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Cytokinins mediate resistance against *Pseudomonas syringae* in tobacco through increased antimicrobial phytoalexin synthesis independent of salicylic acid signalling

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ABSTRACT

Cytokinins are phytohormones that are involved in various regulatory processes throughout plant development, but are also produced by pathogens and known to modulate plant immunity. A novel transgenic approach enabling autoregulated cytokinin synthesis in response to pathogen infection showed that cytokinins mediate enhanced resistance against the virulent hemibiotrophic pathogen *Pseudomonas syringae* pv. *tabaci*. This was confirmed by two additional independent transgenic approaches to increase endogenous cytokinin production and by exogenous supply of adenine and phenylurea derived cytokinins. The cytokinin-mediated resistance strongly correlated with an increased level of bactericidal activities and up-regulated synthesis of the two major antimicrobial phytoalexins in tobacco, scopoletin and capsidiol. The key role of these phytoalexins in the underlying mechanism was functionally proven by the finding that scopoletin and capsidiol substitute *in planta* for the cytokinin signal: phytoalexin pre-treatment increased resistance against *P. syringae*. In contrast to a cytokinin defence mechanism in *Arabidopsis thaliana* based on salicylic acid dependent transcriptional control, the cytokinin-mediated resistance in tobacco is essentially independent from salicylic acid and differs in pathogen specificity. It is also independent of jasmonate levels, reactive oxygen species and high sugar resistance. The novel function of cytokinins in the primary defence response of solanaceous plant species is rather mediated through a high phytoalexin/pathogen ratio in the early phase of infection, which efficiently restricts pathogen growth. The implications of this mechanism for the co-evolution of host plants and cytokinin producing pathogens and practical application in agriculture are discussed.
INTRODUCTION

Phytohormones affect various aspects of growth and differentiation in higher plants and are involved in both biotic and abiotic interactions. Among the plant hormones, ethylene (ET), salicylic acid (SA) and jasmonate (JA) are known for differentially regulating defence responses against biotrophic and necrotrophic pathogens, and are considered as the immunity hormones (Bari and Jones, 2009; Tsuda and Katagiri, 2010). In contrast, only recently the involvement of other hormones in plant pathogen interactions has been recognized. In particular, a crosstalk of abscisic acid, auxin, and gibberellin signalling with the SA-JA-ET backbone of the defence signalling network has been elucidated in Arabidopsis thaliana (Arabidopsis) (Grant and Jones, 2009; Pieterse et al., 2009).

The aminopurine derived cytokinins (CKs) regulate many physiological and developmental processes in higher plants including cell division, leaf senescence, nutrient mobilisation, apical dominance and seed germination (Sakakibara, 2006). Cytokinins are also produced by a range of various microbial pathogens including Pseudomonas syringae (Akiyoshi et al., 1987; Morris et al., 1991) and leaf-mining insects (Engelbrecht, 1968) causing the formation of green islands, galls, growth abnormalities, and manipulation of primary carbon metabolism (Balibrea Lara et al., 2004). For several pathogens it was shown this CK production is essential for infection (Crespi et al., 1994; Hwang et al., 2010). Based on the induction of sink metabolism by CKs (Ehneß and Roitsch, 1997; Walters et al., 2008), it has been suggested that the host physiology is altered in response to CKs to allow the pathogen maximum access to nutrients early in (hemi-) biotrophic interactions (Walters et al., 2008). In contrast, the role of plant derived CKs in defence responses against pathogens that are unable to produce CKs is largely unknown. Elevated CK levels were shown to lead to resistance against different viruses (Sano et al., 1996; Pogany et al., 2004), suppression of hypersensitive responses (HR) (Barna et al., 2008) and the induction of SA in the wounding response (Sano et al., 1996). Only recently, CKs were shown to promote resistance in the model pathosystem Arabidopsis - Pseudomonas syringae pv. tomato DC3000 via TGA3/NPR1-dependent SA signalling (Choi et al., 2010).

The role of CKs in defence responses has been addressed by exogenous CK supply or by different functional approaches involving ectopic expression of CK
biosynthetic genes. However, interpretation of the available literature is complicated by the choice of promoters used for the transgenic approaches: (1) application of constitutive promoters to drive the expression of the bacterial ipt gene dramatically alters growth, development and the metabolic state of the host plant, and thus indirectly affects the interaction (Sakakibara, 2006), (2) application of inducible promoters required stimuli also known to interfere with defence responses such as light (Chandra-Shekara et al., 2006; Griebel and Zeier, 2008) and wounding (Sano et al., 1996). To overcome these limitations we addressed the role of endogenous CK levels in pathogen responses by expression of the ipt gene from the Agrobacterium tumefaciens T-DNA, encoding the rate-limiting enzyme in CK biosynthesis, driven by the synthetic pathogen inducible 4xJERE promoter (Rushton et al., 2002) for autoregulated CK production in response to infection by the hemibiotrophic bacterium P. syringae pv. tabaci (Pst). Local pathogen dependent increased CK levels at the infection site greatly enhanced resistance against Pst in tobacco, which was further substantiated by two alternative independent transgenic approaches to modulate endogenous CK biosynthesis and two different ways of exogenous CK application. This CK induced immunity in tobacco is essentially independent from SA signalling and unrelated to SA levels, and also does not confer resistance against necrotrophic fungi, which is in contrast to the SA dependent transcriptional regulated CK-mediated resistance mechanism in Arabidopsis directed against P. syringae pv. tomato (Choi et al., 2010). The CK induced resistance in tobacco is predominantly mediated by induction of the major antimicrobial phytoalexins of tobacco, scopoletin and capsidiol, resulting in a high phytoalexin/pathogen ratio in the early phase of infection, which efficiently restricts pathogen growth.
RESULTS

Induction of endogenous CK biosynthesis in tobacco leaves enhances resistance against the virulent \emph{P. syringae pv. tabaci}

The hemibiotrophic bacterium \emph{Pst} is infectious for various tobacco cultivars (\emph{Nicotiana tabacum}), visible as chlorotic leaf spots that turn into dark brown to black necrosis. To address the role of CKs in the pathosystem tobacco – \emph{Pst}, we engineered a novel autoregulated system for the pathogen dependent induction of endogenous CK levels in plants. The synthetic pathogen inducible 4x\emph{JERE} promoter (Rushton et al., 2002) was used to drive the expression of the bacterial \emph{ipt} gene from the \emph{A. tumefaciens} T-DNA, encoding isopentenyl transferase that catalyses the rate-limiting step in CK biosynthesis (4x\emph{JERE}:\emph{ipt}). This promoter comprises four repetitions of a 24 bp element and was shown to be jasmonate (J) and elicitor (E) responsive and strongly induced by various pathogens and wounding (Rushton et al., 2002). The following experimental scheme was carried out for local pathogen induced CK production in tobacco leaves (Fig. 1A): \emph{N. tabacum} cv. W38 leaves were pre-infiltrated with \emph{A. tumefaciens} strain LBA4404 harbouring either the 4x\emph{JERE}:\emph{ipt} construct or an empty vector. After 24 h, the infiltrated spots were subjected to a second infiltration either with \emph{PstT} (see methods) or with MgCl$_2$ as mock infection. RNA gel blot analyses revealed a pathogen dependent induction of the \emph{ipt} transgene (Fig. 1B). Higher CK concentrations in the corresponding \emph{ipt} expressing regions corroborated functional transgenic expression (Supplemental Table S1).

In \emph{ipt} expressing regions challenged with \emph{PstT}, pathogen resistance was markedly enhanced. Even 10 days past infection (dpi) only very mild symptoms developed in infiltrated regions (Fig. 1C), while control infiltrations (empty vector) showed disease symptoms already 2 dpi after infection and in many cases the complete infiltrated tissue became necrotic (Fig. 1C). When leaves entered senescence, typically after a period of three to four weeks, the control leaf halves turned completely necrotic, whereas their \emph{ipt} expressing counterparts retained chlorophyll and were clearly visible as green islands, further supporting the stimulation of CK biosynthesis by the 4x\emph{JERE}:\emph{ipt} construct (Fig. 1C). Therefore, induction of \emph{ipt} expression at the site of infection is highly effective in preventing disease development.
Since it has been shown that *A. tumefaciens* may interfere with symptom development after *P. syringae* infection (Rico et al., 2010), we employed two additional transgenic approaches, which confer ectopic elevation of endogenous CK levels independent of prior *A. tumefaciens* infiltration, to confirm the specificity of the observed CK effect. First, we used SAG12:ipt transgenic tobacco plants expressing the *ipt* gene under control of a senescence-induced promoter (Gan and Amasino, 1995). Detached transgenic leaves were subjected to a heat shock to induce senescence and thus to stimulate CK biosynthesis, which were subsequently infiltrated with *PstT*. In such leaves visible symptoms always developed much later and were clearly less pronounced than in the corresponding wild-type leaves (Fig. 1D). Second, we studied development of disease symptoms in *TET:ipt* transgenic tobacco plants expressing *ipt* under control of a chemically-inducible, tetracycline dependent promoter (Redig et al., 1996). Transgenic *TET:ipt* leaves were subjected to local pre-infiltration with tetracycline to induce *ipt* expression or a corresponding mock infiltration which were subsequently infiltrated with *PstT*. Disease symptoms appeared 2 dpi only at mock-treated sites but not at the sites of tetracycline-mediated CK biosynthesis. In tetracycline treated spots weak symptoms could be detected only 7 dpi, whereas mock induced sites were already highly necrotic (Fig. 1E).

To determine the influence of altered CK levels on *PstT* proliferation we re-isolated bacteria from wild-type tobacco leaves following *PstT* infection according to Figure 1A. As shown in Figure 1F, the number of bacteria started to increase strongly 12 h past infection (hpi) in the control incubation, whereas they remained low in samples with elevated CK levels. Therefore, the reduced disease development correlated both with the induction of CK biosynthesis and restricted pathogen growth. Thus, three independent transgenic approaches involving the ectopic induction of *ipt* expression in leaves by different mechanisms clearly demonstrated that the defence response is mediated by induced CK biosynthesis.

**Exogenous supply of CKs also enhances resistance of tobacco against *P. syringae* pv. *tabaci***

In addition to the three transgenic approaches, we tested the effect of feeding varying concentrations (1-18 µM) of kinetin, which is a natural occurring adenine
derived CK (Ge et al., 2005), to detached leaves via petioles for 24 h, followed by infection with *PstT* according to Figure 2A. Kinetin application enhanced the resistance against *PstT* infection in 86% of all treated leaves (275 out of 319; Fig. 2B) and in 95% of the leaves treated with 10 µM kinetin (144 out of 151), which was superior in preventing pathogen proliferation. Both lower and higher doses were less effective, with higher kinetin concentrations (14 µM and 18 µM) causing wilting. This is in agreement with the known CK effect on stomata opening (Meidner, 1967), which apparently increased host sensitivity towards the pathogen, interfering with the positive kinetin effect on pathogen resistance. These data demonstrate that exogenous CK feeding for 24 h enhanced resistance in a dose dependent manner.

Next we analysed whether shorter pulses of kinetin pre-treatment are sufficient. Kinetin (10 µM) was fed to detached leaves for 0.5 h, 1 h, 2 h and 4 h, followed by infection with *PstT* according to Figure 2A. Supplying a kinetin pulse for 4 h was already sufficient to decrease symptom development in 75% of the infected leaves (18 out of 24; Fig. 2C). Shorter pre-treatments were less effective, but still led to about 25% leaves with reduced symptoms (Fig. 2C).

To assess whether the time point of CK treatment relative to the time of infection is critical, the effect of feeding kinetin 24 h prior to infection according to the standard protocol (Fig. 2A) was compared with delayed CK pulses: feeding simultaneously with the time of infection, or 24 h and 48 h thereafter, according to Figure 3A. Figure 3B shows that CK feeding simultaneously with the infection (0 hpi) was as efficient as the 24 h CK pre-treatment (24 hbi) with 77% (23 out of 30) of the infected leaves showing decreased symptom development. A delayed CK application was still effective, although it resulted in a time dependent lower degree of resistance. The number of resistant leaves dropped to 67% (20 out of 30; 24 hpi) and 55% (16 out of 29; 48 hpi), respectively.

For possible practical applications it is important to determine how long the protection of a single CK treatment lasts. Therefore, we fed leaves with 10 µM kinetin for 24 h and infected either immediately thereafter according to the standard protocol, or two days, four days and seven days after the end of the kinetin pulse, according to Figure 3C. Figure 3D shows that the degree of protection gradually decreased upon delayed *PstT* infection following CK pre-treatment. Compared to the standard protocol (0 dpc), 91% of the infected leaves (21 out of 23) showed reduced symptom development when the infection took place 2 days (2 dpc) after the end of the CK
treatment, whereas 57% of the leaves (13 out of 23) still showed reduced symptom
development when leaves were infected even 4 days (4 dpc) and 7 days (13 out of
23; 7 dpc) past the kinetin pulse. Thus, a single kinetin pulse is sufficient to confer at
least a limited resistance for a complete week.

The effect of kinetin was compared with other adenine type CKs (Fig. 2D) to
investigate the specificity of the type of CK in mediating the increased resistance.
Both trans-zeatin and 6-benzyladenine were as efficient as kinetin, each resulting in
97% of resistant leaves, whereas control incubations with adenine were not
effective. Phenyurea derived compounds such as thidiazuron (TDZ) are also known
to exhibit CK activity (Bruce and Zwar, 1966) and 84% of all TDZ treated leaves (96
out of 114) displayed highly reduced symptoms compared to control incubations.
Figure 2E shows that concentrations as low as 2 nM TDZ were sufficient to induce
immunity and a concentration of 200 nM TDZ induced resistance in virtually all
treated leaves (96%, 24 out of 25). Therefore, TDZ proved to be even more potent
than the adenine type CKs.

To address the possible transfer of the identified CK-mediated resistance
mechanism to crop species via application methods that are feasible in agriculture
and do not require transgenic plants, we tested whether the resistance can be
induced by supplying CKs via the leaf surface. Kinetin was supplied by dipping one
leaf half into a kinetin solution (140 \( \mu \)M) (Mothes, 1960) for only 60 to 90 s, whereas
the second leaf half was left untreated as control. This alternative way of kinetin
application reduced symptom development in 81% of the dipped leaf halves (21 out
of 28) compared to non-treated halves (Fig. 2F). Although kinetin supply via the leaf
surface is not as efficient as petiole feeding of detached leaves, a highly elevated
level of resistance could be obtained, demonstrating a high potential for practical
application. Likewise, spraying 140 \( \mu \)M of kinetin to single detached leaves resulted
in resistance against \( PstT \).

To gain insight into the specificity of the CK-mediated resistance mechanism,
the effect of CKs on additional pathosystems was tested. According to Figure 2A
leaves were fed with kinetin for 24 h and infected with the avirulent pathogen \( P.
syringae \) pv. \( phaseolicola \) (\( Psp \)). No effect of the CK pre-treatment on HR
development was observed (Supplemental Fig. S1A). Likewise the number of \( Psp \)
bacteria present in the tissue was not affected. In addition, the spread of the
necrotrophic fungus \( Botrytis cinerea \), is strongly enhanced in CK pre-treated leaves,
evident from the formation of extended necrotic areas (Supplemental Fig. S1B), whereas susceptibility towards *Sclerotinia sclerotiorum* was not affected by the elevated CK levels (Supplemental Fig. S1C). Thus, in contrast to the CK-mediated resistance against the hemibiotrophic bacterium *PstT*, elevated CK levels do not enhance resistance against the two tested necrotrophic fungi. In the case of *B. cinerea*, CK treatment rather had the opposite effect and even enhanced susceptibility of tobacco.

The experiments involving exogenous CK application provide independent and complementing experimental support that CKs in tobacco mediate resistance specifically towards *PstT* in a dose dependent manner, with the possibility for easy transfer of this technology to crop species.

**The CK-mediated resistance mechanism in tobacco is independent of SA signalling, JA levels, ROS, high sugar resistance and extracellular invertase function**

It has been established that SA signalling is generally important for immunity against biotrophs or hemibiotrophs such as *Pst*, while JA signalling generally is relevant for defence responses directed against necrotrophs, although there are exceptions (Tsuda and Katagiri, 2010). Therefore, we determined SA levels following *PstT* infection according to Figure 1A for transient expression of the 4xJERE:ipt construct and Figure 2A for CK feeding. The finding that SA levels were not specifically affected by the various treatments (Table I; Supplemental Table S2) indicates that the CK-mediated resistance is unrelated to SA levels. Further, leaves of a transgenic tobacco line (*N. tabacum* cv. Samsun NN) expressing the *PR1a*:GUS reporter construct (Grüner et al., 2003), a widely used SA marker, were fed with 10 or 40 µM kinetin for 24 h according to Figure 2A. Only feeding with 40 µM kinetin led to weak local induction of the *PR1a* promoter evident by scattered GUS staining (Supplemental Fig. S2A). Likewise, *PR1a* mRNA levels are weakly affected by CK, but stronger by *PstT* infection (Supplemental Fig. S2B). However, the presence of elevated CK levels resulted in synergistic *PstT* induction of the *PR1a* mRNA. Semi-quantitative RT-PCR expression analysis (Supplemental Table S7) was performed to investigate additional markers for SA-JA-ET signalling and secondary metabolite
biosynthesis. This showed that although \textit{EDS1} expression was clearly transiently induced directly following CK treatment (24h K/0) the \textit{NPR1} gene downstream in the SA pathway was not affected. Further, the \textit{PR1} genes are slightly induced by CK treatment, but considerable stronger in combination with \textit{Pst} infection K+Ps/0, corroborating the Northern expression data (Supplemental Fig. S2B). Thus, the additional marker analysis supports our conclusion that the CK-mediated resistance is essentially independent from SA signalling. To further address whether CK-mediated resistance functions through the regulation of SA levels, we tested the effect of CK feeding on \textit{PstT} infection in a transgenic tobacco line expressing the \textit{nahG} gene encoding the SA-degrading salicylate hydroxylase from \textit{Pseudomonas putida}. Indeed the SA levels are strongly reduced in the \textit{nahG} transgenic compared to wild-type (Supplemental Figure S4). Our results corroborated earlier findings that the \textit{nahG} transgenic plants are more susceptible to infection by \textit{Pst} (Delaney et al., 1994). However, the cytokinin treatment was still able to confer an increased resistance against \textit{Pst} (Fig. 4A; Supplemental Figure S3) independent of SA levels (Supplemental Fig. S4, A and B) and accordingly phytoalexin production was stimulated to a similar degree (ratio between kinetin feeding and control) as in wild-type. Thus, different lines of experimental evidence show that the CK-mediated resistance is essentially independent from SA signalling and unrelated to SA levels.

JA is typically not involved in defence responses against \textit{Pst}, but is involved in mediating wounding specific defence responses. Since the infiltration procedures for transient expression of the \textit{4xJERE:ipt} construct and \textit{PstT} infection could potentially result in mechanical wounding of the tissue, we addressed whether JA levels are affected. Supplemental Table S2 and Table I show that JA levels were low and not specifically affected in any of the treatments. The expression analysis for \textit{COI1} and \textit{EIN2} (Supplemental Table S7) not only confirmed that the CK-mediated resistance is independent from JA, but also showed independence from ET signalling. Thus, neither the elevated CK levels nor our experimental approach interfered with the synthesis of the JA defence signal.

Reactive oxygen species (ROS) also play an important role in stress responses. In host plants they cause strengthening of the cell wall, may kill intruders and act as signalling molecules to activate defence pathways (Torres et al., 2006). No difference in the low level of H$_2$O$_2$ accumulation visualised by diaminobenzidine (DAB) staining could be detected after infiltration of \textit{A. tumefaciens} (4xJERE:ipt) or
the corresponding empty vector strain, ruling out an effect of *A. tumefaciens* on the ROS status (Fig. 4B). After infiltration of *PstT* no differences in DAB staining were visible up to 24 hpi and thus prior to symptom development. In contrast, a relatively high amount of H$_2$O$_2$ production was detected in non-*ipt* expressing areas displaying severe necrosis 168 hpi, compared to areas with increased *ipt* levels. Thus, the CK-mediated resistance is not caused by prior accumulation of ROS. Rather, increased ROS levels were the consequence of pathogen spread and concomitantly killed host cells.

Since a mechanism of high sugar resistance (Horsfall and Dimond, 1957) and sugar mediated defence gene induction (Herbers et al., 1996; Ehneß et al., 1997) have been described, we also addressed the role of carbohydrates in the CK-mediated resistance. The levels of the major soluble carbohydrates (glucose, fructose, sucrose) and starch varied significantly in different experiments, but were not specifically altered in leaves with increased CK levels in the presence of *PstT* (Supplemental Tables S3 and S4) and did not correlate with the CK-mediated resistance. Extracellular invertase, a key sucrose cleaving sink enzyme required to supply carbohydrates via an apoplastic pathway (Roitsch and Gonzalez, 2004), has been shown to be required for defence responses against various pathogens (Berger et al., 2007; Bonfig et al., 2010) and to be induced by CKs in various species including tobacco (Ehneß and Roitsch, 1997; Balibrea Lara et al., 2004). Therefore we tested whether a CK induced extracellular invertase is involved in the CK-mediated resistance against *PstT*, employing the tetracycline-inducible promoter system (Balibrea Lara et al., 2004). Figure 4C shows that induction of the extracellular invertase *CIN1* by either local infiltration of tetracycline or feeding of tetracycline via leaf petioles, did not affect subsequent *PstT* infection. However, when CK levels were elevated in leaves of these transgenic plants, by either feeding of kinetin via the petioles or local 4xJERE:*ipt* expression, resistance was strongly enhanced. These results demonstrate that the CK-mediated resistance is not directly or indirectly related to an elevated sugar status and is independent of the metabolic function of extracellular invertase.

**Cytokinins induce the synthesis of bactericidal activities**
Since the CK-mediated resistance strongly interfered with growth of the infiltrated bacteria (Fig. 1E), we analysed the possible involvement of antimicrobial activities in this resistance mechanism. Bioautography experiments with extracts derived from experiments involving transient expression of the 4xJERE:ipt construct according to Figure 1A, failed to detect any antimicrobial activity in the CK-stimulated tissues, indicating the absence of any antimicrobial peptides or proteins active against PstT. Next, disc diffusion assays were performed to address the possible involvement of low molecular weight antimicrobial compounds in the CK induced immunity. Table II demonstrates that extracts derived from samples with elevated CK levels contained antimicrobial activities that are highly active against PstT. In addition, the growth of three other gram-negative bacteria tested, E. coli, P. aeruginosa and Psp, was affected although to different degrees. In contrast, growth of the two tested gram-positive bacteria, B. subtilis and S. aureus, and of the yeast C. maltosa was only slightly inhibited. Growth of the two necrotrophic fungi S. sclerotiorum and B. cinerea was not restricted and growth of the latter even seemed to be promoted, which is in agreement with the finding that the induced antimicrobial activities are inactive against the necrotrophic fungi tested (Supplemental Fig. S1, B and C). Control incubations ruled out that CK had an inherent antimicrobial activity in disc diffusion assays or when directly applied to cultures of PstT, demonstrating that the antimicrobial activities are specifically induced in response to the elevated CK levels.

Cytokinin induced phytoalexin production causes pathogen resistance

Phytoalexins and glucosinolate compounds were shown to be essential in pathogen resistance (Ren et al., 2008; Bednarek et al., 2009; Lu et al., 2009). Since the sesquiterpene capsidiol and the hydroxycoumarin scopoletin are the two prominent antimicrobial compounds in Solanaceae (Brooks et al., 1986; Ahl Goy et al., 1993), we tested by quantitative instrumental analyses whether the levels of these phytoalexins were influenced by the CK status of the plant following transient expression of the 4xJERE:ipt construct according to Figure 1A. Scopoletin and capsidiol levels were 6.4- and 4.6-fold higher, respectively, in ipt expressing samples compared to the corresponding controls (Table III). Therefore, the CK induced biosynthesis for the two major phytoalexins of tobacco, the sesquiterpene capsidiol
and the hydroxycoumarin scopoletin, is in agreement with the CK dependent increase in bactericidal activities.

Next we performed time course analyses of phytoalexin production in relation to bacterial growth in CK feeding experiments according to Figure 2A, to rule out an influence of prior A. tumefaciens infiltration. Cytokinin feeding followed by PstT infection resulted in higher scopoletin levels compared to the control in the early phase of infection (Supplemental Table S5). Consequently, the higher scopoletin levels preceded the increased bacterial growth (Fig. 5A) and at the onset of bacterial growth (12 hpi), the phytoalexin/bacteria ratio is 10-fold higher than that in control infections (Supplemental Fig. S5). Concomitantly, bacterial growth was restricted and even abolished in the late phase of the infection (48-96 hpi) compared to the control treatment (Fig. 5, A and B; Supplemental Tables S5 and S6). Although in control treatments the absolute scopoletin production following PstT infection reached very high levels in the late phase of infection (48-96 hpi), exceeding the maximum scopoletin levels in CK pre-treated leaves by almost 8-fold (Supplemental Table S5), the increased scopoletin production occurred only after the onset of bacterial growth (Fig. 5B). This resulted in a low phytoalexin/bacteria ratio throughout the infection phase (Supplemental Fig. S5) and concomitantly bacterial growth was unrestricted (Fig. 5B; Supplemental Table S6). Thus, two independent experimental approaches showed that the CK-mediated resistance against PstT strongly correlated with increased phytoalexin biosynthesis.

To investigate the regulatory mechanism involved in the CK-mediated increase of scopoletin and capsidiol levels, the mRNA levels for key enzymes involved in the synthesis or remobilisation of both phytoalexins were determined by RNA gel blots. Figure 5E shows, that the expression of 5-epi-aristolochene-synthase (EAS), catalysing a rate limiting step in capsidiol biosynthesis, and cinnamic acid 4-hydroxylase (C4H), catalysing the irreversible conversion of cinnamic acid into p-coumaric acid that is ultimately converted into scopoletin, were induced in response to the induction of the ipt gene, which was corroborated with semi-quantitative RT-PCR (Supplemental Table S7). In contrast, the induction of phenylpropanoid-glucosyltransferase (TOGT), mediating the reversible conversion of the storage form scopolin into scopoletin, was delayed and much less pronounced, indicating that CKs primarily affect synthesis rather than remobilization. Thus, elevated CK levels resulted in increased expression for key enzymes involved in phytoalexin synthesis.
prior to pathogen growth, eventually leading to enhanced scopoletin and capsidiol levels. Secondary metabolite production is not generally increased by elevated CK levels, since *PAL1* expression was not significantly affected, whereas *FLS1* expression (flavonoid synthesis) was even repressed (Supplemental Table S7).

Because of the strong correlation between increased phytoalexin levels and the CK-mediated pathogen resistance, we analysed whether CK induced pathogen resistance is mediated through scopoletin and capsidiol. Loss-of-function analysis in tobacco requires transgenic knock-down regulation of scopoletin and capsidiol biosynthesis, which is limited by incomplete knowledge of the genes involved in the final biochemical conversion, alternative biosynthesis routes and the strength of the knock-down regulation. Alternatively, manipulation of key enzymes in phytoalexin biosynthesis would affect a large range of phenolic compounds resulting in negative side effects. Therefore, we choose a gain-of-function approach by analysing the resistance against *PstT* infection following prior application of the major phytoalexins scopoletin and capsidiol. Based on the scopoletin and capsidiol concentrations determined in W38 leaves following local 4xJERE:ipt expression in response to *PstT* infection (Table III), we infiltrated wild-type W38 tobacco leaves with various concentrations of scopoletin and capsidiol simultaneous with *PstT*. The phytoalexin infiltrations conferred a reduced symptom development (6 dpi) in 69% of the leaves treated with both scopoletin and capsidiol (172 out of 251), independent of the infiltrated concentrations (Fig. 5F), evident by reduced bacterial growth in the treated leaves (Supplemental Table S8). The treatment with phytoalexins did not affect hormone levels in the infiltrated regions, which shows that the late increase in SA levels following CK feeding (Supplemental Fig. 4C) is brought about by CKs rather than a secondary effect of increased phytoalexin levels. Similar to the CK-mediated resistance (Supplemental Fig. S1C), treatment with scopoletin and capsidiol did not reduce symptom development by *B. cinerea* (Supplemental Fig. S6). This showed that phytoalexin infiltration at concentrations present in response to elevated CK levels substituted *in planta* for the CK signal and mimic the CK-mediated pathogen resistance. Thus, the CK induced resistance is predominantly mediated by increased production of antimicrobial phytoalexins.
DISCUSSION

This study demonstrates that CKs induce resistance in tobacco against the virulent hemibiotrophic bacterial pathogen *Pst*, which is predominantly mediated by increased synthesis of the two major antimicrobial phytoalexins, scopoletin and capsidiol, resulting in a high phytoalexin/pathogen ratio in the early phase of infection, efficiently restricting pathogen growth. This CK-mediated resistance was substantiated by three functional independent transgenic approaches, including a novel pathogen inducible expression system, to induce endogenous CK biosynthesis and two different ways of exogenously supplying CKs. The use of the synthetic 4xJERE promoter (Rushton et al., 2002) for autoregulated synthesis of CKs in response to pathogen infection proved to be a valuable tool to dissect the primary role of CKs in defence from indirect effects related to the developmental stage and/or the metabolic status. The high percentage of leaves with increased resistance following CK treatment among at least 1500 individual replicate leaves reflecting more than 6200 infection sites analysed, demonstrate the reproducibility and robustness of the observed CK-mediated pathogen resistance in our study despite the varying greenhouse conditions. Importantly, this resistance mechanism is effective even when initial defence reactions of the host plant failed. The protection is restricted to tissues with elevated CK levels, revealed by the various local treatments on a single leaf, ruling out a systemic effect on other parts of the plant. This study extends the well established role of CKs in regulating various aspects of plant growth and development to triggering the primary plant defence response through increased phytoalexin production.

Cytokinins were recently shown to also induce resistance in the virulent interaction of Arabidopsis with *P. syringe* pv. *tomato* (Choi et al., 2010). However, distinct species specific differences to tobacco are apparent, both in the underlying regulatory mechanism and the specificity of the immunity. In Arabidopsis, the CK-mediated resistance is a solely transcriptional control mechanism that interferes with SA and most likely also JA dependent signalling pathways to enhance resistance against bacterial and also fungal pathogens (Choi et al., 2010). This demonstrates that in Arabidopsis in addition to auxin, abscisic acid, and gibberellins, also CKs cross-communicate with the central ET-SA-JA signal transduction pathways in immunity. In contrast, in tobacco the CK-mediated resistance is induced also in SA
depleted \textit{(nahG overexpressing)} plants, whereas CKs cannot rescue the pathogen susceptibility in \textit{nahG} transgenic Arabidopsis plants. In addition, the CK-mediated resistance in tobacco is independent from SA and JA levels, although the synergistic effect of CKs on pathogen dependent \textit{PR1a} expression is still conserved, and this resistance is rather mediated through an increased antimicrobial phytoalexin production. Another striking difference is that in tobacco CKs conferred resistance only against the bacterial pathogen \textit{Pst}, but not against the two necrotrophic fungi tested, \textit{B. cinerea} and \textit{S. sclerotiorum}, whereas in Arabidopsis the resistance was enhanced against bacterial as well as necrotrophic fungal pathogens.

**Cytokinin induced phytoalexin production causes pathogen resistance in tobacco**

The two main phytoalexins known from tobacco and other solanaceous species are the sesquiterpene capsidiol and the hydroxycoumarin scopoletin (Brooks et al., 1986; Ahl Goy et al., 1993). Their antimicrobial activity against various pathogens is well established and they accumulate to high levels in infected plants (Dropkin et al., 1969; Ward et al., 1974; Kim et al., 2000; El Oirdi et al., 2010). Scopoletin and capsidiol are often restricted to diseased tissues (Guedes et al., 1982) or a ring of apparently healthy cells around lesions (Chong et al., 2002). The determined specificity of the antimicrobial activities induced by CKs is in agreement with the specificity of scopoletin and capsidiol against microbial pathogens. It was shown that capsidiol levels were not affected in SA-overproducing tobacco (Nugroho et al., 2002). Further, the promoter of the tobacco 5-\textit{epi}-aristolochene synthase \textit{EAS4} gene, central for capsidiol synthesis (Bohllmann et al., 2002), strongly responded to pathogen or elicitor treatment, but not to SA, JA, or \textit{H}_2\textit{O}_2 (Yin et al., 1997). This is in agreement with our observation that the CK-mediated resistance is independent from these stress signalling compounds and our conclusion that it is predominantly mediated by the observed elevated levels of capsidiol and scopoletin. Indeed, CK feeding still induced scopoletin production in \textit{nahG} transgenic plants at similar ratios compared to wild type plants. Interestingly, in Arabidopsis phytoalexins were shown to be essential for resistance against fungal pathogens, while SA signalling was not involved in either resistance or phytoalexin production (Ferrari et al., 2007; Lu et al., 2009). Thus, the involvement of phytoalexins in the host range specificity exemplifies
another species specific difference between Arabidopsis and tobacco. Only few previous studies addressed the effect of CK feeding on phytoalexin production. In one case, scopoletin levels in tobacco tissues treated with CK dramatically decreased while scopolin, the presumed storage form, increased significantly (Skoog and Montaldi, 1961). This discrepancy to our findings may originate from the fact that an artificial tissue culture system was used by these authors. In the other case, the levels of phenolic compounds, including the scopoletin precursor p-coumaric acid, were increased in CK overproducing ipt transgenic tobacco plants, whereas SA levels were reduced (Schnablová et al., 2006).

In this work we focussed on the two major phytoalexins of tobacco. The combined scopoletin and capsidiol infiltration conferred stronger pathogen resistance compared to either single treatment, which shows that the bactericidal effect of these phytoalexins is either additive or synergistic. Nevertheless, the increased pathogen resistance resulting from the combined scopoletin and capsidiol infiltration was not as prominent as that mediated by increased CK levels. At least 13 additional phytoalexins have been described for Nicotiana species (Tanaka and Fujimori, 1985; Nugroho and Verpoorte, 2002) and also in other plant species many different phytoalexins are produced following pathogen infection (Bednarek et al., 2005; Pedras et al., 2008). Therefore, it is possible that the two most prominent phytoalexins capsidiol and scopoletin account for just a part of the antimicrobial activity mediated by increased CK levels in tobacco leaves, and that several phytoalexins act in concert to ward off pathogens. Further, an effect of the subcellular phytoalexin compartmentalisation on its antimicrobial activity cannot be ruled out (Rogers et al., 1996). Due to the experimental approach, the phytoalexin infiltration resulted in increased extracellular phytoalexin levels. Considering the time lag until the onset of bacterial growth (12 hpi), it is possible that the infiltrated phytoalexins remaining in the extracellular space are modified, reducing their antimicrobial activity. In addition, diffusion, active transport and metabolism can take place reducing the level of active phytoalexins following infiltration. Indeed, scopoletin infiltration and recovery experiments revealed a substantial reduction of the infiltrated scopoletin amounts to physiological levels, which efficiently inhibited bacterial proliferation. On the other hand, high phytoalexin levels can confer cell death (Rogers et al., 1996; Kim et al., 2005), limiting the concentration range that could be used in the phytoalexin infiltration experiment. Considering these limitations, it is
remarkable that exogenously supplied scopoletin and capsidiol substituted for the CK signal and mimic the CK-mediated pathogen resistance, thereby functionally identifying \textit{in planta} scopoletin and capsidiol as the main antimicrobial phytoalexins in tobacco. Thus, the CK induced pathogen resistance is predominantly mediated by increased antimicrobial phytoalexin production.

Time course analysis of scopoletin production and bacterial growth in CK feeding experiments, showed that the CK-mediated scopoletin production, strengthened by pathogen triggered scopoletin production, resulted in an increased phytoalexin/pathogen ratio in the early phase of infection, which efficiently restricted pathogen growth (Fig. 5, A and C; Supplemental Table S6). Following pathogen infection, a complex signalling network comprising many interacting defence pathways is activated in plants (Sato et al., 2010). Since the bacterial growth initially continued in the presence of increased scopoletin levels after CK feeding (Fig. 5 A), either certain phytoalexin threshold levels are required for bactericidal activities or other defence mechanisms participate in the complete abolishment of bacterial growth in the late infection phase. Probably these additional defence mechanisms are induced following pathogen infection and can only function properly when pathogen growth is already restricted, as observed for the CK-mediated immunity. The weaker pathogen resistance observed following phytoalexin treatment and in CK treated \textit{nahG} transgenics, and the late increase in SA levels in respect to the increased phytoalexin levels following CK treatment in wild-type plants (Table I; Supplemental Figure S4C), suggests that SA dependent pathogen responses act as such an additional defence mechanism to eliminate the pathogens weakened by the bactericidal phytoalexins. In contrast, in control treatments the pathogen triggered scopoletin production reached very high levels, exceeding that in CK pre-treated leaves by almost 8-fold. However, this increase occurred only after the onset of bacterial growth (Fig. 5, B and D), resulting in a low phytoalexin/pathogen ratio throughout the infection phase (Supplemental Fig. S5). Therefore, the scopoletin production occurred too late to restrict pathogen growth (Fig. 5, B and D).

The role of cytokinins in plant stress resistance

The plant hormone CK is emerging as a new important player in stress response signalling and in addition to its role in pathogen resistance it is also
important in promoting the tolerance towards abiotic stress conditions (Tran et al., 2007; Rivero et al., 2009; Ghanem et al., 2011). In agreement with our model on the CK-mediated defence mechanism in tobacco, several bacteria have been described that exert positive effects on plants by promoting plant growth, controlling diseases (Compant et al., 2005; Wu et al., 2009) and/or increasing tolerance to abiotic stress (Mayak et al., 2004). Corresponding to the plant growth-promotion by such biocontrol bacteria, also the production and secretion of different phytohormones has been shown (Boiero et al., 2007; Wu et al., 2009) that include notably CKs (García de Salamone et al., 2001; Ortiz-Castro et al., 2008; Abo-Elyousr and Mohamed, 2009; Wu et al., 2009). The CK production by the plant growth promoting micro-organisms as well as CK signalling in plants were shown to be essential for the beneficial effect on plant growth (Arkhipova et al., 2005; López-Bucio et al., 2007; Ortiz-Castro et al., 2008). So far the results from studies that linked CKs with either resistance or susceptibility to herbivores and pathogens were mostly inconclusive (Dropkin et al., 1969; Beckman and Ingram, 1994; Choi et al., 2010). In our study this beneficial effect of CKs is linked to natural infection conditions and we show that CKs mediate pathogen resistance by increasing phytoalexin production in tobacco.

Infection of crop species by fungal and bacterial pathogens results in dramatic losses in harvest yield, which are usually fought against with pesticides. With respect to their negative impact on human health and increased resistance of microbial pathogens against commercially available pesticides, the practical application of the CK-mediated resistance could complement the protection of various crop species to increase harvest yield and contribute to food safety. Possible applications include transgenic plants harbouring a pathogen inducible ipt gene (4xJERE:ipt), spraying of CKs, or the application of CK producing biocontrol microorganisms. We routinely observed the CK-mediated pathogen resistance under many independent greenhouse experiments, which are subject to varying growth conditions, and under stable growth conditions in controlled growth-chambers. This shows that this novel pathogen resistance mechanism is robust, an important requirement for practical agronomical application. Our data demonstrate that a single, not yet optimized, CK treatment is sufficient to provide at least a limited protection against pathogen infection for a minimum of one week, supporting the possible agronomical practical application.
The species-specific differences in the CK-mediated defence mechanism between Arabidopsis (Choi et al., 2010) and tobacco (this study) shows that the evolution of these defence mechanisms probably occurred independently in several plant species in response to pathogens that make use of the natural role of CKs in the host plant to facilitate infection. This arms race evolution started when the function of CKs in regulating growth and sink-source relationships in the host plant was hijacked by plant pathogens that either produce CKs or manipulate CK production in the infected plant to alter the host physiology, and resembles the zigzag model for the plant immune system (Jones and Dangl, 2006) in pathogen-associated molecular patterns (PAMP) triggered immunity (PTI) and effector triggered immunity (ETI) (Tsuda and Katagiri, 2010). As a consequence, CK-mediated defence mechanisms co-evolved in plants to counter the infection by such pathogens. Finally, agonistic microorganisms with plant growth promoting characteristics evolved the ability to produce CKs to enhance the overall plant defence capacity or prime the host plants for a faster defence response. A lack in the plants ability to respond to infection via HR, which would stop the progress of biotrophic pathogens, could result in uncontrolled spread of bacteria. Interestingly, despite the absence of HR, the elevated CK levels induced bactericidal activities and restricted bacterial growth. During pathogen defence, the plant needs to balance two apparently conflicting demands, efficiently abolish the pathogen infection and limit the negative impacts of immune responses on tissue integrity, photosynthetic capacity and plant primary metabolism (Sato et al., 2010). Thus, the CK-mediated pathogen resistance could have evolved as an important immunity mechanism that limits negative effects on plant fitness during pathogen defence responses.
MATERIALS and METHODS

Plant growth conditions

All plants used in the present study were grown under greenhouse conditions at 20-24 °C achieved by additional heating or cooling during cold or warm outside temperatures, respectively, and a 16 h/8 h day/night cycle by supplemented lighting (Envirolite 200W 6400K Fluorescent lamps).

Cloning of the 4xJERE:ipt construct

The ipt fragment was PCR amplified from the pUC19-IPT plasmid (provided by T. Schmülling) with the primers IPTfwd and IPTrev (Supplemental Table S9) adding restriction sites for XhoI and SacI. The PCR fragment was cloned into a pGEM-T Easy cloning vector (Promega, Germany) to obtain pGEM-T Easy-ipt. To place the ipt gene under the control of the 4xJERE promoter (Rushton et al., 2002), the GUS fragment from the pBT10-GUS vector (Sprenger-Haussels and Weisshaar, 2000) was replaced by ipt from pGEM-T Easy-ipt using XhoI and SacI to create the pBT10-4xJERE:ipt plasmid. The 4xJERE:ipt expression cassette was subsequently cloned into the pCambia1380 vector (Cambia, Australia) using the restriction enzymes BglII and HindIII, creating the vector pCam1380-4xJERE:ipt (4xJERE:ipt).

Pathogen experiments

The virulent hemibiotrophic pathogen strain P. syringae pv. tabaci was transformed with plasmid pMP4655 (Bloemberg et al., 2000) to confer tetracycline resistance (PstT), required for experiments involving tetracycline inducible plant gene expression and all P. syringae infections were performed with this PstT strain for comparison. An exponentially growing culture of PstT containing 20 mg L\(^{-1}\) tetracycline was harvested and adjusted to 10\(^6\) to 10\(^7\) cfu mL\(^{-1}\) in 10 mM MgCl\(_2\). Approximately 100 µL of the suspension were infiltrated into the abaxial side of leaves with a needleless syringe. Determination of bacterial growth was carried out as described before (Griebel and Zeier, 2008). The avirulent strain P. syringae pv. phaseolicola was grown accordingly in cultures containing 50 mg L\(^{-1}\) rifampicin and
re-suspended in 10 mM MgCl₂ after harvesting. For the fungal infection experiments, fully developed leaves from three different varieties (*N. tabacum* cv. Xanthi, Xanthi nc, W38) were cut with a razor blade and immediately placed into water, 10 μM kinetin or 40 μM kinetin. After 24 h they were inoculated either by infiltration with *B. cinerea* spore suspension (ca. 10⁵ spores mL⁻¹) or by placing a 50 μL drop of spore suspension onto the abaxial side of the leaf. For the latter method, spores were preincubated in 3 g L⁻¹ Gamborg B5 Medium (in 10 mM KH₂PO₄ buffer, pH 7) supplemented with 25 mM glucose for 2 h to stimulate germination. Leaves were placed in sealed petri dishes with moist sterile filter paper to ensure high humidity and stored under ambient light conditions.

From each plant, four leaves of similar developmental stage were used for the different treatments and the replicates from each treatment were randomly distributed among the individual plants from one experimental series. For each treatment at least three individual experimental series were performed. Likewise, for the detached leaf experiments (petiole feeding) four leaves from each plant were taken and randomly distributed for the different treatments. Although the overall symptom development and final disease phenotype at the end of the infection phase was always similar and the changes in susceptibility are evident throughout symptom development following *Pst* infection, the speed of the symptom development varied for each experimental series. The symptom development following *PstT* infection was binned into seven classes (0= no symptoms; 0.5= reduced chlorosis (up to 75 %); 1= >75 % chlorosis; 2= ≤10 % necrosis; 3= 10-50% necrosis; 4= 51-75 % necrosis; 5= 76-100 % necrosis) reflecting different degrees of visible pathogen response, which was subsequently used to determine the effect of the different treatments compared to control plants.

**Determination of bacterial growth in leaf tissue**

From *PstT* infiltrated areas, leaf discs were punched out using a cork borer (0.4 cm diameter). Each leaf disc was homogenized in 1 mL sterile 10 mM MgCl₂. 100 μL of serial dilutions were plated in three replicates on LB medium supplemented with 20 mg L⁻¹ tetracycline. Colony formation was determined after 36 to 48 h incubation at 28 °C. Statistical significance for differences between treatments was analysed using the unpaired Student’s t-test.
Induction of the *ipt* gene in plants

Before leaf infiltration, the 4xJERE:ipt strain (*pCam1380-4xJERE:ipt* in *A. tumefaciens* strain LBA 4404) was pre-cultured in 5 mL LB-liquid medium, subsequently diluted 1:100 in 50 mL LB-liquid medium containing 50 mg L\(^{-1}\) kanamycin, 200 mM MES (pH 5.5) and 40 μM acetosyringone for 24 h each. Cells were harvested at OD\(_{600} = 0.8\), resuspended in 10 mM MgCl\(_2\), 200 mM MES (pH 5.5) and 200 μM acetosyringone, and kept at room temperature for 2 h in darkness. For transient *ipt* expression in tobacco plants before pathogen infection, approximately 350 μL of the suspension were infiltrated into marked regions on one half of 9-10 weeks old *N. tabacum* cv. W38 leaves. For all controls, the *A. tumefaciens* strain LBA 4404 harbouring an empty vector (control) was infiltrated into defined areas on the other half of the same leaf. After 24 h, all pre-treated regions were inoculated with *PstT*. Induction of the *SAG12:ipt* construct by heat treatment of detached leaves and of the *TET:ipt* construct by chlortetracycline were carried out as described previously (Balibrea Lara et al., 2004).

Exogenous application of CKs

For petiole feeding, 7-10 week old leaves from *N. tabacum* wild-type cv. SR1 plants were placed in water (control) or in aqueous solutions of either kinetin or the indicated CK (Duchefa, Netherlands). For all feeding experiments, the leaves were immediately transferred to water after infiltration of *PstT* and symptom development was continuously observed for up to 14 days. Alternatively, leaves were dipped for 60 s or 90 s into kinetin solution containing 0.005 % Silwet L-77 (Lehle Seeds, USA) and kept with petioles in water 24 h before infection and afterwards.

Exogenous application of phytoalexins

Stock solutions of capsidiol (20 mM in EtOH) and scopoletin (50 mM in MeOH) were diluted to 100 μM in H\(_2\)O. Approximately, 100 μL of either scopoletin, capsidiol or scopoletin + capsidiol were infiltrated in 9-10 weeks old *N. tabacum* cv. W38.
leaves 1 to 2 h before infiltration with \( PstT \) \((10^7 \text{ cfu mL}^{-1})\). Control treatments contained the corresponding EtOH and/or MeOH concentration. In total three biological replicates experiment were performed yielding similar results, and the infiltration spots were randomised to exclude positional effects on symptom development, which was continuously observed for up to 10 days.

**Determination of antimicrobial activities**

Total antimicrobial activity was tested using a paper–disc diffusion assay (Murray et al., 1995). The presence of antimicrobial proteins was determined by bioautography (Vigers et al., 1991).

**Instrumental analyses**

Cytokinins (Novák et al., 2008), soluble sugars (Bonfig et al., 2006), scopoletin (Thoma et al., 2003), SA and JA (Thoma et al., 2003) in the infiltrated leaf areas were analysed according to published procedures. For capsidiol determination, leaf material was frozen and ground in liquid nitrogen. Ground material was mixed three times with 75 % ethanol for 1 h and centrifuged at 5000 rpm for 10 min. Supernatants from all three extractions were combined and dried using a SpeedVac concentrator (Eppendorf, Germany). After drying, the residue was dissolved in 10 mL methanol (Roth, Germany) for further analysis. The analysis of capsidiol was done by GC-MS using a Trace Ultra gas chromatograph (Thermo Fisher Scientific; Waltham, USA) and a Zebron ZB-5-capillary column (30 m x 0.25 mm x 0.25 μm; Phenomenex, USA) with helium as carrier gas at a constant gas flow of 1.0 mL min\(^{-1}\). Injector temperature was 250 °C, and samples were injected under splitless conditions. Transferline temperature was 300 °C. For mass spectrometry a Trace DSQ TM (Thermo Electron, Waltham, USA) was used with an ion source temperature of 200 °C and ionization at 70 eV (EI mode). Data were acquired under the control of the Finnigan Xcalibur version 1.4 software package (Thermo Electron, Waltham, USA). Both analyses were repeated thrice. Statistical significance for differences between treatments was analysed using the unpaired Student’s t-test.

**Histochemical determinations**
The distribution of β-glucuronidase activity and H₂O₂ in leaves was determined according to published procedures (Grüner et al., 2003; Barna et al., 2008).

**Northern blotting**

For total RNA isolation, 100 mg leaf material was ground in liquid nitrogen and mixed with 1 mL TRIR reagent (Thermo Fisher Scientific, Germany). After chloroform extraction, the RNA was precipitated with isopropanol. The RNA pellet was washed in 3 M LiCl (removal polysaccharides) and subsequently dissolved in RNase free water. For gel electrophoresis, the RNA was separated on 1.2 % (v/v) denaturing (formamide) agarose gels. The resulting RNA was transferred to nitrocellulose membranes (Macherey-Nagel, Germany) in 20X SSC, which were fixed by baking the membranes for 2 h at 80 °C. The DNA probes to detect expression for the respective genes were labelled with α³²P-dATP with the HexaLabel DNA Labelling Kit (MBI-Fermentas, Germany). The primers used to amplify the DNA probes from cDNA generated from *N. tabacum* total RNA are shown in Supplemental Table S9.

**Supplemental Data**

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

**Supplemental Figure S1.** Cytokinins are not affecting the avirulent interaction with *Psp* or resistance against necrotrophic fungi.

**Supplemental Figure S2.** *PR1a* expression is induced by CKs and *PstT* infection.

**Supplemental Figure S3.** Cytokinins induce resistance independent of SA.

**Supplemental Figure S4.** SA levels are depleted in *nahG* transgenic plants and not induced following CK-treatment.

**Supplemental Figure S5.** Cytokinins induce a high phytoalexin/bacteria ratio early in the infection phase.

**Supplemental Figure S6.** Application of capsidiol and scopoletin does not restrict proliferation of *B. cinerea*. 
Supplemental Table S1. Cytokinin levels are increased following 4xJERE:ipt expression.

Supplemental Table S2. SA and JA levels are not specifically affected by ipt expression.

Supplemental Table S3. Free sugar levels are not specifically affected by CK, low sugar levels.

Supplemental Table S4. Free sugar levels are not specifically affected by CK, high sugar levels.

Supplemental Table S5. Scopoletin production is stimulated by CK feeding.

Supplemental Table S6. Proliferation of PstT is reduced by CK feeding.

Supplemental Table S7. Semi-quantitative RT-PCR expression analysis.

Supplemental Table S8. Proliferation of PstT is reduced by combined scopoletin and capsidiol feeding.

Supplemental Table S9. Primers used in this study.

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Authors Contributions

T.R., D.G., and U.S. conceived and designed the experiments. D.G., U.S, U.R., N.P., M.N., T.G., and T.E. performed the experiments. D.G., M.N., U.S., E.G., T.R., and T.E. analysed the data. N.P., O.N., M.S., J.Z., H.P., and T.R. contributed reagents/materials/analysis tools. M.N., U.S., E.G., T.R., and D.G. wrote the article.
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FIGURE LEGENDS

**Figure 1.** Accumulation of CKs by overexpressing *ipt* causes resistance against *PstT*.

A, Experimental scheme for transient 4xJERE:ipt expression in tobacco leaves followed by *PstT* infection. Tobacco leaves were pre-infiltrated with *A. tumefaciens* containing (*At(ipt)*, blue line) or without the 4xJERE:ipt construct (*At(0)*, black line), followed by a second infiltration with either *PstT* (*Pst*, red line) or a mock infiltration with MgCl₂ (grey line) after 24 h. Numbers refer to time points of sampling for CK determination (Supplemental Table S1), JA and SA (Supplemental Table S2), free sugar (Supplemental Tables S3 and S4), antimicrobial activities (Table II), or phytoalexins (Table III). B, RNA blot analysis of tobacco tissue transiently transformed with 4xJERE:ipt. 28S rRNA for loading control is shown in bottom panel. C, Effect of transient 4xJERE:ipt expression and subsequent *PstT* infection on symptom development and green island formation. Tobacco leaves pre-infiltrated with *A. tumefaciens* with (*ipt*) or without (0) the 4xJERE:ipt construct followed by a second infiltration of *PstT* (*Pst*) or mock infiltration (0). The infiltrated areas are marked by circles. Pictures were taken at the indicated days past infection (dpi). D, Effect of senescence induced *ipt* expression in SAG12:ipt plants and subsequent *PstT* (red) infection on symptom development (left) in comparison to wild-type (right). E, Effect of tetracycline induced *ipt* expression in TET:ipt plants and subsequent *PstT* infection on symptom development. Leaves of the transgenic TET:ipt tobacco line were pre-infiltrated with tetracycline (TET, yellow) or water (0, grey) followed by a second infiltration with *PstT* (*Pst*, red). F, Effect of transient 4xJERE:ipt expression on the proliferation of *PstT*. Tobacco leaves were pre-infiltrated with *A. tumefaciens* with (*ipt*) or without (0) the 4xJERE:ipt construct followed by a second infiltration with *PstT*. Bacteria were re-isolated from leaf discs, plated and quantified. Mean values ± standard deviation of three independent replicates.

**Figure 2.** Exogenously supplied CKs enhance resistance against *PstT*. 
A, Experimental scheme of exogenous applications of CKs by petiole feeding (green bar) and dipping (yellow bar) prior to infection with *PstT* (red arrow). The petioles from both CK-treated and control leaves were kept in water for symptom development. B, Effect of feeding varying kinetin concentrations (1-18 µM) 24 h prior to *PstT* infection on symptom development 7 dpi. C, Effect of short pulse feeding (0.5-4 h) with 10 µM kinetin prior to *PstT* infection on symptom development 13 dpi. D, Comparison of efficiency to induce resistance (12 dpi) against *PstT* for different adenine derived CKs applied in 10 µM concentration 24 h prior to infection. E, Dose dependent (2 fM to 200 nM) ability of phenylurea derived CK thidiazuron (TDZ) to induce resistance against *PstT* (6 dpi) by petiole feeding. F, Induction of resistance against *PstT* (11 dpi) by dipping leaf-halves for 60 s (left) and 90 s (right) in 140 µM kinetin 24 h before infection. Leaves were infiltrated with *PstT* at three sites per leaf half (B-F).

**Figure 3.** Exogenously supplied CKs mediate resistance before as well as after *PstT* infection.

A, Experimental scheme of varying starting times of the 24 h kinetin pulse (10 µM) relative to infection with *PstT* (24 hbi to 48 hpi). B, Effect of varying starting times of 24 h kinetin pulse (10 µM) relative to infection with *PstT* (24 hbi to 48 hpi) on symptom development 5 dpi. C, Experimental scheme for analysis of continuance of CK induced resistance with infections at different time-points past CK feeding. D, Cytokinin induced resistance effect (12 dpi) in leaves infected with *PstT* at the end of CK application (0 dpc) and several days past CK pulse (2-7 dpc) in comparison to water control. Leaves were infiltrated at three sites with *PstT* (left) and 10 mM MgCl₂ (right) (B,D).

**Figure 4.** Cytokinins induce resistance independent of SA, JA, ROS and extracellular invertase activity.

A, Effect of 24 h kinetin application (10 µM) before *PstT* infection on symptom development in *nahG* overexpressing tobacco leaves in comparison to control, 8 dpi.
Leaves were infiltrated at two sites with \( PstT \) (left) and 10 mM MgCl\(_2\) (right). B, Effect of transient \( 4x\text{JERE:ipt} \) expression and \( PstT \) infection on \( \text{H}_2\text{O}_2 \) formation. Tobacco leaves were pre-infiltrated with \( A. \text{tumefaciens} \) with \( (\text{ipt}) \) or without \( (0) \) the \( 4x\text{JERE:ipt} \) construct followed by a second infiltration with \( PstT \) (\( Pst \)). Leaves were stained with DAB to visualize \( \text{H}_2\text{O}_2 \) formation 24 h after pre-infiltration of \( A. \text{tumefaciens} \) (0 hpi, left), 24 h after infiltration of \( PstT \) (24 hpi, middle), and at the time of symptom development 168 h after \( PstT \) infiltration (168 hpi, right). C, Effect of \( CIN1 \) expression in \( \text{TET:}CIN1 \) tobacco leaves induced by 24 h feeding of 1 mg L\(^{-1}\) TET, in comparison to 24 h application of 10 µM kinetin prior to infection and water control on symptom development 10 dpi. Leaves were infiltrated with \( PstT \) at two sites per leaf half.

**Figure 5.** Cytokinin induced phytoalexin production causes pathogen resistance.

A, Experimental data for time course analysis of scopoletin production (blue lines with circles, nmol g\(^{-1}\) fw) in relation to bacterial growth (red lines with squares, cfu mL\(^{-1}\)) in \( PstT \) infected leaves pre-treated with CKs. Note the different scale for the scopoletin levels compared to Figure 5B. B, Experimental data for scopoletin production and bacterial growth in \( PstT \) infected leaves (control). C, Model for CK-mediated phytoalexin production based on Figure 5A. Cytokinin feeding mediates increased phytoalexin levels resulting in a high phytoalexin/bacteria ratio, which restricts bacterial growth in the early infection phase. In the late infection phase bacterial growth is abolished, probably due to additional defence mechanisms triggered by the bacterial infection. D, Model for pathogen induced (control) phytoalexin production based on Figure 5B. Phytoalexin production is triggered by the bacterial infection, but the increase occurs after the onset of bacterial growth. This results in a low phytoalexin/bacteria ratio throughout the infection phase and consequently unrestricted bacterial growth. E, RNA blot analyses of tobacco tissue transiently transformed with \( 4x\text{JERE:ipt} \) for expression levels \( EAS, C4H \), and \( TOGT \). 28S rRNA for loading control is shown in bottom panels. F, Phytoalexin infiltration confers pathogen resistance, evident by reduced symptom development 6 dpi. W38 leaves were infiltrated with different concentrations of capsidiol and scopoletin (200-400 µM, yellow) or according controls (grey), simultaneous with \( PstT \) bacteria (10\(^7\) cfu mL\(^{-1}\), red).
TABLES

Table I. SA and JA levels are not specifically affected by CK feeding.

| Sample    | SA (ng g⁻¹ fw) | JA (ng g⁻¹ fw) |
|-----------|----------------|----------------|
| 0-0-0     | 25.3 ± 5.8     | 20.0 ± 11.9    |
| 24-0-0    | 53.1 ± 24.5    | 29.5 ± 6.7     |
| 24-Kin-0  | 70.9 ± 10.6    | 17.5 ± 9.5     |
| 36-0-0    | 109.4 ± 24.3   | 20.7 ± 8.4     |
| 36-Kin-0  | 275.6 ± 13.3 * | 33.6 ± 16.3    |
| 36-0-Pst  | 440.1 ± 283.2 +| 26.2 ± 12.2    |
| 36-Kin-Pst| 1032.5 ± 610.7 +| 38.5 ± 20.3    |
| 48-0-0    | 65.7 ± 31.5    | 36.9 ± 11.6    |
| 48-Kin-0  | 185.1 ± 127.1  | 40.3 ± 21.2    |
| 48-0-Pst  | 807.0 ± 315.0 +| 58.2 ± 43.4    |
| 48-Kin-Pst| 980.2 ± 103.6 +| 54.7 ± 25.6    |

According to the experimental scheme shown in Figure 2A, samples were obtained at different time points (h) from tobacco leaves pre-treated with kinetin (Kin) or water (0) followed by infiltration with PstT (Pst) or a mock infiltration (0). Sample = sampling time -1st infiltration -2nd infiltration. Mean values of two technical replicates ± standard deviation from three biological replicates. For JA determination, the levels for one biological replicate were below the level of detection in most individual samples and this replicate was omitted from the analysis. * = significantly different after CK feeding from control treatment (p<0.05). + = significantly different after Pst infection from control treatment (p<0.05).
Table II. Disc diffusion assay for antimicrobial activities.

| Sample number according to Figure 1A | Sample | Gram-positive bacteria | Gram-negative bacteria | Fungi |
|--------------------------------------|--------|------------------------|------------------------|-------|
|                                       |        | B. sub | S. aur | E. coli | P. aer | Psp | PstT | C. mal | B. cin | S. scl |
| 1 0h-0-0                             | 1      | 1 2   | 0 1 | 1 1 | 1 1 | 1 0 | 0     |
| 2 24h-ipt-0                          | 2      | 2 2 | 1 2 | 2 2 | 2 1 | 1 d.o. | 0     |
| 3 24h-0-0                            | 1      | 1 1 | 0 2 | 1 2 | 1 1 | 1 0 | 0     |
| 8 48h-ipt-Pst                        | 3      | 3 3 | 7 4 | 8 9 | 3 3 | 2 d.o. | 0     |
| 9 48h-0-Pst                          | 2      | 2 2 | 1 2 | 3 3 | 2 0 | 0     |

According to the experimental scheme shown in Figure 1A, samples were obtained at different time point (h) from tobacco leaves pre-infiltrated with A. tumefaciens with (ipt) or without (0) the 4xJERE:ipt construct followed by infiltration of PstT or a mock infiltration (0). Sample = sampling time -1st infiltration -2nd infiltration. B. cin = Botrytis cinerea, B. sub = Bacillus subtilis, C. mal = Candida maltosa, E. coli = Escherichia coli, P. aer = Pseudomonas aeruginosa, Psp = Pseudomonas syringae pv. phaseolicola, PstT = Pseudomonas syringae pv. tabaci-tet, S. aur = Staphylococcus aureus, S. scl = Sclerotinia sclerotiorum, d.o. = disc overgrown. Values are means of two independent experiments.
### Table III. Scopoletin and capsidiol production is stimulated by CK.

| Sample number according to Figure 1A | Sample | Scopoletin (nmol g⁻¹ fw) | Ratio (ipt/0) | Capsidiol (nmol g⁻¹ fw) | Ratio (ipt/0) |
|-------------------------------------|--------|---------------------------|--------------|--------------------------|---------------|
| 1                                   | 0h-0-0 | 3.1 ± 1.21                |              | 4.3 ± 0.98               |               |
| 2                                   | 24h-ipt-0 | 7.4 ± 2.94             | 1.10         | N.D.                    | N.D.          |
| 3                                   | 24h-0-0 | 6.7 ± 3.64               |              | N.D.                    |               |
| 4                                   | 30h-ipt-Pst | 11.3 ± 4.33         | 1.20         | N.D.                    | N.D.          |
| 5                                   | 30h-0-Pst | 9.4 ± 4.85              |              | N.D.                    |               |
| 6                                   | 36h-ipt-Pst | 24.3 ± 1.56           | 1.90         | N.D.                    | N.D.          |
| 7                                   | 36h-0-Pst | 12.8 ± 1.56             |              | N.D.                    |               |
| 8                                   | 48h-ipt-Pst | 78.8 ± 10.28        | 6.40         | 74.5 ± 6.04             | 4.63          |
| 9                                   | 48h-0-Pst | 12.3 ± 1.85             |              | 16.1 ± 2.66             |               |

According to the experimental scheme shown in Figure 1A, samples were obtained at different time point (h) from tobacco leaves pre-infiltrated with *A. tumefaciens* with (ipt) or without (0) the 4xJERE:ipt construct followed by infiltration of *PstT* or a mock infiltration (0). Sample = sampling time -1st infiltration -2nd infiltration. N.D. = not determined. Mean values of two technical replicates ± standard deviation from one biological sample.
Figure 1. Accumulation of CKs by overexpressing ipt causes resistance against PstT.

A, Experimental scheme for transient 4xJERE:ipt expression in tobacco leaves followed by PstT infection. Tobacco leaves were pre-infiltrated with A. tumefaciens containing (At(ipt), blue line) or without the 4xJERE:ipt construct (At(0), black line), followed by a second infiltration with either PstT (Pst, red line) or a mock infiltration with MgCl₂ (grey line) after 24 h. Numbers refer to time points of sampling for CK determination (Supplemental Table S1), JA and SA (Supplemental Table S2), free sugar (Supplemental Tables S3 and S4), antimicrobial activities (Table II), or phytoalexins (Table III). B, RNA blot analysis of tobacco tissue transiently transformed with 4xJERE:ipt. 28S rRNA for loading control is shown in bottom panel.
C, Effect of transient 4xJERE:ipt expression and subsequent PstT infection on symptom development and green island formation. Tobacco leaves pre-infiltrated with A. tumefaciens with (ipt) or without (0) the 4xJERE:ipt construct followed by a second infiltration of PstT (Pst) or mock infiltration (0). The infiltrated areas are marked by circles. Pictures were taken at the indicated days past infection (dpi). D, Effect of senescence induced ipt expression in SAG12:ipt plants and subsequent PstT (red) infection on symptom development (left) in comparison to wild-type (right). E, Effect of tetracycline induced ipt expression in TET:ipt plants and subsequent PstT infection on symptom development. Leaves of the transgenic TET:ipt tobacco line were pre-infiltrated with tetracycline (TET, yellow) or water (0, grey) followed by a second infiltration with PstT (Pst, red). F, Effect of transient 4xJERE:ipt expression on the proliferation of PstT. Tobacco leaves were pre-infiltrated with A. tumefaciens with (ipt) or without (0) the 4xJERE:ipt construct followed by a second infiltration with PstT. Bacteria were re-isolated from leaf discs, plated and quantified. Mean values ± standard deviation of three independent replicates.
A

24h
4h
2h
1h
0.5h
90sec

Symptom development

Feeding  Dipping  Water  Infection with *Pseudomonas*

B

7 dpi

H$_2$O  1 µM  2.5 µM  5 µM  7 µM  10 µM  14 µM  18 µM
Kinetin

C

13 dpi

H$_2$O  0.5 h  1 h  2 h  4 h
Kinetin

D

12 dpi

H$_2$O  Adenine  Kinetin  6-BAP  trans-Zeatin

E

6 dpi

H$_2$O  2 fM  200 fM  20 pM  200 pM  2 nM  20 nM  200 nM
TDZ
Figure 2. Exogenously supplied CKs enhance resistance against *PstT*.

A, Experimental scheme of exogenous applications of CKs by petiole feeding (green bar) and dipping (yellow bar) prior to infection with *PstT* (red arrow). The petioles from both CK-treated and control leaves were kept in water for symptom development. B, Effect of feeding varying kinetin concentrations (1-18 µM) 24 h prior to *PstT* infection on symptom development 7 dpi. C, Effect of short pulse feeding (0.5-4 h) with 10 µM kinetin prior to *PstT* infection on symptom development 13 dpi. D, Comparison of efficiency to induce resistance (12 dpi) against *PstT* for different adenine derived CKs applied in 10 µM concentration 24 h prior to infection. E, Dose dependent (2 fM to 200 nM) ability of phenylurea derived CK thidiazuron (TDZ) to induce resistance against *PstT* (6 dpi) by petiole feeding. F, Induction of resistance against *PstT* (11 dpi) by dipping leaf-halves for 60 s (left) and 90 s (right) in 140 µM kinetin 24 h before infection. Leaves were infiltrated with *PstT* at three sites per leaf half (B-F).
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Figure 4. Cytokinins induce resistance independent of SA, JA, ROS and extracellular invertase activity.

A, Effect of 24 h kinetin application (10 µM) before PstT infection on symptom development in nahG overexpressing tobacco leaves in comparison to control, 8 dpi. Leaves were infiltrated at two sites with PstT (left) and 10 mM MgCl₂ (right). B, Effect of transient 4xJERE:ipt expression and PstT infection on H₂O₂ formation. Tobacco leaves were pre-infiltrated with A. tumefaciens with (ipt) or without (0) the 4xJERE:ipt construct followed by a second infiltration with PstT (Pst). Leaves were stained with
DAB to visualize H$_2$O$_2$ formation 24 h after pre-infiltration of A. tumefaciens (0 hpi, left), 24 h after infiltration of PstT (24 hpi, middle), and at the time of symptom development 168 h after PstT infiltration (168 hpi, right). C, Effect of CIN1 expression in TET:CIN1 tobacco leaves induced by 24 h feeding of 1 mg L$^{-1}$ TET, in comparison to 24 h application of 10 µM kinetin prior to infection and water control on symptom development 10 dpi. Leaves were infiltrated with PstT at two sites per leaf half.
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A, Experimental data for time course analysis of scopoletin production (blue lines with circles, nmol g⁻¹ fw) in relation to bacterial growth (red lines with squares, cfu mL⁻¹) in *Pst* infected leaves pre-treated with CKs. Note the different scale for the scopoletin
levels compared to Figure 5B. B, Experimental data for scopoletin production and bacterial growth in *PstT* infected leaves (control). C, Model for CK-mediated phytoalexin production based on Figure 5A. Cytokinin feeding mediates increased phytoalexin levels resulting in a high phytoalexin/bacteria ratio, which restricts bacterial growth in the early infection phase. In the late infection phase bacterial growth is abolished, probably due to additional defence mechanisms triggered by the bacterial infection. D, Model for pathogen induced (control) phytoalexin production based on Figure 5B. Phytoalexin production is triggered by the bacterial infection, but the increase occurs after the onset of bacterial growth. This results in a low phytoalexin/bacteria ratio throughout the infection phase and consequently unrestricted bacterial growth. E, RNA blot analyses of tobacco tissue transiently transformed with 4xJERE:ipt for expression levels EAS, C4H and TOGT. 28S rRNA for loading control is shown in bottom panels. F, Phytoalexin infiltration confers pathogen resistance, evident by reduced symptom development 6 dpi. W38 leaves were infiltrated with different concentrations of capsidiol and scopoletin (200-400 µM, yellow) or according controls (grey), simultaneous with *PstT* bacteria (10^7 cfu mL^-1, red).