A genome-wide association study unravels cytokinin as a major component in the root defense responses against *Ralstonia solanacearum*

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Highlight

Using Genome-Wide Association Analysis we identify cytokinin as an important component in early root defense responses against the bacterial wilt pathogen *Ralstonia solanacearum*. 
Abstract

Bacterial wilt caused by the soil-borne pathogen *Ralstonia solancearum* is economically devastating, with no effective methods to fight the disease. This pathogen invades plants through their roots and colonizes their xylem, clogging the vasculature and causing rapid wilting. Key to preventing colonization are the early defense responses triggered in the host’s root upon infection, which remain mostly unknown. Here, we have taken advantage of a high-throughput *in vitro* infection system to screen natural variability associated to the root growth inhibition phenotype caused by *R. solanacearum* in Arabidopsis during the first hours of infection. To analyze the genetic determinants of this trait, we have performed a Genome-Wide Association Study, identifying allelic variation at several loci related to cytokinin metabolism, including genes responsible for biosynthesis and degradation of cytokinin. Further, our data clearly demonstrate that cytokinin signaling is induced early during the infection process and cytokinin contributes to immunity against *R. solanacearum*. This study highlights a new role of cytokinin in root immunity, paving the way for future research that will help understanding the mechanisms underpinning root defenses.

**Keywords:** Bacterial wilt, cytokinin, defense, GWAS, hormones, immune system, root, *Ralstonia solanacearum*, salicylic acid
Introduction

Plant hormones are extremely important for the regulation of the plant defense against pathogens (Pieterse et al., 2012). Several studies have shown that the accumulation of salicylic acid (SA) induces plant defense against biotrophic pathogens (Shigenaga & Argueso, 2016), whereas jasmonic acid (JA) and ethylene are essential against necrotrophs. The crosstalk between JA and abscisic acid (ABA) induces plant defense against herbivores and insects (Pieterse et al., 2012). The synergistic or antagonistic interaction between the different hormone signaling pathways enables the plant to fine-tune defense responses to the pathogen that are effective while minimizing damage or yield penalties (Pieterse et al., 2009).

Cytokinin is a plant hormone traditionally associated with plant growth and development, (Mok 1994; Sa et al., 2001; Wybouw and De Rybel, 2019) with an emerging role in plant immunity. Cytokinin has been shown to participate in defense against various plant pathogens, including fungi (Argueso et al., 2012; Gupta et al., 2020), bacteria (Choi et al., 2010; Naseem et al., 2012; Pieterse et al., 2012) and viruses (Clarke et al., 1998; Pogány et al., 2004). Furthermore, application of exogenous cytokinin results in an increase of callose production in Arabidopsis thaliana (henceforth, Arabidopsis) infected with Pseudomonas syringae or treated with the flagellin-derived defense elicitor flg22 (Choi et al., 2010). Tight regulation of cytokinin levels is essential to determine the precise signaling outcome. Treatments with low concentrations of exogenous cytokinin result in greater susceptibility to infection with the oomycete Hyaloperonospora arabidopsidis in Arabidopsis, and also to infection with Blumeria graminis in wheat. In contrast, treatments with higher levels of cytokinin increase resistance of plants to these and other pathogens (Argueso et al., 2012; Babosha, 2009; Gupta et al., 2020).

Importantly, cytokinin signaling in immunity is greatly intertwined with SA signaling. It has been observed that increased resistance to H. arabidopsidis induced by cytokinin treatment is mediated by SA accumulation and the activation of SA-dependent defense genes (Choi et al., 2010; Argueso et al., 2012). Mechanistically, it has been shown that the ARR2 (ARABIDOPSIS RESPONSE REGULATOR), a major transcription factor of the cytokinin signal transduction pathway, physically interacts with TGA3, a transcription factor from the SA signaling pathway (Choi et al., 2010). The interaction of these two transcription factors is...
regulated by NPR1 (NON-EXPRESSOR OF PATHOGENESIS-RELATED PROTEINS 1), leading to changes in PRI expression and plant immune status (Choi et al., 2010). More recently, it has been shown that cytokinin treatment of tomato leaves induces resistance against fungi in an SA-dependent manner. (Gupta et al., 2020). Although sparse, current evidence indicates that the crosstalk between cytokinin and SA signaling pathways is very important for plant immune responses (Choi et al., 2010; Argueso et al., 2012).

The majority of studies analyzing the role of cytokinin in plant defense have been performed using foliar pathogens (Clarke et al., 1998; Pogány et al., 2004; Choi et al., 2010; Pieterse et al., 2012; Naseem et al., 2012; Argueso et al., 2012; Gupta et al., 2020). In contrast, the role of cytokinin in root defenses remains mostly unexplored.

*Ralstonia solanacearum* is a natural soil-borne bacterial vascular pathogen that infects many plant species, including Arabidopsis, and it is the causative agent of bacterial wilt, a disease of devastating economic impact worldwide. *R. solanacearum* invades plants through the roots moving centripetally until it reaches the xylem. Xylem colonization allows movement of the bacteria up into the stem, causing a rapid and permanent obstruction of the vasculature (Hayward, 1991; Planas-Marquès et al., 2020). Many genetic tools are available to study this pathogen, since it has been widely used as a model species for plant-pathogen interactions in the last decades (Mansfield et al., 2012).

Transcriptomic analyses of Arabidopsis roots infected with the bacterial pathogen *R. solanacearum* show expression of cytokinin biosynthetic genes at early time points (6 hours post-infection) (Zhao et al., 2019), while cytokinin-degrading genes are expressed at later stages of infection (after 72 hours) in *Medicago truncatula* roots infected with *R. solanacearum* (Moreau et al., 2014). Interestingly, Arabidopsis plants lacking the redundant negative regulator of cytokinin signaling ARR6 showed increased resistance to the fungal pathogen *Plectosphaerella cucumerina* but increased susceptibility to *R. solanacearum* (Bacete et al., 2020). However, these phenotypes did not occur as a direct result of the interactions between cytokinin signaling and classical hormone-based defense pathways.

In previous work, we set up an in vitro system to study Arabidopsis early root phenotypes caused by *R. solanacearum* infection: root growth inhibition, root hair formation and root tip cell death (Lu et al., 2018). This robust method revealed genetic determinants of the
interaction both from the bacterial virulence and plant defense sides at very early stages of infection, which were masked in classic pathogenicity assays. Here, we have taken advantage of the high-throughput potential of this in vitro system to screen the natural variation of the root growth inhibition phenotype across 430 Arabidopsis accessions representative of the worldwide genetic variation of this species and determine the gene(s) responsible for this trait using Genome-Wide Association (GWA) mapping.

Thanks to the large number of Arabidopsis accessions that have been sequenced and genotyped, this model plant has great potential for GWAS analyses (Atwell et al., 2010a). Previous studies using GWAS and natural genetic variation have detected genetic variants associated with resistance to abiotic stress (Bac-Molenaar et al., 2015; Kalladan et al., 2017; Satbhai et al., 2017; Li et al., 2019), root development (Meijón et al., 2014), or flowering time (Aranzana et al., 2005). GWAS has also been shown to be a very powerful tool to unravel genomic regions associated with the natural variation of disease resistance of various plants against different pathogens, for example Arabidopsis against Pseudomonas syringae (Aranzana et al., 2005; Atwell et al., 2010; Iakovidis et al., 2016), Xanthomonas campestris (Huard-Chauveau et al., 2013) and Botrytis cinerea (Corwin et al., 2016; Thoen et al., 2016) or Glycine max against Fusarium virguliforme (Wen et al., 2014), among others. Importantly, GWAS has been recently used to study the temperature-dependent genetic variation that underscores resistance of Arabidopsis against Ralstonia solanacearum (Aoun et al., 2017). Finally, GWAS has also highlighted the importance of hormonal crosstalk between SA and ABA on the JA pathway involved in defense in Arabidopsis (Proietti et al., 2018). Taking advantage of GWAS, we have identified cytokinin signaling as an important component in the root growth inhibition phenotype caused by R. solanacearum in Arabidopsis, contributing to root defenses against the pathogen.
Material and methods

Plant material

A collection of 430 Arabidopsis thaliana ecotypes (Table S1) provided by the Molecular Plant Biology Stock from the Gregor Mendel Institute (Vienna, Austria) was used for GWAS. The Arabidopsis mutant lines used in this study have been previously described in Kiba et al., 2013 (cyp735a1, cyp735a2) and Caesar et al., 2011 (ahk2, ahk3, ahk4/cre1). Transgenic line TCSn::GFP has been described in Zürcher et al., 2013. The eds16 TCSn::GFP line was obtained by crossing the eds16 mutant (Dewdney et al., 2000) to the TCSn::GFP transgenic line and screening F2 plants for presence of TCSn::GFP by selection on MS plates supplemented with BASTA and for eds16 using PCR primers that can detect the eds16 mutation (eds16 Fwd: CCTGAGAGACTATTCCAAAGGAC; eds16 Rev: ACTCTGAAGATGGTCACTTCC). Homozygous seeds were used in all the assays.

Plant and bacteria growth conditions for GWAS.

Seeds were surface sterilized for 2 h in opened 1.5-mL Eppendorf tubes in a sealed box containing chlorine gas generated from 125 mL of 10% w/v sodium hypochlorite and 3.5 mL of 37% hydrochloric acid. For stratification, sterile seeds were kept at 4°C for 72 h in the dark. After that, seeds were put on agar plates containing MS (Duchefa Biochemie B.V., Haarlem, the Nederlands) and 0.8% Agar (Becton, Dickinson and Co., Franklin Lakes, NJ, USA). The placement of the seeds was guided by a printout of a seed-planting grid schematic (Fig. S1) placed below the plate. Each plate contained two accessions with six seeds per accession. To account for positional effects within and between the Petri dishes, we plated 12 seeds for each accession over two plates in a permuted block design. Plates were positioned in racks that oriented the plates in a vertical position to a growth chamber constantly kept at 21°C and a 16 h light/8 h dark cycle, with a light intensity of 120 μmol·m⁻²·s⁻¹ during the light period. Plants were inoculated as described in the section below “in vitro inoculation assays”.

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Image acquisition.
Root images were obtained using CCD flatbed scanners (EPSON Perfection V600 Photo, Seiko Epson CO., Nagano, Japan). The BRAT (Busch-lab Root Analysis Toolchain) image acquisition tool on a standard desktop computer running Ubuntu Linux allowed the simultaneous control of the scanners (Slovak et al., 2014). Scans were performed with a resolution of 1200 x 1200 dpi, resulting in an image size of 6000 x 6000 pixels (36 MP) for each of our 12 x 12-cm agar plates. To enhance image quality, scanning was performed in a dark room and with the scanner lid open.

Genome-Wide Association Mapping.
We measured median and mean total root length values of 430 Arabidopsis accessions after R. solanacearun infection using BRAT (n =12) to conduct GWA using an accelerated mixed model (EMMAX) (Kang et al., 2010) followed by EMMA (Kang et al., 2008) for the most significant associations among all accessions studied. The GWA was performed on a cluster, with algorithms identical to the ones used in the GWAPP Web interface (Seren et al., 2012). SNPs with minor allele counts greater or equal to 10 were considered. The significance of SNP associations was determined at 5% FDR threshold computed by the Benjamini-Hochberg-Yekutieli method (Benjamini and Hochberg, 1995).

Broad sense heritability calculation.
All individuals that were measured were used to calculate the broad-sense heritability (H2 = VG/VP), which is defined as the proportion of phenotypic variation (VP) due to genetic variation (VG) (estimated from the between-line phenotypic variance).

Gene ontology analysis.
The GO-finder website (https://go.princeton.edu/) was used for GO analysis. Genes solely "Inferred from Electronic Annotation associations" were excluded from the analysis.

In vitro inoculation assays.
Seeds were surface sterilized with a solution containing 30% bleach and 0.02% Triton-X 100 for 10 min, washed five times with Milli-Q water and sown (20 seeds/plate) on agar plates containing MS (Duchefa Biochemie B.V., Haarlem, the Nederlands) and 0.8% Agar (Becton, Dickinson and Co., Franklin Lakes, NJ, USA). Sown plates were stratified at 4°C in the dark
for two days. Plates were then transferred to chambers and grown vertically for 7 days under constant conditions of 21-22°C, 60% humidity and a 16h light/8h dark photoperiod with a light intensity of 120 μmol·m⁻²·s⁻¹ during the light period.

*R. solanacearum* GMI1000 was grown at 28°C in solid or liquid rich B medium (0.1% yeast extract, 1% bacto peptone and 0.1% casamino acids) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). For inoculation, *R. solanacearum* GMI1000 was collected by centrifugation (1,500 rcf, 5 min) from overnight liquid cultures at 28°C, resuspended with water and adjusted to a final OD₆₀₀ of 0.001 corresponding to 10⁶ CFU/ml. Arabidopsis seedlings grown on plates as detailed above were inoculated with 5 μl of the bacterial solution, which was applied 1 cm above the root tip, as described previously (Digonnet et al., 2012). Plates were then sealed with micropore tape (3M Deutschland GmbH, Neuss, Germany) and transferred to a controlled growth chamber at 25°C, 60% humidity and a 12h light/12h dark photoperiod with a light intensity of 120 μmol·m⁻²·s⁻¹ during the light period.

For the analysis of root growth inhibition and root hair formation, pictures were taken 48-72 hours post inoculation (hpi) with an Olympus DP71 stereomicroscope (Olympus, Center Valley, PA, USA) at 11.5x. To analyze GFP root expression, root from seedlings grown on plates were collected 48 hpi and photographed with an epifluorescence microscope Leica DM6 (Leica, Wetzlar, Germany). In order to quantify GFP fluorescence the Leica Application Suite X (LAS X) software was used. A 0.1 cm section of the maturation zone was selected and GFP intensity was quantified as relative units and presented as the average of all roots measured. Three independent biological replicate were performed and for each replica, 24 (Fig. 2) or 10 (Fig. 5) roots per condition were used.

**Exogenous cytokinin and salicylic acid application.**

For *R. solanacearum in vitro* root inoculation assays that included exogenous application of hormones, 7-day-old seedlings were transferred from MS agar plates to fresh MS agar plates supplemented with different hormone concentrations (25 and 50 nM Kinetin; 1.5 and 7.5 μM SA) from Duchefa Biochemie (Haarlem, the Netherlands). Roots were inoculated 24 hours later as described above.
Pathogenicity assays.

*R. solanacearum* pathogenicity tests were carried out using the soil-drench method (Monteiro et al., 2012). Briefly, Arabidopsis was grown for 4 weeks on Jiffy pots (Jiffy Group, Lorain, OH, USA) in a controlled chamber at 22°C, 60% humidity and an 8h light/16h dark photoperiod. Jiffys were drilled 3 times with a wooden stick and immediately submerged for 30 min into a solution of overnight-grown *R. solanacearum* adjusted to OD$_{600}$=0.1 corresponding to $10^8$ CFU/ml with distilled water (35 ml of bacterial solution per plant). Inoculated plants were transferred to trays containing a thin layer of soil drenched with the same *R. solanacearum* solution and kept in a chamber at 27°C, 60% humidity and 12h light/12h dark. Plant wilting symptoms were recorded every day and expressed according to a disease index scale (0: no wilting, 1: 25% wilted leaves, 2: 50%, 3: 75%, 4: death) (Figure S5). At least 30 plants per condition were used in each assay, and at least three replicates were performed for every experiment.

Quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR).

Roots were collected from *R. solanacearum*-infected or water-treated Arabidopsis plants at 0, 24 and 48 hpi. Briefly, roots from approximately 40 seedlings were cut and pooled. Roots were rapidly washed in water and dried before freezing in liquid nitrogen. Samples were stored at -80 °C. RNA was extracted using the Maxwell 16 LEV Plant RNA Kit (Promega, Australia) according to the manufacturer’s recommendations. RNAs were treated with DNase-free RNase (Promega, Australia) and the concentration measured with a ND-8000 Nanodrop. cDNA was synthesized from 2 µg RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to manufacturer’s instructions. According to the SYBR Green PCR mix instructions (Roche, Switzerland), 2.5 µL of cDNA were used in a final reaction volume of 10 µL in the LightCycler 480 System (Roche, Switzerland). Melting curves and relative quantification of target genes were determined using the software LightCycler V1.5 (Roche, Switzerland). The amplification program was set to an initial step of 10 min at 95°C followed by 45 cycles using 95°C for 10 seconds, 60°C for 30 seconds and 72°C for 30 seconds. All samples were run in triplicate for each biological replicate, and the target gene was normalized to the endogenous control gene Arabidopsis tubulin beta-1 chain (*At1g75780*). To visualize the data, we calculated the fold change of each biological replicate at 24 h and 48 h samples by normalizing to the ΔCt of time point 0 hpi of the mock and infected samples separately. The statistical analysis of the normalized data was
performed using “rstatix” R package (ver. 0.6.0). To test for differences in gene expression between mock and infected samples, the normalized data was tested for normality and homogeneity of variances. If these two requirements were fulfilled, the parametric t-test was performed for each time point to compare between mock and infected samples. All primer sequences used were obtained from previous publications and are listed in Table S6. qPCR analysis conforms to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009).

Cytokinin analysis (LC-MS/MS)

Arabidopsis plants were grown in pots with sand for 5 weeks in a controlled chamber at 22°C, 60% humidity and an 8h light/16h dark photoperiod. with a light intensity of 120 μmol·m⁻²·s⁻¹ during the light period. Sand from pots were drilled 3 times with a wooden stick and immediately irrigated with bacterial solution of overnight-grown R. solanacearum adjusted to OD₆₀₀=0.1 with distilled water (50 ml of bacterial solution per plant). Trays with plant-pots infected were transferred a chamber at 27°C, 60% humidity and 12h light/12h dark. Then, after 4-7 days post-inoculation, inoculated roots were washed with distilled water and dried with filter paper. After that, the root samples were weighed and stored at -80°C degrees. 4 biological replicates with 20 mg each was used for each time (0, 4 and 7dpi). Cytokinin levels were measured as described previously (Poitout et al., 2018).

Results

Genome-Wide Association mapping reveals several loci associated with cytokinin metabolism in Arabidopsis roots infected with R. solanacearum

Infection of Arabidopsis roots with R. solanacearum GMI1000 in vitro results in root growth inhibition. We previously observed natural variation of this phenotype across a small population of Arabidopsis accessions (Lu et al., 2018). To identify loci responsible for this natural variation we performed a Genome-Wide Association Study (GWAS) using a collection of 430 Arabidopsis accessions representative of the worldwide genetic variation of this species (Figure S1 and Table S1). Arabidopsis seeds were sown on agar plates following the scheme presented in Figure S1 to ensure randomization. After 7 days, seedlings were inoculated 1 cm over the root tip with a 5 μl droplet of a 10⁶ CFU/ml suspension of R.
solanacearum GMI1000. Images of seedlings were then acquired using scanners every day for 5 days to measure root length and to monitor impact on root growth caused by *R. solanacearum* infection *in vitro*. Differences in root length between accessions were monitored after infection and subsequently analyzed.

To identify sequence variation in genomic regions associated with the variation of the root growth inhibition phenotype caused by the *R. solanacearum* root infection, we conducted GWA mapping using the Arabidopsis 250K single nucleotide polymorphism (SNP) chip data (Horton *et al.*, 2012) with a mixed model correcting for population structure (Seren *et al.*, 2012) and the root growth data described in Table S2. Because we were interested in the root growth responses upon *R. solanacearum* root infection, we focused our analysis on root growth rates. The broad sense heritability (H^2) of these traits ranged from 10% to 55% with an average of 36% (Table S3). We observed 20 unique SNPs significantly associated with the root growth responses to *R. solanacearum* infection using a 5% Benjamini-Hochberg threshold (Table S4). The most significant of these associations (SNP 15401974, chromosome 5; P-value: 1.64*10^-9; FDR: 5.6*10^-5) was found for two root growth rate measurements: the mean of the relative root growth rate between day 2 and day 3 (Figure 1a) and the median of the relative root growth rate between day 4 and day 5 (Figure 1b). Because this SNP displayed the most significant p-value and was found in traits relating to two different days of the time course, we concluded that it might be important in explaining the root growth phenotypic variation between accessions. While this SNP is located within the 5 Kb upstream region of multiple genes (*At5g38450, and At5g38460*) (Figure S2), the highest level of linkage disequilibrium in any gene of this region with the top SNP can be observed for a SNP in the *At5g38450* gene (Pearson coefficient of correlation r=0.39). This gene encodes a cytokinin hydroxylase (*CYP735A1*) that catalyzes the biosynthesis of the cytokinin trans-zeatin (Takei, Yamaya, & Sakakibara, 2004a). Another analysis guided our focus towards the cytokinin pathway: when conducting a gene ontology enrichment analysis of genes in 10 Kb proximity of SNPs associated with root growth rate upon infection (EMMAX P-value < 10^-6) (Table S4), we found the process cytokinin catabolism to be significantly enriched (P-Value: 0.00025; FDR: 4.86%; Table S5). These included two additional genes associated with cytokinin metabolism are, the cytokinin oxidases *At2g19500 (CKX2)*, and *AT4G29740 (CKX4)*. *CKX2* is upstream of a SNP significantly associated with mean relative root growth rate between day 2 and day 3 (SNP 8436350; chromosome 2; P-value 8.89*10^-7; FDR=0.015) (Figure 1c) and *CKX4* is upstream of a SNP marginally associated with median
root growth rate between day 2 and day 3 (SNP 14577216; chromosome 4; PVAL $6.55 \times 10^{-7}$; FDR=0.101) (Figure 1d). Both genes encode for proteins that catalyze the degradation of cytokinins (Mok & Mok, 2001).

Next, we analyzed the level of expression of CYP735A1, CKX2 and CKX4 in Arabidopsis Col-0 roots. This ecotype was selected for further analysis because it has been widely used for pathogenicity assays using R. solanacearum, many genetic resources are available and it is susceptible to the widely-available GMI1000 strain, with a clearly observable root inhibition phenotype that appears at early stages of infection (Lu et al., 2018; Figure S3). Quantitative PCR was used to compare plants infected with R. solanacearum and mock-treated plants after 0, 24 and 48 hours post-treatment. R. solanacearum infection consistently induced expression of two of these three genes (CYP735A1 and CKX2) after 48 hours post-inoculation (hpi) (Figure 1e-g), indicating a potential involvement of cytokinin signaling in plant root defenses against this bacterial pathogen.

**Early R. solanacearum infection induces cytokinin signaling in Arabidopsis roots.**

Next, we analyzed whether R. solanacearum infection resulted in an increase of cytokinin content in Arabidopsis Col-0 roots. For this, we measured the levels of cytokinins trans-zeatin, cis-zeatin and Isopentenyladenine, as well as total cytokinins using LC-MS/MS. We could observe a significant increase in trans- and cis-zeatin, as well as total cytokinins after infection (Figure 2a-d). We could only detect significant increases at later stages of infection (4 and 7 dpi), probably due to the sensitivity constraints of the measurement method.

In order to more specifically investigate the early effects of R. solanacearum infection on root cytokinin signalling, we took advantage of a more sensitive approach by analyzing expression of the synthetic Arabidopsis cytokinin reporter TWO COMPONENT SIGNALING SENSOR new (TCSn) fused to GFP (TCSn::GFP) (Zürcher et al., 2013). Arabidopsis seedlings stably expressing TCSn::GFP were grown vertically on MS medium during 7 days and then, roots were inoculated with R. solanacearum. (see methods) Infection resulted in a strong induction of GFP expression driven by the cytokinin signaling reporter TCSn in the vasculature of the root maturation zone (Figure 2b-c). The intensity of the GFP induction caused by R. solanacearum infection at 48 hpi was 4 times higher than in the water control,
which clearly indicated that cytokinin signalling is engaged in root responses to *R. solanacearum* invasion (Figure 2d).

**Plants affected in cytokinin biosynthesis and perception display enhanced susceptibility towards *R. solanacearum*.**

If cytokinin levels and cytokinin signaling are important for root defense responses against *R. solanacearum*, it would be expected that impairment of cytokinin biosynthesis results in enhanced susceptibility to the pathogen. To address this question we performed pathogenicity assays on knock-out mutants of the cytokinin biosynthetic enzymes CYP735A1 and CYP735A2, which do not display any apparent phenotype (Kiba, Takei, Kojima, & Sakakibara, 2013). For this, 4-week-old Arabidopsis plants were inoculated with *R. solanacearum* GMI1000 by soil drenching and symptoms were evaluated over time following a disease index scale (Lu et al., 2018). Both *cyp735a1* and *cyp735a2* showed earlier wilting disease symptoms and were dramatically more susceptible to *R. solanacearum* than wild-type plants (Figure 3a). This clearly indicates that cytokinin biosynthesis is involved in defense responses against *R. solanacearum*.

Based on this, we hypothesized that cytokinin perception would be equally important for immune responses against *R. solanacearum*. To test this idea, we performed pathogenicity assays on knock-out mutants of the sensor histidine kinases AHK2, AHK3 and CRE1/AHK4, which act as cytokinin receptors (Higuchi et al., 2004; Ueguchi et al., 2001). All three cytokinin receptor mutants *ahk2*, *ahk3* and *cre1/ahk4*, which grow normally on soil (Higuchi et al., 2004; Ueguchi et al., 2001), displayed enhanced susceptibility to *R. solanacearum* infection (Figure 3b), indicating that perception of cytokinin is an important component of defense responses during *R. solanacearum* infection.

**Exogenous cytokinin application partially reverts *R. solanacearum*-induced early root phenotypes.**

Our next goal was to determine whether exogenous cytokinin application could counteract the effects caused by *R. solanacearum* infection using the *in vitro* early root phenotypes as a measurable output (root growth inhibition and root hair production) (Lu et al., 2018). For this, 7-day-old seedlings grown *in vitro* were transferred to fresh MS media supplemented
with different concentrations of the natural cytokinin kinetin (0, 25 and 50 nM). After 24 hours, roots were pin-inoculated with *R. solanacearum* 1 cm above the root tip and root growth inhibition and root hair production were monitored over time. Interestingly, kinetin supplementation (both 25 and 50 nM concentrations) resulted in partial reversion of the root growth inhibition phenotype caused by *R. solanacearum in vitro* (Figures 4a and b). Whereas untreated inoculated seedlings stopped growing 24 hpi, kinetin-treated inoculated seedlings kept growing, although to a lesser extent than non-infected roots. In addition, root hair production resulting from *R. solanacearum* infection was also inhibited by the kinetin pre-treatment (Figure 4c). This effect was more pronounced when using a higher kinetin dose (50 mM), were no root hairs were observed. In contrast, the lower dose resulted in delayed but visible root hair production. We can then conclude that kinetin pre-treatment at concentrations between 25 and 50 nM can alleviate the early root *in vitro* phenotypes caused by *R. solanacearum* infection, without causing toxicity on the plants. This indicates that cytokinin contributes to the onset of early responses that take place upon *R. solanacearum* infection in the root.

**Salicylic acid contributes to cytokinin signalling in Arabidopsis roots in response to *R. solanacearum* infection.**

A crosstalk between cytokinins and salicylic acid has been previously shown to regulate plant defenses against pathogens infecting leaves, such as *Pseudomonas syringae* (Choi et al., 2010), *Hyalopenospora arabidopsidis* (Argueso et al., 2012), *Botrytis cinerea* and *Oidium neolycopersici* (Gupta et al., 2020). However, whether a crosstalk between these hormones in root defenses takes place has not been determined. Previous *R. solanacearum* pathogenicity tests have not detected differences in susceptibility between wild type and SA-deficient plants (*sid2* mutant or *NahG* transgenic lines, carrying an SA-degrading enzyme) (Hirsch, Deslandes, Feng, Balagué, & Marco, 2002; Hernández-Blanco et al., 2007 and Hanemian et al., 2016). However, exogenously applied SA had a clear effect on the root phenotypes induced by *R. solanacearum* infection *in vitro*. Seven-day-old seedlings were transferred for 24 hours to MS media supplemented with different SA concentrations (0, 1, 5 and 7.5 µM). We observed that SA concentrations above 1 µM (5 and 7.5 µM) caused root growth inhibition of up to 50% of untreated seedlings (Figure S4a), as reported in previous studies (Pasternak et al., 2019). Therefore, we performed *R. solanacearum in vitro* inoculation assays only on seedlings pre-treated with 1 µM SA, which did not cause any obvious effect on root growth.
growth before inoculation. Seven-day-old seedlings were transferred to MS media supplemented with 1 µM SA and 24 hours later, roots were inoculated with *R. solanacearum* and monitored over time for root growth inhibition and root hair production. Exogenous application of 1 µM SA partially reverted the root inhibitory phenotype caused by *R. solanacearum* infection (Figure 5a). On the other hand, 1 µM SA did not have any significant effect on root hair production (Figure S4b). Root hair production was partly inhibited only when higher SA concentrations (5, 7.5 and 1 µM) were exogenously supplied prior to *R. solanacearum* infection (Figure S2b). Considering that these concentrations affect root growth under normal conditions (Figure S2a), root hair inhibition may be a pleiotropic growth/development phenotype derived from SA toxicity rather than the result of SA modulation of defense responses to *R. solanacearum*.

To ascertain whether SA contributes to the cytokinin signalling involved in the response of Arabidopsis Col-0 roots to *R. solanacearum* root infection, we tested if impairing SA signaling would result in a decrease of cytokinin signaling outputs. For this, we quantified expression of *TCSn::GFP* in transgenic lines in a wild-type or an *eds16* mutant background, which is impaired in SA biosynthesis upon pathogen challenge (Dewdney *et al.*, 2000). *TCSn::GFP* expression after infection is reduced when SA signaling is suppressed, compared to wild-type. This can be observed at the whole root level (Fig. 5b) and in a zoomed area of the root maturation zone (Fig. 5c). Fluorescence quantification shows that in *eds16* mutant plants, *TCSn::GFP* expression is 25% lower than in a *TCSn::GFP* wild-type background (Fig. 5d). This indicates that SA signaling affects cytokinin signaling in response to *R. solanacearum* infection, pointing towards a potential cytokinin-SA crosstalk occurring in roots in response to infection with soilborne pathogens. Further research in this area will clarify the possibility of a cytokinin-SA crosstalk during responses to pathogens in roots.

**Discussion**

**Role of cytokinin in the interaction between *R. solanacearum* and Arabidopsis**

In this study we have taken advantage of GWAS to understand the genetic nature of the root phenotypic changes induced by *R. solanacearum* on Arabidopsis roots during early stages of infection. GWAS has been previously used to understand the basis of resistance against *R. solanacearum* in Arabidopsis under different temperatures and inoculation conditions (Aoun
et al., 2017). The study by Aoun and colleagues used wilt disease index rates over time as a trait to underscore temperature-dependent genetic diversity. At lower temperatures (27°C) the main resistance QTL identified was RPS4/RRS1-R, a plant immune receptor pair with a very well-known role in resistance of Arabidopsis to R. solanacearum (Deslandes et al., 2002; Le Roux et al., 2015; Sarris et al., 2015). In addition to that, this study revealed a new potential susceptibility gene at higher temperatures (30°C) strictosidine synthase-like 4 (SSL4), which encodes for a protein with structural similarities to animal proteins involved in immunity (Aoun et al., 2017). This study highlights the power of GWAS in revealing new potential sources of resistance to be engineered in crops.

Our study focuses on the same Arabidopsis-R. solanacearum pathosystem but from a different angle. At the very early stages of infection (2-3 days post-inoculation) R. solanacearum infection results in quick root growth inhibition, root hair formation and root meristem cell death, which can be easily observed and screened in in vitro inoculation assays. We detected natural variation associated to these phenotypes among a small subset of accessions representative of Arabidopsis diversity (Lu et al., 2018). Based on that observation and on the fact that the initial stages of plant colonization by R. solanacearum are poorly understood, despite being important for establishment of the bacteria inside the plant, we took advantage of GWAS to analyze the genetic diversity associated to one of these traits: root growth inhibition. Root hair formation and root meristem cell death were not included in GWAS because the technology at hand did not allow precise measuring of these traits.

Using GWAS, we screened root growth inhibition at different time points after infection on a large number of Arabidopsis accessions and focused on three candidate loci in the close proximity to SNPs that are significantly associated with this phenotype (Fig. 1a-c). These three genes are involved in the metabolism of cytokinin: CYP735A1, in biosynthesis (Takei, Yamaya, & Sakakibara, 2004b), and CKX2 and CKX4, in cytokinin degradation (Mok & Mok, 2001). Gene expression analysis by qPCR showed that the expression of these genes in Col-0 roots was consistently induced by R. solanacearum at 48 hpi (Fig. 1e-f). Although the cytokinin-degrading genes CKX2 and CKX4 have not been investigated further in this work, they might participate in modulating the increased cytokinin levels in response to R. solanacearum infection.
Our data are in line with previous data underscoring a potential role of cytokinins in plant defense against *R. solanacearum*. RNA sequencing results showing induction of genes involved in cytokinin synthesis (*CYP735A2, LOG2* and *LOG6*), degradation (*CKX2, CKX3* and *CKX5*), and response regulation (*ARR3, ARR4, ARR5, ARR7* and *ARR16*) in Arabidopsis Col-0 roots at early time points after *R. solanacearum* infection (Zhao, Wang *et al.* 2019). Moreover, genes controlling cytokinin metabolism (*LOG* and *CKX*), signaling (*ARR*s) and perception (*CRE1*) have been shown to be differentially expressed in the susceptible A17 *Medicago truncatula* genotype roots after infection with *R. solanacearum* (Moreau *et al.*, 2014). Importantly, Arabidopsis plants deficient in ARR6 show altered cell wall composition and are more susceptible to infection with *R. solanacearum* (Bacete *et al.*, 2020). Interestingly, Aoun *et al.* (2017) found two cytokinin-related genes among their top SNPs obtained upon infection with *R. solanacearum* at high temperatures (30°C): the signal receptor AHK3 and the cytokinin response factor CRF2. In agreement with this, we have found *ahk3* knock-out mutants are more susceptible to *R. solanacearum* than the wild-type control (Fig. 3b).

Coupled to these increases in cytokinin-regulated gene expression, we observed an activation of cytokinin signaling in the root 48 hours after inoculation with *R. solanacearum*, as evidenced by expression of the reporter *TCSn::GFP* (Fig. 2e-g). Together, these data indicate that *R. solanacearum* triggers cytokinin production in the root by means of induction of gene expression of cytokinin biosynthetic genes, which is accompanied by activation of cytokinin signaling; in parallel, cytokinin degradation genes are upregulated, to ensure a timely response and a tight regulation of cytokinin levels in the plant as has been described in the literature (Rashotte *et al.*, 2003; Brenner *et al.*, 2012).

Furthermore, we could observe an increase of cytokinin levels in the root after infection (Fig. 2a-d), albeit at later stages of infection, since at early stages reliable detection was challenging. When assessing which cytokinin forms were most abundant, we could detect that *R. solanacearum* resulted in an increase in both *trans*- and *cis*-zeatin levels, whereas the levels of isopentenyladenine did not show significant changes. The fact that *trans*-zeatin was among the most abundant forms was not surprising, since it is one of the most active forms of cytokinin in plants (Sakakibara, 2006). In contrast, *cis*-zeatin has always been regarded as an isomer with lower activity in plants. In fact, the study of *cis*-zeatin in the context of plant-pathogen interactions has only been addressed in the *Nicotiana tabacum-Pseudomonas*
syringae pathosystem, where the exogenous addition of this isomer promotes the resistance of the plant against the pathogen (Großkinsky et al., 2013). Our data indicate that cytokinin may play a role in root defense against *R. solanacearum*, with *cis*- and *trans*-zeatin as two potentially important cytokinin forms for this defense function.

Taking advantage of the genetic resources available for Arabidopsis, we tested whether cytokinin synthesis and/or perception participated in defense against *R. solanacearum*. For this, we carried out pathogenicity tests, comparing a variety of mutants with defects in cytokinin perception (*ahk2*, *ahk3* and *ahk4/cre1*) and biosynthesis (*cyp735a1* and *cyp735a2*). Our data clearly showed that both cytokinin synthesis and perception participate in defense against *R. solanacearum*, as defects in either pathway result in enhanced susceptibility towards the pathogen (Fig. 3).

Furthermore, application of low concentrations of the cytokinin kinetin partially reversed the phenotypes caused by *R. solanacearum* infection in Arabidopsis roots (Fig. 4). A plausible explanation could be that exogenous cytokinin application induces the expression of defense-related genes in the root, as we have shown here (Fig. 1f-g) similar to what has been previously reported for leaves (Rashotte et al., 2003; Choi et al., 2010; Argueso et al., 2012). In fact, high doses of cytokinin were shown to induce resistance in Arabidopsis against the oomycete *H. arabidopsidis* (Argueso et al., 2012), against *P. syringae* in Arabidopsis (Choi et al., 2010) or even against virus replication in *Phaseoulus vulgaris* (Clarke et al., 1998). In our root system, low doses of cytokinin were sufficient to partially prevent the root phenotypes caused by *R. solanacearum*. We did not use high doses of cytokinin because they have been shown to inhibit primary root (To et al., 2004; Argyros et al., 2008) growth.

**The impact of SA on the cytokinin signalling involved in the response of Arabidopsis roots to *R. solanacearum* infection.**

Previous research, performed mostly in shoot tissue, showed that the role of cytokinins in plant immunity is deeply related to SA signaling, with a clear crosstalk between the two signaling pathways taking place (Choi et al., 2010; Argueso et al., 2012; Gupta et al., 2020). Choi et al. demonstrated that the SA-dependent TGA3 transcription factor binds to the response regulator ARR2, which is modulated by cytokinin signaling, to generate a complex that binds to the PR1 promoter and promotes defense against *P. syringae* (Choi et al., 2010).
Also, it has been shown that cytokinin regulates plant immunity against the oomycete *H. arabidopsidis* through the elevation of defense responses that are dependent on SA (Argüeso *et al.*, 2012).

Here, we addressed whether SA had any impact on cytokinin signalling induced as part of the defense responses against root-invading pathogens and root immunity. Although the SA-deficient plants show the same level of susceptibility to *R. solanacearum* as the wild type (Hirsch, Deslandes, Feng, Balagué, & Marco, 2002; Hernández-Blanco *et al.*, 2007 and Hanemian *et al.*, 2016), previous reports indicate that SA may participate in defense against this pathogen. For instance, RRS1-R-mediated defense in Arabidopsis ecotype Niederzenz-1 is orchestrated by SA (Deslandes *et al.*, 2002). Additionally, SA partly contributes to the enhanced tolerance to *R. solanacearum* observed in the Arabidopsis mutant *wat1* (*WALLS ARE THIN1*) (Denancé *et al.*, 2012). Further, SA participates in defense of other plant species to *R. solanacearum*, such as in tomato resistant varieties Hawaii 7996 and CRA 66 (Baichoo & Jaufeerally-Fakim, 2016) and tobacco (Lowe-Power *et al.*, 2016; Liu *et al.*, 2017).

It has been previously reported that treatments with SA or its analog BTH (benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester) are potent activators of plant defenses against various pathogens both in leaves (Achuo *et al.*, 2003; Herman *et al.*, 2008; War *et al.*, 2011; Azami-Sardooei *et al.*, 2013; Bektas & Eulgem, 2015; Kouzai *et al.*, 2018) and in roots (Attard *et al.*, 2010; Chuberre *et al.*, 2018). We found that exogenous SA application results in a partial reversion of the *in vitro* root phenotypes caused by *R. solanacearum* infection (Fig. 5a), similar to that we observed after cytokinin treatment (Fig. 4a-b). Our results indicate that SA might also contribute to root defenses against *R. solanacearum* at early stages of infection.

Although evidence is still limited, our results point towards the existence of a SA-cytokinins crosstalk in Arabidopsis roots after infection, since *R. solanacearum*-triggered expression of the cytokinin marker *TCSn::GFP* is significantly reduced by SA depletion in the *eds16* mutant when compared to wild type plants (Fig. 5b-d). These data demonstrate that in the context of root infection, SA levels affect cytokinin signaling and in turn, cytokinin signaling could modulate SA levels, although evidence proving the effects of cytokinin on SA signaling is still limited in this system. This indicates that crosstalk between the cytokinin and SA pathways in response to pathogens could also take place in response to *R. solanacearum*.
in roots and might affect defense response outcomes. Whether cytokinins, SA and their crosstalk have a more general role in immunity against root-invading pathogens remains will be interesting to explore in the future.

Together, our data demonstrate that cytokinins participate in defense against *R. solanacearum* and are involved in the early root phenotypes caused by the pathogen at early stages of infection. While it is known that cytokinins plays a very important role in defense against bacteria, fungi or viruses (Clarke *et al.*, 1998; Pogány *et al.*, 2004; Choi *et al.*, 2010; Groβkinsky *et al.*, 2011; Argueso *et al.*, 2012), our findings highlight a novel role of cytokinin in root immunity. Defenses in the root remain vastly unexplored and our study adds evidence indicating that pathogen perception in the root activates cytokinin metabolism and signaling, which modulates plant immunity contributing to plant defense.
Acknowledgements

We are grateful to Hitoshi Sakakibara (RIKEN Center, Yokohama, Japan) for the *cyp735a1* and *cyp735a2*; to Klaus Harter (Center for Plant Molecular Biology, Tübingen, Germany) for *ahk2, ahk3* and *ahk4/cre1*. We thank Saul Lema and Marc Planas-Marquès for inspiring discussions on the topic. We thank Ling Zhang (Salk Institute) for the calculation of broad sense heritability estimates and Martí Bernardo (CRAG) for his help with data visualization and statistics. This work was supported by the Spanish Ministry of Economy and Competitiveness with grant numbers RyC 2014-16158, AGL2016-78002-R and PID2019-108595RB-I00/AEI/10.13039/501100011033 to N.S.C. and through the “Severo Ochoa Programme for Centres of Excellence in R&D” (SEV-2015-0533). We also acknowledge financial support from the CERCA Programme/Generalitat de Catalunya, from the Austrian Academy of Sciences through the Gregor Mendel Institute (WB, SBS) and from funding for CTA and HMB by the United States Department of Agriculture (USDA) grant number COL00781.

Author contribution

A. A-D Designed the research, performed the research, analyzed and interpreted data and wrote the manuscript.
S.B.S. Performed the research, analyzed data and edited the manuscript.
R. dP.-J. Performed the research and analyzed data.
H.M.B. Performed the research, analyzed data and edited the manuscript.
C.G. Performed the research and analyzed data.
C.T.A. Designed the research, interpreted data and edited the manuscript.
O.N. Performed the research and analyzed data.
W.B. Designed the research, interpreted data and edited the manuscript.
M.V. Designed the research, analyzed and interpreted data and edited the manuscript.
N.S.C. Designed the research, analyzed and interpreted data and wrote the manuscript.
**Data availability statement**

The data that support the findings of this study are available from the corresponding author, N.S.C., upon reasonable request.
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Figure legends

Figure 1: GWA analysis reveals association of cytokinin metabolism genes with root growth inhibition caused by *R. solanacearum* infection of *Arabidopsis* roots. a-d) Manhattan plots of GWA results for root growth traits. Different colors represent different chromosomes. The horizontal dashed lines correspond to a nominal $P < 0.05$ significance threshold after Benjamini-Hochberg correction. Solid red boxes highlight the SNPs with the highest $P$-values in: a) mean relative root growth rate between day 2 and day 3 ($P$-value $2.42 \times 10^{-7}$), b) median root growth rate between day 2 and day 5 ($P$-value $1.64 \times 10^{-9}$), c) mean relative root growth rate between day 2 and day 3 ($P$-value $8.89 \times 10^{-7}$) and c) median root growth rate between day 2 and day 3 ($P$-value $6.55 \times 10^{-7}$). Fold change values of the quantitative PCR analysis of e) *CYP735A1*, f) CKX2, g) CKX4 using *TUBULIN* as control. Asterisks indicate statistically significant differences between 48 and normalizing by the values of the time point control (0 hours post-inoculation (hpi)). The asterisk indicates statistical significance ($p$) in a paired Student's t-test (* corresponds to a $P$-value < of $p < 0.05$) following the parametric t-test analysis. ** corresponds to a $P$-value of $p < 0.01$ and *** corresponds to a $P$-value of $p < 0.001$). Twelve plants were used for each condition.

Figure 2: Early *R. solanacearum* infection induces cytokinin signaling in *Arabidopsis* Col-0 roots. Four week old Col-0 plants were inoculated with *R. solanacearum* and a) total cytokinin, b) *trans*-zeatin, c) *cis*-zeatine and d) isopentenyladenine concentrations were analyzed in inoculated root tissues at the indicated times (0, 4 and 7 dpi) by LC-MS/MS using four biological replicates. Error bars correspond to standard errors. Asterisks indicate statistically significant differences between 4 and 7 dpi versus the control (0 dpi) in a paired Student's t-test (*** corresponds to a $P$-value of $p < 0.001$). (e-g) Six-day-old seedlings stably expressing the cytokinin signaling marker *TCSn::GFP* were inoculated with *R. solanacearum* or water, and 48 hours post-inoculation roots were observed under an epifluorescence microscope. *TCSn::GFP* signal in e) whole roots and f) root maturation zone. g) Quantification of the fluorescence intensity on the maturation zone (f) corresponds to the average GFP intensity from 24 individual roots per condition, calculated using the LAS X software. The experiment was repeated 3 times with similar results. Error bars correspond to standard errors. Asterisks indicate statistically significant differences between 48 hpi versus the control (0 hpi) in a paired Student's t-test (** corresponds to a $P$-value of $p < 0.01$).

Figure 3: Cytokinin biosynthesis and perception are important for the plant response against *R. solanacearum* root infection. Four-week-old plants were soil-drench inoculated with *R. solanacearum* and symptoms were measured over time using a disease index on a scale
of 1 to 4 (0 = no wilting, 1 = 25% wilted leaves, 2 = 50%, 3 = 75%, and 4 = death). a) Wild-type Col-0 and trans-zeatin biosynthesis mutants cyp735a1 and cyp735a2, b) Wild-type Col-0 and cytokinin sensor histidine kinase mutant genes (ahk2, ahk3 and ahk4/cre1). Each experiment was repeated at least three times obtaining similar results, using twenty-four plants per experiment. Error bars correspond to standard errors. Asterisks indicate statistically significant differences between wildtype and different mutant line in a paired Student's t-test (* corresponds to a P-value of p < 0.05, ** corresponds to a P-value of p < 0.01 and *** corresponds to a P-value of p < 0.001).

**Figure 4: Exogenous cytokinin application partially reverts R. solanacearum-induced early root phenotypes.** Seven-day-old Col-0 seedlings were grown for 24 hours in MS medium supplemented with kinetin (0, 25 and 50 nM) and were inoculated with R. solanacearum or water. a) An image of representative plants were obtained using a stereoscope 72 hours after infection or mock treatment, b) root growth was measured at the indicated time points and c) Images of representative roots were obtained using a stereoscope at the indicated time points after infection. Error bars correspond to standard errors. Asterisks indicate statistically significant differences between 24, 48 and 72 hpi in a paired Student's t-test (** corresponds to a P-value of p < 0.01 and *** corresponds to a P-value of p < 0.001).

**Figure 5: SA contributes to cytokinin signalling in roots in response to R. solanacearum infection.** a) Seven-day-old Col-0 wild-type seedlings were grown for 24 hours in MS medium supplemented with SA (0 and 1 µM) and were then inoculated with R. solanacearum. Root growth was measured. Error bars correspond to standard errors. Asterisks indicate statistically significant differences between 24, 48 and 72 hpi in a paired Student's t-test (** corresponds to a P-value of p < 0.01). b-d) Six-day-old seedlings stably expressing the cytokinin signaling marker TCSn::GFP in Col-0 and eds16 background were inoculated with R. solanacearum, and 48 hours post-inoculation roots were observed under an epifluorescence microscope. TCSn::GFP signal in b) whole roots and c) root maturation zone. This experiment was performed two times using 10 plants per genotype in each experiment. d) Quantification of the fluorescence intensity on the maturation zone (c) corresponds to the average GFP intensity from 10 individual roots per condition calculated using the LAS X software. The experiment was repeated 3 times with similar results. Error bars correspond to standard error. Asterisks indicate statistically significant differences between 48 hpi versus the control (0 hpi) in a paired Student's t-test (** corresponds to a P-value of p < 0.01).
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