 2014. "Cytokinins for Immunity Beyond Growth, Galls and Green Islands." *Trends in Plant Science* 19: 481–484.

Reusche, M., J. Klaskova, K. Thole, et al. 2013. "Stabilization of Cytokinin Levels Enhances *Arabidopsis* Resistance Against *Verticillium longisporum*." *Molecular Plant-Microbe Interactions* 26: 850–860.

Shanks, C. M., J. H. Rice, Y. Zubo, G. E. Schaller, T. Hewezi, and J. J. Kieber. 2016. "The Role of Cytokinin During Infection of *Arabidopsis thaliana* by the Cyst Nematode *Heterodera schachtii*." *Molecular Plant–Microbe Interactions* 29: 57–68.

Siddique, S., Z. S. Radakovic, C. M. De La Torre, et al. 2015. "A Parasitic Nematode Releases Cytokinin That Controls Cell Division and Orchestrates Feeding Site Formation in Host Plants." *Proceedings of the National Academy of Sciences of the United States of America* 112: 12669–12674.

Siemens, J., I. Keller, J. Sarx, et al. 2006. "Transcriptome Analysis of *Arabidopsis* Clubroots Indicate a Key Role for Cytokinins in Disease Development." *Molecular Plant–Microbe Interactions* 19: 480–494.

Spallek, T., P. Gan, Y. Kadota, and K. Shirasu. 2018. "Same Tune, Different Song—Cytokinins as Virulence Factors in Plant–Pathogen Interactions?" *Current Opinion in Plant Biology* 44: 82–87.

Svolacchia, N., and S. Sabatini. 2023. "Cytokinins." *Current Biology* 33: R10–R13.

To, J. P., G. Haberer, F. J. Ferreira, et al. 2004. "Type-A *Arabidopsis* Response Regulators Are Partially Redundant Negative Regulators of Cytokinin Signaling." *Plant Cell* 16: 658–671.

Tokunaga, H., M. Kojima, T. Kuroha, et al. 2012. "*Arabidopsis* Lonely Guy (LOG) Multiple Mutants Reveal a Central Role of the LOG-Dependent Pathway in Cytokinin Activation." *Plant Journal* 69: 355–365.

Torres-Martinez, H. H., S. Napsucialy-Mendivil, and J. G. Dubrovsky. 2022. "Cellular and Molecular Bases of Lateral Root Initiation and Morphogenesis." *Current Opinion in Plant Biology* 65: 102115.

Vissenberg, K., N. Claeijs, D. Balcerowicz, and S. Schoenaers. 2020. "Hormonal Regulation of Root Hair Growth and Responses to the Environment in *Arabidopsis*." *Journal of Experimental Botany* 71: 2412–2427.

Wang, S., Q. Sun, L. Yang, Y. Zhu, Y. Yuan, and J. Hua. 2017. "A Role of Cytokinin Transporter in *Arabidopsis* Immunity." *Molecular Plant-–Microbe Interactions* 30: 325–333.

Yin, R., K. Xia, and X. Xu. 2023. "Spatial Transcriptomics Drives a New Era in Plant Research." *Plant Journal* 116: 1571–1581.

Yu, X., Z. Liu, and X. Sun. 2023. "Single-Cell and Spatial Multi-Omics in the Plant Sciences: Technical Advances, Applications, and Perspectives." *Plant Communications* 4: 100508.

Zhao, C., H. Wang, Y. Lu, et al. 2019. "Deep Sequencing Reveals Early Reprogramming of *Arabidopsis* Root Transcriptomes Upon *Ralstonia solanacearum* Infection." *Molecular Plant–Microbe Interactions* 32: 813–827.

Zhou, J. M., and Y. Zhang. 2020. "Plant Immunity: Danger Perception and Signaling." *Cell* 181: 978–989.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.