

Methylated Cytokinins from the Phytopathogen *Rhodococcus fascians* Mimic Plant Hormone Activity¹[OPEN]

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Cytokinins (CKs), a class of phytohormones that regulate plant growth and development, are also synthesized by some phytopathogens to disrupt the hormonal balance and to facilitate niche establishment in their hosts. *Rhodococcus fascians* harbors the *fasciation* (*fas*) locus, an operon encoding several genes homologous to CK biosynthesis and metabolism. This pathogen causes unique leafy gall symptoms reminiscent of CK overproduction; however, bacterial CKs have not been clearly correlated with the severe symptoms, and no virulence-associated unique CKs or analogs have been identified. Here, we report the identification of monomethylated *N*⁶-(Δ^2 -isopentenyl)adenine and dimethylated *N*⁶-(Δ^2 -isopentenyl)adenine (collectively, methylated cytokinins [MeCKs]) from *R. fascians*. MeCKs were recognized by a CK receptor and up-regulated type-A *ARABIDOPSIS THALIANA* *RESPONSE REGULATOR* genes. Treatment with MeCKs inhibited root growth, a hallmark of CK action, whereas the receptor mutant was insensitive. MeCKs were retained longer in planta than canonical CKs and were poor substrates for a CK oxidase/dehydrogenase, suggesting enhanced biological stability. MeCKs were synthesized by *S*-adenosyl methionine-dependent methyltransferases (MT1 and MT2) that are present upstream of the *fas* genes. The best substrate for methylation was isopentenyl diphosphate. MT1 and MT2 catalyzed distinct methylation reactions; only the MT2 product was used by FAS4 to synthesize monomethylated *N*⁶-(Δ^2 -isopentenyl)adenine. The MT1 product was dimethylated by MT2 and used as a substrate by FAS4 to produce dimethylated *N*⁶-(Δ^2 -isopentenyl)adenine. Chemically synthesized MeCKs were comparable in activity. Our results strongly suggest that MeCKs function as CK mimics and play a role in this plant-pathogen interaction.

The balance of phytohormones, such as cytokinins (CKs) and auxins, is finely controlled to maintain proper plant growth and development but is often disturbed following pathogen infection (Robert-Seilaniantz et al., 2007; Pieterse et al., 2012). As a virulence strategy, many phytopathogens synthesize phytohormones that cause aberrant organogenesis and modulate primary carbon

metabolism that ultimately aids disease establishment (Jameson, 2000; Robert-Seilaniantz et al., 2007). For several pathogens, CK production is essential for virulence, and they carry genes for CK biosynthesis in a harbored plasmid (Jameson, 2000). Fungal pathogens employ CKs to form green islands with delayed senescence, whereas bacterial pathogens develop gall structures (Sakakibara et al., 2005; Walters et al., 2008; Giron et al., 2013). *Rhodococcus fascians* is a gram-positive actinomycete that causes symptoms ranging from leaf deformation to differentiated shooty outgrowths known as leafy galls in more than 150 different plant species (Goethals et al., 2001; Stes et al., 2011). In ornamental plants, such infections reduce their value and contribute to economic losses worldwide (Putnam and Miller, 2007). Leafy gall symptoms are reminiscent of CK overproduction and can be partially induced by exogenous application of CKs (Thimann and Sachs, 1966; Eason et al., 1996). Although several CKs have been isolated from *R. fascians* culture filtrates, a clear correlation with pathogenesis is lacking partially owing to the low concentration of bacterial CKs (Eason et al., 1996). A synergistic action by a mixture of bacterially produced CKs has been proposed, leading to persistent accumulation of CKs locally (Pertry et al., 2009). Nevertheless, to date, no virulence-associated CK

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V.R. and H.S. designed the research; V.R., N.U., M.K., T.K., and H.S. performed the research; Y.T. and J.K. performed the NMR analysis; V.R., N.U., M.K., Y.T., J.K., and H.S. analyzed the data; V.R. and H.S. wrote the article.

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analogues have been identified that could contribute to the infection symptoms.

Naturally occurring CKs are adenine derivatives with different side chains at the N⁶ position. Major plant CKs are N⁶-prenylated adenine derivatives such as N⁶-(Δ^2 -isopentenyl)adenine (iP), trans-zeatin (tZ), cis-zeatin (cZ), and dihydrozeatin, collectively known as isoprenoid CKs (Sakakibara, 2006). Among them, iP and tZ are the major CKs in *Arabidopsis* (*Arabidopsis thaliana*). iP is synthesized by adenosine phosphate-isopentenyl transferase (IPT) using dimethylallyl diphosphate (DMAPP) and adenosine phosphate as substrates (Sakakibara, 2006). tZ is formed by hydroxylation of the trans-end of the prenyl side chain of the iP nucleotide. CK homeostasis is governed by both biosynthesis and catabolism and has an important regulatory role in plant growth (Sakakibara, 2006; Werner et al., 2006). CK oxidase/dehydrogenase (CKX) is responsible for an irreversible reaction cleaving the unsaturated isoprenoid side chain that results in the formation of adenine and the corresponding aldehyde (Werner et al., 2006). In *Arabidopsis*, CKs are perceived by a subset of sensory His kinases, ARABIDOPSIS HIS KINASE2 (AHK2) to AHK4, which undergo a His-Asp phosphorelay leading to induction of direct target genes including type-A ARABIDOPSIS RESPONSE REGULATOR (ARR) genes (Kieber and Schaller, 2010). This two-component signaling system has been implicated in mediating basal and pathogen-induced plant immunity (Choi et al., 2010; Argueso et al., 2012). For instance, infection of *Arabidopsis* plants by *R. fascians* reportedly activates type-A ARR5 expression with increased expression of AHK3 and AHK4, resulting in mitotic cell divisions that arrest the infected leaves in a meristematic state to establish a nutrient-rich niche (Depuydt et al., 2008, 2009; Pertry et al., 2010; Stes et al., 2011). As the infection progresses, IPT genes are switched off, whereas the expression of all CKX genes are strongly induced in symptomatic tissues (Depuydt et al., 2008).

The virulence determinant of *R. fascians* is located within the *fasciation* (*fas*) locus, an operon encoding several genes involved in CK metabolism, indicating that CKs are essential for this plant-pathogen interaction (Stes et al., 2011). *fas4* encodes IPT that catalyzes the rate-limiting step of CK biosynthesis and is vital for virulence (Stes et al., 2013). Interestingly, two methyltransferase-like genes are present upstream of the *fas* gene, whose functions have been unknown. Despite the presence of the *fas* genes in *R. fascians*, fewer known CKs have been detected compared with other gall-causing pathogens such as *Pantoea agglomerans*, *Agrobacterium tumefaciens*, and *Pseudomonas savastanoi* (Goethals et al., 2001). Further, the leafy gall phenotype is unique, not invoked by any of the above-mentioned pathogens, implying that the virulence of *R. fascians* might not be due to typical CKs alone (Goethals et al., 2001). *R. fascians* has long been hypothesized to produce CK analogues using similar or modified substrates (Goethals et al., 2001; Galis et al., 2005; Stes et al., 2011), but no such molecules have been discovered so far. Here, we report the identification and mode of

biosynthesis for methylated cytokinins (MeCKs) as hormone mimics from *R. fascians*. These compounds are synthesized by two methyltransferases and FAS4. Their CK-like activity and higher in planta stability suggest a role for the methylated analogues as CK mimics that foster efficient pathogenesis.

RESULTS

Isolation and Structural Determination of MeCKs

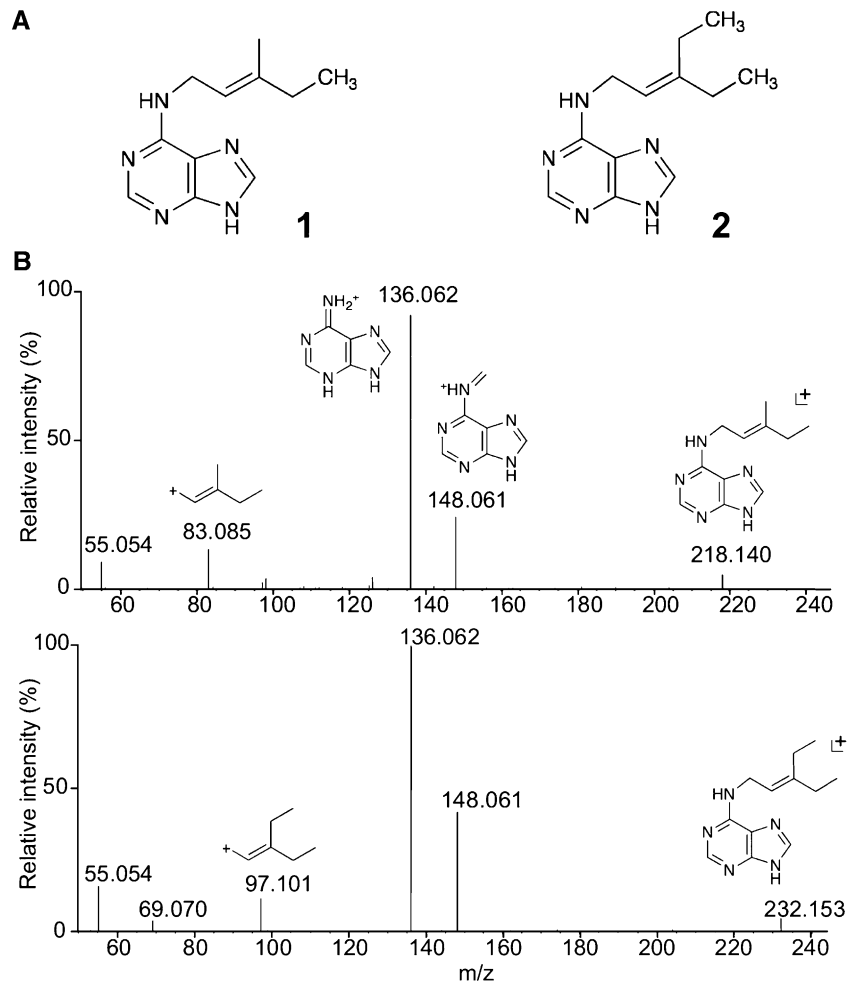
Upstream of the *fas* genes, there are two putative methyltransferase-like genes, *mt1* and *mt2*, but their function and relation to CK biosynthesis has remained unknown (Stes et al., 2011). To examine the involvement of the *mt* genes in the synthesis of CKs, *fas4mt1mt2*, *fas4mt1*, and *fas4mt2* were overexpressed in *Escherichia coli*, and liquid culture filtrates were analyzed by liquid chromatography (LC)-quadrupole tandem mass spectrometry (MS). In the filtrates of *fas4mt1mt2* and *fas4mt2*, two new CK-like peaks were detected; one peak (peak 2) corresponds to a compound with a mass-to-charge ratio (*m/z*) of 218 and the other peak (peak 3) has a *m/z* of 232 (Supplemental Fig. S1, C and E–G). Peak 2 was also detected in small amounts in the filtrate of *fas4mt1* (Supplemental Fig. S1D). These compounds were purified from large-scale *E. coli* cultures. Characterization by Orbitrap MS and NMR analyses revealed that the compounds obtained from the *fas4mt2* and *fas4mt1mt2* filtrates were monomethylated N⁶-(Δ^2 -isopentenyl)adenine (1MeiP) and dimethylated N⁶-(Δ^2 -isopentenyl)adenine (2MeiP), respectively (Fig. 1).

To ascertain whether MeCKs are involved in the infection process, tobacco (*Nicotiana tabacum*) plants infected with *R. fascians* were analyzed. In our experimental conditions, leafy galls became visible after 6 to 7 d post infection (dpi). Overall, the levels of all CKs were significantly higher in plants after infection than in the uninfected control plants, in which iP and MeCKs were below the quantification limits (Fig. 2; Supplemental Table S1). Our analyses detected 1MeiP and 2MeiP in the infected leaves of tobacco (Fig. 2). The MeCKs were not detected on 1 dpi, but from 2 dpi, the MeCK levels increased in the infected plants (Fig. 2). The levels of 2MeiP and iP were similar, reaching their highest concentrations at 3 dpi and then gradually decreasing (Fig. 2), whereas 1MeiP was only found at a low concentration. Total MeCKs were 33% of the total active CK concentration at 3 dpi. A steady decrease in the levels of tZ was observed in both infected and uninfected plants (Fig. 2). On the other hand, methylthio derivatives of CK, such as 2-methylthio-N⁶-(Δ^2 -isopentenyl)adenosine and 2-methylthio-cis-zeatin, were below the detection limit in this experiment. These results indicate that infection of *R. fascians* causes the accumulation of MeCKs preceding leafy gall formation.

MeCKs Can Mimic CK Action

Symptoms of *R. fascians* infections are characteristic of CK overproduction and are correlated with a strong

Figure 1. MeCKs produced by *R. fascians mts* and *fas4*. A, Chemical structures of 1MeiP and 2MeiP isolated from filtrates of *E. coli* cultures expressing *R. fascians fas4mt2* and *fas4mt1mt2*, respectively. 1, 1MeiP, $^1\text{H NMR}$ (DMSO-d_6): δ (ppm) = 12.86 (brs, 1H), 8.17 (s, 1H), 8.01 (s, 1H), 7.61 (s, 1H), 5.32 (t, 6.2 Hz, 1H), 4.08 (brs, 2H), 1.98 (q, 6.93 Hz, 2H), 1.70 (s, 3H), and 0.95 (t, 7.39 Hz, 3H). 2, 2MeiP, $^1\text{H NMR}$ (DMSO-d_6): δ (ppm) = 12.91 (brs, 1H), 8.17 (s, 1H), 8.06 (s, 1H), 7.67 (s, 1H), 5.25 (t, 6.2 Hz, 1H), 4.08 (brs, 2H), 2.13 (q, 7.5 Hz, 2H), 1.99 (q, 7.3 Hz, 2H), 0.97 (t, 7.4 Hz, 3H), and 0.94 (t, 7.4 Hz, 3H). B, Fragmentation patterns of MeCKs. MS spectra of 1MeiP (top) and 2MeiP (bottom) were analyzed with a hybrid quadrupole-Orbitrap mass spectrometer in the positive ion mode. Predicted fragmented structures are shown above the peaks.



CK response. To examine whether the MeCKs have CK activity, the Arabidopsis wild type and the *ahk3ahk4* double mutant, in which CK perception is deficient, were grown in the presence of 100 nM iP, tZ, 1MeiP, and 2MeiP, and root growth was monitored. Treatment with MeCKs significantly inhibited primary root growth, as did iP and tZ treatments (Fig. 3). The *ahk3ahk4* double mutant was insensitive to both iP and MeCK treatments (Fig. 3).

A previous study demonstrated that heterologous expression of Arabidopsis *AHK4* in budding yeast (*Saccharomyces cerevisiae*) complements the yeast *synthetic lethal of N-end rule1 (sln1)* mutation in a CK-dependent manner (Inoue et al., 2001). Normal yeast growth was observed in the presence of iP and MeCKs, whereas the mutant yeast cells with the empty vector were able to grow only in the presence of Gal (Supplemental Fig. S2).

Type-A *ARR* genes are the primary transcriptional targets of CK signaling and are specifically induced in response to exogenous CKs (To et al., 2004). The effect of MeCKs on inducing the expression of two type-A-*ARR* genes, *ARR5* and *ARR6*, was examined (Fig. 4). Quantitative reverse transcription-PCR analysis showed that *ARR* gene expression was induced by

treatment with 0.1 μM MeCKs, and the dose dependency was similar with iP (Fig. 4). Together, these results show that the MeCKs have activity equivalent to canonical CKs.

In Planta Stability of MeCKs

R. fascians infection triggers a homeostasis mechanism in the host plant directed to reduce excess CKs, as shown by the down-regulation of plant *IPT* genes and activation of CK-degrading CKX enzymes (Depuydt et al., 2008). To monitor the fate of MeCKs in the homeostasis mechanism, we examined their in planta stability (Fig. 5). Wild-type Arabidopsis plants supplied with equal amounts of ^{13}C -labeled iP and MeCKs were found to retain a significantly higher percentage of MeCKs (approximately 20%–30%) after 60 min, whereas levels of [^{13}C]iP were reduced to approximately 10% (Fig. 5A), suggesting that MeCKs are more stable in planta compared with iP. Additionally, an in vitro assay with partially purified recombinant CKX2 showed that MeCKs are poor substrates for this enzyme when compared with iP (Fig. 5B). Similar to cZ, which is barely degraded by CKX, 2MeiP

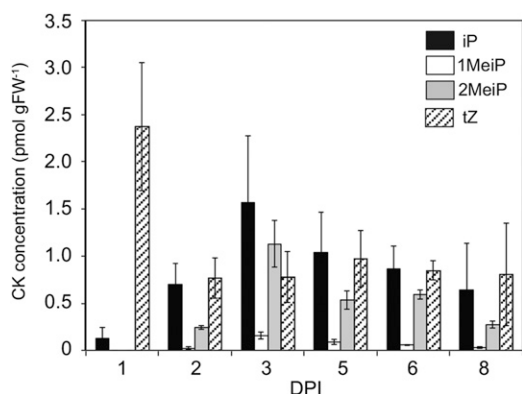


Figure 2. CK concentrations in tobacco plants after infection with *R. fascians*. Error bars represent the SD of three biological replicates. The complete data set, including cZ, is presented in Supplemental Table S1. FW, Fresh weight.

was not degraded by CKX2, and 1MeiP was less suitable as a substrate than iP and tZ (Fig. 5B) as measured by the reduction of the electron acceptor 2,6-dichloroindophenol. These results show that methylation of iP could circumvent or delay the homeostasis mechanisms leading to hyperaccumulation of CKs in host plants.

Biosynthesis of CK Mimics

To investigate the biosynthesis of MeCKs, recombinant MTs and FAS4 proteins were purified. The recombinant MTs (MT1 and MT2) were assayed for methyltransferase activity in vitro using *S*-adenosyl methionine (SAM), various CKs and their conjugates, iP, iP riboside, iP riboside 5'-monophosphate, tZ, tZ riboside, and tZ riboside 5'-monophosphate, and isoprenoid diphosphates such as DMAPP, isopentenyl diphosphate (IDP), and 1-hydroxy-2-methyl-2(*E*)-butenyl 4-diphosphate as substrates. Interestingly, the only substrate that was methylated in our assay was IDP (Fig. 6A). Methylation by MT1 and MT2 led to different reaction products with IDP, as evidenced by their different retention times (Fig. 6B). Both MT1 and MT2 reaction mixtures had very low amounts of the dimethylated IDP ($m/z = 273$; Fig. 6C). When both MT1 and MT2 enzymes were present together in the reaction, dimethylated IDP was formed in higher amounts (Fig. 6C). These results were further confirmed by gas chromatography (GC)-MS analysis of the corresponding alcohols obtained after dephosphorylation by alkaline phosphatase (Supplemental Fig. S3). The MT2 reaction produced 3-methyl-2-penten-1-ol, as confirmed by comparison with a synthetic standard (Supplemental Fig. S3, C and D). A mixture of MT1 and MT2 enzymes led to 3-ethyl-2-penten-1-ol, also confirmed by comparison with a synthetic standard (Supplemental Fig. S3, E and F). The MT1 product could not be identified due to non-correspondence with any available candidate standards. Our results clearly showed that MT1 and MT2 transform IDP in different ways, leading to monomethylated

products, and when present together, they give rise to dimethylated IDP. Notably, the monomethylated products of MT2 have mass fragments corresponding to allyl alcohol ($m/z = 71.1$; Supplemental Fig. S3, B–D), suggesting that the MT reaction also isomerizes IDP.

Next, to investigate the order of methylation, we used a sequential enzymatic reaction assay, where one of the MTs was added first, followed by addition of the other MT to the same reaction mixture. When MT2 was added first and allowed to react for a day followed by MT1, both isomers of the methylated IDP were observed, although the amount of the MT2 product was higher (Supplemental Fig. S4A). In this case, a very low level of the dimethylated product was observed (Supplemental Fig. S4B). When the order of addition was reversed, only the MT1 product was observed as a small peak (Supplemental Fig. S4A). Here, an intense peak of the dimethylated product could be seen (Supplemental Fig. S4B). This result shows that the dimethylated product is formed only when MT2 reacts on the MT1 product and not vice versa; in other words, MT2 can utilize IDP as well as the MT1 product as a substrate.

To understand whether the *R. fascians* FAS4 enzyme can utilize the methylated IDPs as substrates, we linked the FAS4 reaction to follow the MT reaction. The MT2 reaction followed by FAS4 and phosphatase produced 1MeiP riboside (1MeiPR) and small amounts of 2MeiP riboside (2MeiPR; Supplemental Fig. S5). This finding is in line with our earlier results where 1MeiP was purified from *fas4mt2*-expressing *E. coli* filtrates. When FAS4 was added to MT1 reaction mixtures, no product was observed, indicating that the MT1 product is not utilized by FAS4. Interestingly, the product of the sequential reaction of MT1 followed by MT2 was readily utilized by FAS4 to form 2MeiPR as a single product (Supplemental Fig. S5). Similarly, in the reaction where MT2 was added first followed by MT1 and later FAS4, 1MeiPR was produced (Supplemental Fig. S5). These results clearly show that FAS4 can utilize the MT2 product directly to synthesize 1MeiPR but cannot use

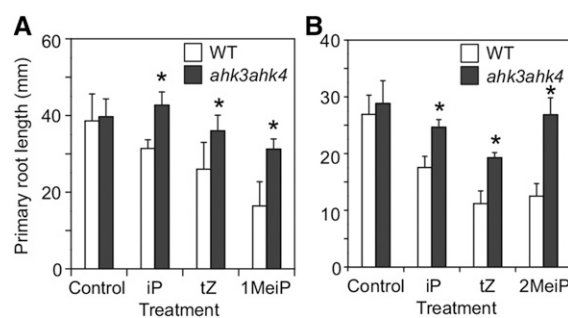


Figure 3. Effect of MeCKs on primary root growth in Arabidopsis. The Arabidopsis wild type (WT) and the *ahk3ahk4* mutant were germinated and grown on Murashige and Skoog agar plates containing 100 nM iP, tZ, or 1MeiP (A) and 100 nM iP, tZ, or 2MeiP (B) for 7 d. The lengths of the primary roots were measured. Asterisks represent statistical significances between the wild type and the mutant (one-way ANOVA, $P < 0.05$). Error bars represent the SD of 10 biological replicates.

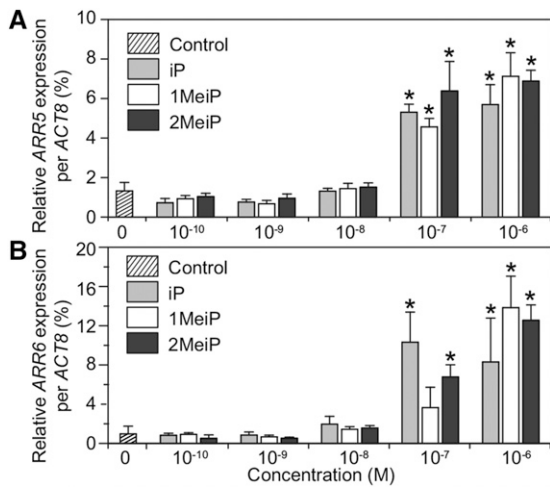


Figure 4. Induction of CK-responsive gene expression by MeCKs. Two-week-old Arabidopsis wild-type seedlings were treated with iP, 1MeiP, or 2MeiP at the indicated concentrations by vacuum infiltration for 1 h. Expression levels of *ARR5* (A) and *ARR6* (B) were analyzed by quantitative reverse transcription-PCR. The expression levels were normalized using *ACTIN8* (*ACT8*) as the internal control. Error bars represent the SD of four or five biological replicates. Asterisks indicate statistically significant differences compared with a mock treatment (one-way ANOVA, $P < 0.05$).

the MT1 product; however, the MT1 product is used as a substrate by MT2 to form dimethylated IDP, which is then utilized by FAS4 to synthesize 2MeiPR (Supplemental Figs. S4 and S5). Thus, methylation occurs prior to prenyl transfer, and only one of the monomethylated IDPs is utilized by FAS4.

Chemical Synthesis of CK Mimics

1MeiP and 2MeiP were chemically synthesized starting from commercially available 2-butanone and 3-pentanone, respectively (Supplemental Fig. S6), and were compared with pathogen-derived MeCKs (Supplemental Fig. S7). The chemically synthesized MeCKs were tested for their inhibition of root elongation compared with the pathogen-derived MeCKs and iP (Supplemental Fig. S8). Both synthesized MeCKs and pathogen-derived MeCKs reduced root elongation in a manner similar to iP. Root elongation in the *ahk3ahk4* mutant did not change in any of the treatments. There was no significant difference among various CK treatments. These results demonstrate that synthetic MeCKs are comparable in activity to MeCKs obtained from *R. fascians*.

DISCUSSION

Many pathogens produce phytohormones such as jasmonic acid (JA), CKs, abscisic acid, and auxins (Crocoll et al., 1991; Costacurta and Vanderleyden, 1995; Mittal and Davis, 1995; MacMillan, 2001). *R. fascians* is known to secrete CKs and indole-acetic acid, an auxin, but the amounts of these phytohormones are low when compared with other gall-causing pathogens (Galis et al., 2005;

Depuydt et al., 2008). The low concentration of bacterial CKs might be one reason that no new CKs or CK analogs have been reported so far. In this study, two MeCKs were identified as new CK analogs from *R. fascians* using highly sensitive MS analysis and were isolated from *E. coli* cultures expressing the relevant *fas* genes. In an older study, 6-(3-methylpent-trans-2-enylamino) purine (Fig. 1A) was chemically synthesized as a zeatin analog and was shown to induce cell expansion in radish (*Raphanus sativus*) cotyledons (Letham, 1972). In our study, we identified the bacterially produced 1MeiP and 2MeiP and elucidated the biosynthetic pathway.

Methylation plays an important role in plants and is commonly used to modify metabolites. The carboxyl groups of JA, salicylic acid, GAs, and indole-acetic acid are methylated to form the corresponding methyl esters by the SABATH family of methyltransferases (Qu et al., 2010). Methylation facilitates enhanced movement by diffusion through cell membranes, and some methylated phytohormones such as methyl JA and methyl salicylic

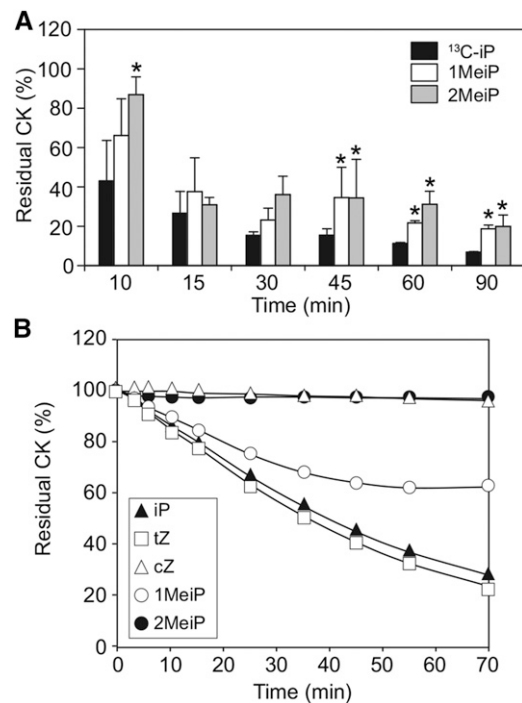


Figure 5. Stability of MeCKs. A, Comparison of the metabolism of CKs in planta. Two-week-old Arabidopsis wild-type seedlings were treated with $1 \mu\text{M}$ ^{13}C -labeled iP, 1MeiP, or 2MeiP by vacuum infiltration for 1 h. The seedlings were then placed in water and harvested at the indicated times. The residual amount of supplied CKs in the seedling tissues was analyzed. Asterisks represent statistically significant differences among the various treatments (one-way ANOVA, Tukey's honestly significant difference posthoc test, $P < 0.05$). Error bars represent the SD of three biological replicates. B, Reactivity of MeCKs to CKX in vitro. The indicated CK compounds ($50 \mu\text{M}$) were incubated with recombinant Arabidopsis CKX2 in the presence of 2,6-dichlorophenolindophenol. Reduction of 2,6-dichlorophenolindophenol was monitored by measuring the A_{600} at the indicated times.

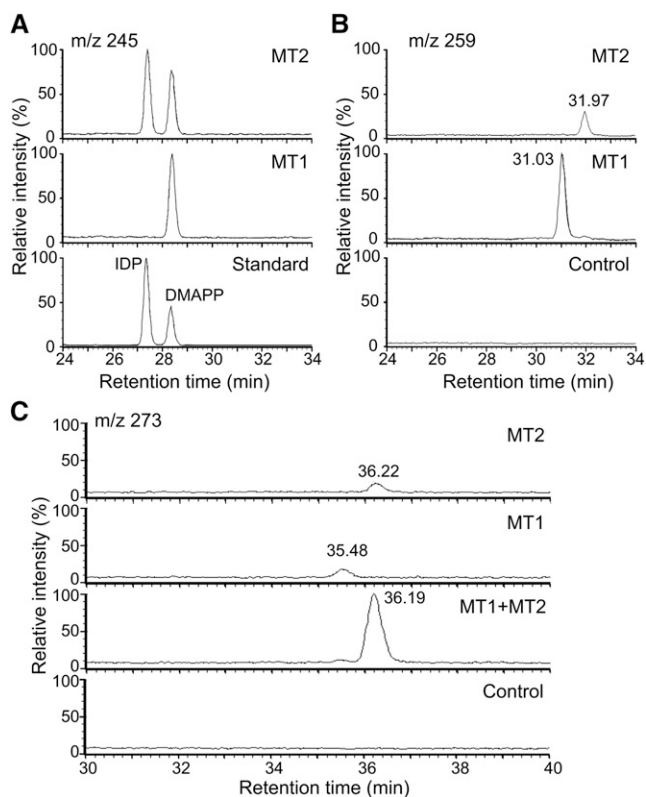


Figure 6. Characterization of MT1 and MT2 substrates. LC-MS chromatograms of in vitro MT assay reaction products. The reaction mixtures containing SAM, IDP, and DMAPP and MT enzymes in MT buffer were incubated at 30°C for 20 h. The reaction products were analyzed by LC-MS with selected ion modes: the chromatograms of $m/z = 245$, which corresponds to IDP and DMAPP (A); $m/z = 259$, which corresponds to a monomethylated prenyl substrate (B); and $m/z = 273$, which corresponds to a dimethylated prenyl substrate (C). In A, the chromatogram of the standards is shown in the bottom section, and the highest peak in each chromatogram is indicated as 100. In B and C, the chromatograms of the control (–MT enzymes) are shown in the bottom sections, and the highest peak in each section is indicated as 100.

acid are biologically active as plant defense signals in vivo (Reymond and Farmer, 1998). We hypothesize that by secreting MeCKs, the pathogen can also facilitate movement across cellular membranes without a specific transport system. Nevertheless, whether bacterial MeCKs are transported or metabolized into corresponding conjugates requires further investigation. It is interesting to note that previous studies reported the accumulation of CK-methylthio derivatives in *Arabidopsis* after *R. fascians* infection as well as in *R. fascians* culture (Pertry et al., 2009, 2010; Stes et al., 2013). However, in our study, these CK derivatives were below the detection limit in the recombinant *E. coli* cultures expressing *mts* and *fas4* as well as in the *R. fascians*-infected tobacco plants. One possible explanation for this discrepancy is genotype variation of the *R. fascians* strains used in our respective studies, resulting in a different capacity for CK-methylthio derivative production. However, in all studies, infection of *R. fascians*

induced leafy gall formation. In addition, in vitro studies demonstrated that MT1 and MT2 catalyze the methylation of the prenyl-donor substrate for FAS4. Thus, the two MTs are not involved in the synthesis of CK-methylthio derivatives.

Functional analyses demonstrated that MeCKs mimic CK action. Their recognition by the CK receptor *AHK4* and the nonresponsiveness of the *ahk3ahk4* double mutant clearly showed that MeCKs are perceived in a manner similar to classical CKs. The *Arabidopsis ahk3ahk4* mutants are known to respond poorly toward *R. fascians* infection (Pertry et al., 2009), an observation that is in line with our findings. MeCKs also induced the expression of downstream *ARR* genes, corroborating a previous observation that bacterial infection induces expression of the *ARR* genes (Pertry et al., 2010). Further, MeCKs were retained at higher levels than iP, suggesting a delay in compensatory homeostasis mechanisms. This hypothesis is further supported by our result that MeCKs are not good substrates for CKX2, an enzyme whose transcript strongly accumulates in *R. fascians*-infected *Arabidopsis* (Depuydt et al., 2008). On the other hand, in vivo experiments showed a decrease in the MeCK levels with time (Fig. 5) that could be due to either the action of other CKX enzymes or metabolic conversion into the corresponding glucosides, nucleotides, or nucleosides that are common modifications of CKs in planta (Sakakibara, 2006).

R. fascians virulence is primarily due to the CKs derived from the *fas* operon (Stes et al., 2011). *fas1*, encoding a putative cytochrome P450 monooxygenase, and *fas4* are absolutely essential for virulence, as evidenced by the *fas* mutants characterized in the strain D188 (Crespi et al., 1992, 1994). FAS2 and FAS3 are hypothesized accessory proteins that generate the reducing power for FAS1 (Goethals et al., 2001). FAS5 is homologous to CKX, although the actual substrate is still elusive (Pertry et al., 2010; Stes et al., 2011). FAS6, a phosphoribohydrolase, is probably involved in generating CK bases essential for symptom maintenance

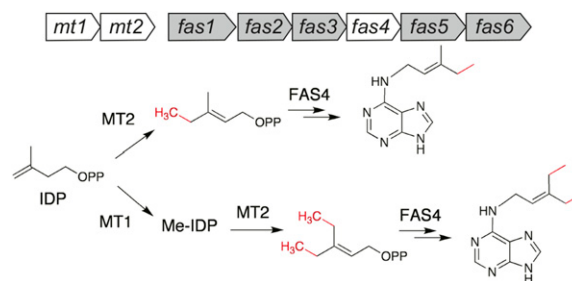


Figure 7. A model of reaction schemes for MeCK production by *R. fascians*. MeCK biosynthesis starts from IDP, which is methylated and then utilized by FAS4. Gene organization of the *fas* operon and *mt1* and *mt2* are shown above the schemes. The MT1 and MT2 products are different, and only the MT2 product is directly utilized by FAS4 as shown. The MT1 product (methylated [Me]-IDP) is dimethylated by MT2, and the dimethylated product is used as a substrate by FAS4 to yield 2MeiPR 5'-monophosphate. The nucleotides are hypothesized to be converted to nucleobases by FAS6 or by bacterial metabolism. PP, Diphosphoric acid.

(Pertry et al., 2010). Our study also suggests a role for *mts*, which is upstream of the *fas* genes, in *R. fascians* pathogenicity. Both *mt1* and *mt2* mutants of *R. fascians* are nonpathogenic and incapable of inhibiting seedling growth in tobacco (Pertry, 2009). The proximal location of methyltransferase-like genes is also reported for *Streptomyces turgidiscabies*, another phytopathogen that carries the *fas* operon, although function of the genes is not known (Joshi and Loria, 2007). Our results show that MTs are essential for MeCK biosynthesis and that IDP is the most suitable substrate for MT1 and MT2. Interestingly, FAS4 failed to use IDP as a substrate, but methylated IDP was readily utilized. The *R. fascians ipt* gene (*fas4*) has low (approximately 30%) overall amino acid identity with *ipt* sequences of other hyperplasia-inducing pathogens like *P. savastanoi* and *A. tumefaciens*. (Crespi et al., 1992; Temmerman et al., 2000). The FAS4 protein is unique in utilizing methylated IDP as a substrate, corroborating its different enzymatic capability. IDP is isomerized to its more active isomer, DMAPP, for canonical CK biosynthesis (Sakakibara, 2006); however, in this study, DMAPP was not a good substrate for *R. fascians* MTs. MeCKs are thus synthesized via a biochemical pathway similar to that of CKs but with different substrates (Fig. 7).

We identified 3-methyl-2-pentene diphosphate as the reaction product of MT2 but could not identify the reaction product of MT1. Because the IDP plus MT1 product does not have mass fragments corresponding to allyl alcohol (Supplemental Fig. S3G), MT1 is not expected to have isomerization activity. Based on these observations, 3-methyl-3-pentene diphosphate or 3-methylidene pentane diphosphate are possible candidates.

Upon pathogen attack, a complex phytohormone signaling network is activated in plants causing defensive responses. Pathogens either suppress or hijack these pathways by secreting effectors or phytohormone mimics to promote disease establishment. The best-studied example of such molecules is the JA mimic, coronatine produced by *Pseudomonas syringae* (Mittal and Davis, 1995; Fonseca et al., 2009), that stimulates opening of the stomata and also suppresses SA-dependent defenses (Brooks et al., 2005). *R. fascians* is not known to enter through stomata and is usually present on the exterior of the infection sites (Goethals et al., 2001) but secretes highly stable MeCKs. Whether these CK mimics affect the signaling of other phytohormones remains to be elucidated. Synthetic and/or natural phytohormone mimics are vital to the agrochemical industry. Using chemical genomics approaches, many such synthetic compounds have been identified (Fonseca et al., 2014). Natural phytohormone mimics are valuable for elucidating pathogen biochemical pathways and for providing insights into the coevolutionary arms race.

MATERIALS AND METHODS

Bacterial Strains

Rhodococcus fascians 6162 (ATCC 35014, Tilford 1936) was obtained from the RIKEN BioResource Center Microbe Division (Japan Collection of

Microorganisms). All experiments using the living strain were conducted in the permitted area of the BioResource Center. Four-week-old tobacco (*Nicotiana tabacum*) 'Petit Havana SR1' was infected by local application of a bacterial culture to the stump of the cut shoot apical meristem (Temmerman et al., 2000).

Overexpression of *Rhodococcus* spp. Genes in *Escherichia coli*

For the isolation of MeCKs, the coding regions of *fas4*, *mt1* (National Center for Biotechnology Information accession no. JN093097, Region:76291.0.77142), and *mt2* (National Center for Biotechnology Information accession no. JN093097, Region:77182.0.78033) were amplified with extracted DNA from the *R. fascians* strain using gene-specific primers (Supplemental Table S2). The PCR products were ligated into the pCOLD-IV vector (Takara). The BL21 (DE3) strain harboring pG-Tf2 (Takara) was used as the *E. coli* host. Expression of these three proteins was induced in modified M9 medium (Takei et al., 2001) for 20 h at 15°C. CKs were purified from the cell-free medium.

Isolation and Purification of MeCKs

The cell-free *E. coli* supernatants were filtered through 0.45- μ m membrane filters before passing through solid phase extraction cartridges (OASIS HLB [6 g] followed by filtration through OASIS MCX [6 g]; Waters). The MCX cartridge was equilibrated with 1 M formic acid. After passing the filtrates, the cartridge was successively washed with methanol followed by 0.35 M NH₄OH. The CKs were eluted with 0.35 M NH₄OH in 60% (v/v) methanol. The eluent was dried and purified on Sephadex-LH20 (GE Healthcare). The fractions were further purified by semipreparative HPLC (Waters Alliance 2695 HPLC system) with an ODS column (Symmetry C18, 5.0 μ m, 19 \times 100 mm; Waters).

Mass Fragmentation Analyses

Purified MeCKs from *E. coli* liquid culture filtrates were analyzed by Q-Exactive (Thermo Scientific), a hybrid quadrupole-Orbitrap mass spectrometer in the positive ion mode, and fragmented structures were predicted with Mass Frontier (Thermo Scientific).

NMR Analyses

Spectra were recorded on an AVANCE II-700 spectrometer (Bruker) equipped with an inverse triple resonance cryogenic probe with a z-axis gradient operating at 700.154 MHz for ¹H (176.061 for ¹³C). All samples were inserted in 5-mm-diameter tubes and maintained at 298 K.

CK Quantification

Extraction and determination of CKs in plant material were performed as described previously (Kojima et al., 2009).

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia was used as the wild type. The CK receptor *ahk3ahk4* double mutant was characterized previously (Higuchi et al., 2004). Plants were grown on vertical one-half-strength Murashige and Skoog agar plates supplemented with 1% (w/v) Suc at 22°C under fluorescent lights (100 μ mol m⁻² s⁻¹, 16-h-light/8-h-dark cycle).

CK Activity Assays

Stock solutions of CKs were prepared in dimethyl sulfoxide (DMSO). Root growth assays used *Arabidopsis* plants germinated on vertical one-half-strength Murashige and Skoog agar plates containing 1% Suc supplemented with the corresponding CK (100 nM) diluted in water. Expression analysis of ARR genes used 2-week-old seedlings that were vacuum infiltrated with diluted CKs for 1 h. Entire seedlings were frozen in liquid N₂ immediately and stored at -80°C until further analysis. For measuring CK stability in planta, 2-week-old seedlings were dipped in the corresponding CK solution in a 12-well plate taking care to dip the roots fully. After 1 h of uptake, the seedlings were transferred to water, and samples of whole plants were collected at selected

time intervals. The *sln1* yeast (*Saccharomyces cerevisiae*) assay was performed as previously described (Inoue et al., 2001).

Gene Expression Analyses

RNA was extracted with an RNeasy Plant Mini kit (QIAGEN). First-strand complementary DNA was synthesized by a Superscript III First-Strand Synthesis System (Life Technologies) using total RNA with oligo(dT)₂₀ primers. The gene expression analysis was performed by quantitative reverse transcription-PCR with the StepOne Plus Real-Time PCR System (Applied Biosystems) using gene-specific primers (Supplemental Table S2) and the KAPA SYBR FAST qPCR Kit (Kapa Biosystems) according to the manufacturer's protocol.

MS Analysis of Isoprenoid Phosphates

Analyses were performed by LC-MS equipped with an electrospray interface (Alliance 2695/ZQ2000; Waters) with an AS11-HC column (250 × 2 mm, 13 μm; Dionex) as described (Kollas et al., 2002). The capillary was held at 350°C at a voltage of 3.5 kV, and the peaks were detected by single ion recording in negative mode. For the analyses of MT products by GC-MS, the in vitro assay mixture was treated with alkaline phosphatase (Takara) for 30 min. The reaction was then purified by extraction with diethyl ether, dried over anhydrous MgSO₄, evaporated, and suspended in *n*-hexane for analysis. GC-MS was performed on a 7890A GC system equipped with a 5975C triple-axis mass selective detector (Agilent Technologies).

Recombinant Enzymes and Enzyme Assays

The coding regions of *mt1*, *mt2*, and *fas4* were ligated into the pCOLD1 (Takara) vector to express the corresponding His-tagged recombinant proteins. BL21 (DE3) harboring pG-Tf2 (Takara) was the host. Detailed procedures for purification of the recombinant proteins were described previously (Takei et al., 2001). The recombinant MT proteins were assayed for methyltransferase activity as follows: MT buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 10 mM MgCl₂, 1 mg mL⁻¹ bovine serum albumin, and 0.5 mM dithiothreitol), 2.5 mM SAM (Wako Chemicals), 100 μM IDP, DMAPP, or 1-hydroxy-2-methyl-2(*E*)-butenyl 4-diphosphate, and 40 μg of recombinant enzyme for a 100-μL reaction mixture. Arabidopsis CKX2 recombinant enzyme was prepared as described (Frébertová et al., 2007). The CKX assay was conducted as described (Laskey et al., 2003) with two independent assays.

Chemical Synthesis of 1MeiP and 2MeiP

The procedure was modified from Ogura et al. (1970). NaH (60% [w/v] in mineral oil, 8.6 mmol) was washed with hexane (10 mL) three times. After removing the hexane, the mixture was cooled on ice after the addition of 4 mL of dry tetrahydrofuran. To this, diethyl methoxycarbonylmethyl phosphonate (8.6 mmol) was slowly added in 1-mL aliquots to the dry tetrahydrofuran to avoid a violent reaction. Subsequently, the corresponding methyl alkyl ketone (8.6 mmol) was added and stirred at room temperature for 2 h. After completion, the reaction mixture was extracted into hexane and dried over anhydrous MgSO₄ overnight, and the solvents evaporated to yield 1. The products were confirmed by NMR analysis: methyl-3-ethylpent-2-enoate, ¹H NMR (CDCl₃) δ (ppm) = 5.61 (s, 1H), 3.68 (s, 3H), 2.62 (q, 7.5 Hz, 2H), 2.19 (q, 7.4 Hz, 2H), 1.07 (t, 7.5 Hz, 3H), and 1.07 (t, 7.2 Hz, 3H) and methyl (*E*)-3-methylpent-2-enoate (purified by distillation), ¹H NMR (CDCl₃) δ (ppm) = 5.64 (s, 1H), 3.66 (s, 3H), 2.14 (q, 7.5 Hz, 2H), 2.14 (s, 3H), and 1.04 (t, 7.5 Hz, 3H).

To prepare the alcohol, the corresponding alkenoate (2.1 mmol) in dry diethyl ether (5 mL) was added drop-wise to a solution of LiAlH₄ (3.2 mmol) in dry ether (7–8 mL) cooled on ice and then stirred overnight at room temperature. The next day, ice-cold water was added drop-wise to the cold reaction mixture, followed by 10 mL of 10% (w/v) NaOH, and the precipitate was filtered. The filtrate was extracted in ether and dried to obtain the alcohols: 3-ethylpent-2-en-1-ol, ¹H NMR (CDCl₃) δ (ppm) = 5.36 (t, 6.9 Hz, 1H), 4.17 (d1, 6.4 Hz, d2 6.0 Hz, 2H), 2.10 (q, 7.6 Hz, 2H), 2.06 (q, 7.4 Hz, 2H), 1.02 (t, 7.4 Hz, 3H), and 0.99 (t, 7.66 Hz, 3H) and (*E*)-3-methylpent-2-en-1-ol, ¹H NMR (CDCl₃) δ (ppm) = 5.41 (t, 6.9 Hz, 1H), 4.16 (dd, 5.2 Hz, 2H), 2.04 (q, 7.4 Hz, 2H), 1.68 (s, 3H), and 1.02 (t, 2.5 Hz, 3H). The next step in preparing tetracyclated adenosine followed by the Mitsunobu reaction was done according to a published procedure (Taravov et al., 2011) using diisopropyl azodicarboxylate instead of diethyl azodicarboxylate for the synthesis of *N*⁶-acetyl-2',3',5'-tri-*O*-acetyl-*N*⁶-(3-ethylpent-2-enyl)-adenosine.

This step was followed by an enzymatic reaction with *E. coli* purine nucleoside phosphorylase and alkaline phosphatase (Takei et al., 2003) to obtain 1MeiP and 2MeiP as the final products, respectively. An outline is shown in Supplemental Figure S6.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number JN093097 for *mt1* and *mt2*.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Detection of CK-like compounds in liquid culture filtrates of *E. coli* coexpressing *fas4* and *mts*.

Supplemental Figure S2. CK-dependent growth of yeast *sln1Δ* expressing Arabidopsis AHK4.

Supplemental Figure S3. Mass spectra of methylated prenyl compounds in GC-MS with electron ionization.

Supplemental Figure S4. Sequential reaction assay.

Supplemental Figure S5. Reaction products generated by the *R. fascians* FAS4 recombinant protein using the MT products as substrates.

Supplemental Figure S6. Chemical synthesis of 1MeiP and 2MeiP.

Supplemental Figure S7. Fragmentation patterns of purified and synthesized MeCKs.

Supplemental Figure S8. Effect of chemically synthesized MeCKs on primary root growth in Arabidopsis.

Supplemental Table S1. CK concentrations in tobacco plants after infection with *R. fascians*.

Supplemental Table S2. Primers used in this study.

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