Remodeling of Cytokinin Metabolism at Infection Sites of *Colletotrichum graminicola* on Maize Leaves

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When inoculated onto maize leaves at the onset of senescence, the hemibiotroph *Colletotrichum graminicola* causes green islands that are surrounded by senescing tissue. Taking advantage of green islands as indicators of sites of the establishment of successful infection and of advanced high-performance liquid chromatography tandem mass spectrometry methodology, we analyzed changes in the patterns and levels of cytokinins (CK) at high spatial and analytical resolution. Twenty individual CK were detected in green islands. Levels of *cis*-zeatin-9-riboside and *cis*-zeatin-9-riboside-5'-monophosphate increased drastically, whereas that of the most prominent CK, *cis*-zeatin-*O*-glucoside, decreased. The fungus likely performed these conversions because corresponding activities were also detected in *in vitro* cultures amended with CK. We found no evidence that *C. graminicola* is able to synthesize CK entirely *de novo* in minimal medium but, after adding dimethylallyl diphosphate, a precursor of CK biosynthesis occurring in plants, a series of *trans*-zeatin isoforms (i.e., *trans*-zeatin-9-riboside-5'-monophosphate, *trans*-zeatin-9-riboside, and *trans*-zeatin) was formed. After applying CK onto uninfected leaves, transcripts of marker genes for senescence, photosynthesis, and assimilate distribution were measured by quantitative reverse-transcribed polymerase chain reaction; furthermore, pulse-amplitude modulation chlorophyll fluorometry and single-photon avalanche diode analyses were conducted. These experiments suggested that modulation of CK metabolism at the infection site affects host physiology.

Cytokinins (CK) control crucial aspects of plant development such as postembryonic organogenesis (Pernisova et al. 2009); shoot, root (Kyojuka 2007), and cambial meristem activities (Matsumoto-Kitano et al. 2008); and senescence (Buchanan-Wollaston 1997; Kim et al. 2006). Evidence has accumulated that CK are produced not only in roots but also in aerial organs (Sakakibara 2006). CK constitute a structurally complex group of compounds derived from adenine that can carry diverse isoprenoid or aromatic side chains at the *N*⁶ position (Mok and Mok 2001; Sakakibara 2006). Isoprenoid CK

are usually categorized according to their structure and physiological activity into four functionally different groups, including i) bioactive nucleobases and ribosides (iP, iPR, Z, ZR, *cis*Z, *cis*ZR, DHZ, and DHZR) (for structures and abbreviations see Supplementary Table S1), ii) inactive or weakly active *N*-glucosides (iP7G, iP9G, Z7G, Z9G, *cis*Z7G, *cis*Z9G, DHZ7G, and DHZ9G), iii) inactive or weakly active *O*-glucosides (ZOG, ZROG, *cis*ZOG, *cis*ZROG, DHZOG, and DHZROG), and iv) nucleotides representing the first products of CK biosynthesis (iPRMP, ZRMP, *cis*ZRMP, and DHZRMP). Conventionally, the nucleobases are known as the active forms of CK that are recognized by membrane-bound receptors to mediate intracellular signaling (Kieber and Schaller 2010). Ribosylated forms (nucleosides) are considered transport forms of CK, whereas ribosylphosphates (nucleotides) may constitute a reservoir of precursors for potential side-chain modifications. The *O*-glucosides of CK are regarded as storage forms that can be reversibly converted into their corresponding nucleobases (Frebort et al. 2011; Letham and Palni 1983; Mok and Mok 2001). *N*-glucosylation of CK is irreversible, rendering such forms probably irretrievably inactivated (Frebort et al. 2011; Letham and Palni 1983; Mok and Mok 2001).

The most typical representative of isoprenoid CK and the most active in a number of bioassays is zeatin, which occurs as two isomers, *cis* and *trans*, referring to the position of the terminal hydroxyl group at the isoprenoid side chain. In the past, *cis*-zeatin (*cis*Z) and its derivatives were largely overlooked, being considered as adjunct to CK homeostasis, with only weak biological impact. The relevance of *cis*Z-type CK has just recently begun to emerge, due to the impressive progress in CK analysis, based on ultra-performance liquid chromatography (UPLC) in combination with sensitive and selective detection (tandem mass spectrometry [MS/MS]) (Kojima et al. 2009; Novak et al. 2008). The *cis*Z-type CK occur in high concentrations exceeding those of corresponding *trans*Z (*Z*) types in many plants. In a comprehensive screening of over 150 land plants, *cis*Z has been found to be ubiquitous throughout the plant kingdom. In many monocotyledons, including maize, *cis*Z-type CK accounted for approximately 90% of the total CK pool, with *cis*Z-*O*-glucoside (*cis*ZOG) and its 9-riboside (*cis*ZROG) being the most prevalent (Gajdosova et al. 2011). Although it is still not clear whether *cis*Z plays a critical role in plant development, the distinct isoprenoid origins of *Z* and *cis*Z (Kasahara et al. 2004) suggest that plants are able to modulate independently, individually, and specifically the levels of these CK isoforms. In addition to *cis*-*trans* isomerization, further consequences of structural CK diversity for plant physiolo-

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ogy are just beginning to emerge, including the role of CK di- and triphosphates as biosynthetic precursors; interconversion of free bases, ribosides, and nucleotides; side-chain hydroxylation; and reduction (Sakakibara 2006).

Beyond their significance for plant development, CK are also involved in pathogenic and symbiotic interactions between plants and microbes, including bacteria (Costacurta and Vanderleyden 1995; Frugier et al. 2008; Pertry et al. 2010; Sakakibara et al. 2005), protista (Ludwig-Müller and Schuller 2008), and fungi (Barker and Tagu 2000; Vadassery et al. 2008). It has been proposed that CK may play a role in the interactions of plants with biotrophic fungal pathogens (i.e., rusts and mildews) (Ashby 2000). In the latter pathosystems, particular symptoms called green islands occur. It is unknown whether biotrophic fungi either synthesize and actively secrete CK at infection sites or stimulate the host to increase CK levels, leading to locally increased invertase activity and creation

of a carbohydrate sink, the delay of senescence, and the appearance of green islands (Walters and McRoberts 2006). In order to understand the impact of alterations in CK levels and composition on pathogenicity, analyses have to be performed at high spatial resolution (i.e., exactly at the sites of infection), at which CK-mediated modulation of plant metabolism may occur. However, experimental proof for the impact of fungal pathogens on CK profiles at the infection site is scarce because previous investigations were carried out with extracts from whole, infected plant organs. Because infection sites represent only a minor part of the total leaf tissue at early stages of pathogenesis, infection-mediated changes in the CK profiles observed thus far may reflect only a part of the alterations.

We have previously shown that green islands occur also in the compatible interaction of *Zea mays* with the hemibiotrophic ascomycete *Colletotrichum graminicola* (Behr et al. 2010). In this pathosystem, green islands develop only on infected leaves at early senescence, whereas on mature leaves, a different symptom appears, which is called anthracnose. Although the fungus causes extended tissue collapse in anthracnose lesions, only little damage to the host plant occurs in green islands during initial stages of pathogenesis. Furthermore, in green islands, the fungal infection delays the decline of photosynthetic activity in a highly localized fashion (i.e., exactly at the infection site). Quantitative reverse-transcribed polymerase chain reaction (qRT-PCR) indicated that the transcript levels of the *incw1* gene, encoding a maize cell-wall-bound invertase, were drastically increased in green islands. We reasoned that increased invertase activity induced a local carbon sink supporting fungal growth during the biotrophic phase of pathogenesis (Behr et al. 2010). In combination with specific access to green islands (Behr et al. 2010), recent improvements of analytical methods (Dobrev and Kaminek 2002; Hoyerova et al. 2006; Kojima et al. 2009; Novak et al. 2008) have made it possible to detect and quantify a considerably increased number of CK than in previous studies.

In this article, we combine precise tissue excision with high-resolution CK analyses to investigate the entire CK profile and quantify the amounts and changes of individual CK at fungal infection sites in green islands. Furthermore, we examine the capability of *C. graminicola* to convert exogenously applied CK in vitro and assess the capability of the fungus to synthesize CK de novo.

RESULTS

CK mimic fungal activity in delaying senescence and the decline of photosynthesis.

The hemibiotroph *C. graminicola* is able to delay senescence of host leaves at infection sites, resulting in appearance of green islands. We applied small droplets of Z, *cis*Z, and *N*⁶-benzyladenine (BA) solutions onto detached and still green maize leaves. All applied CK delayed senescence, as made evident by the maintenance of green spots at the sites of application. The green spots were surrounded by yellow senescing leaf tissue that had developed during 3 days of incubation (Fig. 1A). BA appeared to be the most effective CK, followed by Z and *cis*Z. Leaves that were already yellow at the time of treatment did not show re-greening with any of the CK at any concentration tested (not shown).

We used pulse-amplitude modulation (PAM) chlorophyll fluorometry and single-photon avalanche diode (SPAD) analyses to determine whether the apparent delay of senescence corresponded to altered photosynthetic activity and chlorophyll contents, respectively. Leaves from untreated controls that had reached the early stages of senescence at the beginning of the experiment had lost photosynthetic activity within 4 days (Fig.

Fig. 1. Effects of cytokinin (CK) treatment on senescence, photosynthesis, and chlorophyll content of maize. **A**, Treatment of detached senescing leaves with *N*⁶-benzyladenine (BA), *trans*-zeatin (Z), and *cis*-zeatin (*cis*Z) at the indicated concentrations. Photograph was taken at 3 days postinoculation; **B**, and **C**, 28-day-old whole plants were sprayed with BA, Z, and *cis*Z at the indicated concentrations. The still-attached third leaves were used to determine photosynthetic yield by **B**, pulse-amplitude modulation chlorophyll fluorometry and **C**, chlorophyll content by single-photon avalanche diode analysis.

1B). Application of Z, *cisZ*, and BA delayed or completely abolished this process. Correspondingly, SPAD analyses showed that treatment with each of these compounds resulted in a slower decline of the relative chlorophyll contents (Fig. 1C). These results indicated that, in maize, CK are sufficient to cause occurrence of green islands and to delay the decay of photosynthetic activity and the degradation of chlorophyll during leaf senescence. In this regard, Z appeared more effective than *cisZ*.

Effects of CK on expression of markers

of photosynthesis, senescence, and assimilate distribution.

We had previously employed qRT-PCR assays for maize genes encoding invertases (*incw1* to *incw4*, *ivr1*, and *ivr2*), and marker proteins for senescence (*see1*, *see2b*, and *ccp1* encoding proteases) and photosynthesis (*me1*, *pep1*, *cp29*, *RbcS1*, and *RbcS2* encoding the C4-specific isoforms of malic enzyme and phosphoenolpyruvate carboxylase, chlorophyll *a/b*-binding protein, and two isoforms of the small subunit of ribulose-1,5-biphosphate carboxylase, respectively) (Behr et al. 2010). Fungal infection caused specific transcriptional responses of some of these genes, most notably of *incw1*, encoding a cell wall invertase. Here, we investigated whether a treatment of whole plants with Z or *cisZ* may evoke similar transcriptional responses of these genes. For this purpose, leaf number 3, which was at an early stage of senescence when sprayed, and leaf number 5, which was mature, were analyzed. The transcript levels of *see1* and *see2b* were slightly increased by Z and *cisZ* on leaf number 3, and those of *ccp1* only by *cisZ* (Supplementary Fig. S 1). These effects vanished on leaf number 5. Treatment with both CK resulted in reduced transcript abundances of the photosynthesis marker genes, except for *pep1* and *cp29* on leaf number 3. Among the genes encoding invertases, the transcript levels of *incw1* were increased on leaf number 3 after CK treatment. Transcript abundances of the other invertase genes either showed no distinct response to CK application or decreased.

C. *graminicola* infection locally changes CK profiles.

We quantified CK levels at a spatial resolution comparable with that previously attained in qRT-PCR assays analyzing transcript levels at infection sites (Behr et al. 2010). Extracts obtained from tissue samples excised from infected leaf number 3, representing green islands and the area surrounding them, were analyzed by high-performance liquid chromatography (HPLC) MS/MS and were compared with those from a noninfected control leaf of the same age. In general, the *O*-glucosides constituted the majority of the total CK contents, whereas the nucleobases and the nucleosides were found at lower concentrations (Fig. 2A). The nucleotides and *N*-glucosides occurred only in trace amounts. In the noninfected leaf number 3, the levels of *cis*-isomers of zeatin-type CK considerably exceeded those of the corresponding *trans*-isomers (Fig. 2B; Supplementary Table S2), confirming previous data reported for maize (Gajdosova et al. 2011; Veach et al. 2003). The most prevalent CK derivative was *cisZOG*, representing 89% of total CK. Infection caused a decrease of total CK contents of $46 \pm 4\%$ in green islands and $33 \pm 11\%$ in tissue surrounding such infection sites (Fig. 2A). This likely resulted from considerably diminished levels of *O*-glucosides whereas the nucleobases remained relatively constant. The nucleosides and nucleotides were also increased, especially those derived from *cisZ*. For some individual CK derivatives, drastic changes were observed (Fig. 2B). The *cisZ*-9-riboside (*cisZR*) and *cisZ*-9-riboside-5'-monophosphate (*cisZRMP*) contents increased substantially by 733 ± 5 and $1,660 \pm 25\%$, respectively, at green islands compared with the noninfected control. Conversely, the *cisZOG* content decreased

by $54 \pm 6\%$. Evidently, for some CK (e.g., *cisZR*), clear differences existed between tissue sampled from green islands and the surrounding tissue, indicating an infection-site-directed concentration gradient over a distance of up to 5 mm.

Fig. 2. Qualitative and quantitative cytokinin (CK) determination at fungal infection sites. CK analysis by high-performance liquid chromatography tandem mass spectrometry used extracts obtained from number 3 leaves harvested at 4 days postinoculation that were either untreated (control) or inoculated with *Colletotrichum graminicola* (gi, sen). Infected leaves were analyzed by using excised tissue samples originating either from infection sites visible as green islands (gi) or the noninfected senescent tissues surrounding them (sen). **A**, CK combined in subgroups. **B**, Individual CK showing concentrations altered after fungal infection. Asterisks above the columns indicate significant differences between gi and sen when compared with the corresponding controls.

CK metabolism of *C. graminicola* in vitro.

The above results suggested a conversion of certain CK at fungal infection sites by either host or fungal enzymes or both. To determine the capability of *C. graminicola* to alter CK profiles at the infection site, we took three approaches to analyze whether or not *C. graminicola* is able to convert or de novo synthesize CK in fungal in vitro cultures. In the first approach, we used a cell-free extract prepared from maize leaves to expose the fungus to a naturally occurring mixture of CK, similar to what the fungus encounters during pathogenesis. In total, six variants were analyzed by HPLC MS/MS, three using extracts from mature green leaves and three using extracts from yellow, senescing leaves. As expected, CK contents were generally higher in the former (Fig. 3A). For each of the two extracts (i.e., from both green and yellow leaves), in two of the

Fig. 3. In vitro conversion of maize-derived cytokinins (CK) by *Colletotrichum graminicola*. Media contained extracts of maize leaves that were young and green (g) or senescing and yellow (y) when harvested for preparation. C-0 and C-7 = noninoculated controls at the beginning and after 7 days, respectively; F-7 = culture of *C. graminicola* after 7 days of incubation. CK analysis by high-performance liquid chromatography tandem mass spectrometry used the supernatants. **A**, CK combined in subgroups. **B**, Individual CK showing concentrations altered by fungal activity. Asterisks on top of the columns indicate significant differences for gF-7 and yF-7 (supernatants of fungal cultures at 7 days) when compared with the corresponding noninoculated controls (gC-7 and yC-7).

three treatments, CK were analyzed in noninoculated media at the beginning (0 days postinoculation [dpi]) and at the end (7 dpi) of the incubation period. These noninoculated controls showed to which extent CK decomposed under the experimental conditions used. Both media (i.e., those made from green as well as those from yellow leaves) showed some decline for total CK contents during an incubation period of 7 days (Fig. 3A). In the third treatment, both extracts (i.e., from green as well as yellow leaves) were inoculated with *C. graminicola* and analyzed after 7 days. Here, fungal metabolic activity led to an additional reduction of total CK contents. These decreases amounted to $56 \pm 4\%$ in the medium prepared from green leaves and to $11 \pm 17\%$ in the medium from senescing leaves, as compared with the corresponding noninoculated controls measured at day 7. In marked contrast, the contents of CK nucleobases, representing the active forms of CK, were significantly increased by the fungus (Fig. 3B). This effect was strongest for *cisZ*, which increased by $1,744 \pm 5$ and $3,867 \pm 13\%$ in extracts prepared from green and yellow leaves, respectively, compared with the corresponding noninoculated controls. These increases were accompanied by strong decreases in some of the CK O-glucosides, nucleosides, and nucleotides, suggesting that CK O-glucosides were transformed to the corresponding nucleobase. Interestingly, most of these changes were again associated with the *cis*-isoforms (i.e., *cisZOG*, *cisZR*, and *cisZRMP*).

In the second approach, we used minimal medium that was amended with individual commercially available CK (Z, *cisZ*, *trans*-zeatin-9-riboside [ZR], *cisZR*, *trans*-zeatin-9-riboside-5'-monophosphate [ZRMP], and *cisZRMP*) to test whether *C. graminicola* is able to convert these CK in vitro and, if so, which products are formed. HPLC MS/MS analyses were performed for each of these CK in the presence or absence of the fungus, and before and 7 days after inoculation (Fig. 4; the first designation of CK on the top of each box refers to the amended CK, the second indicates the CK measured). In addition, CK profiles were analyzed in the supernatants and in the mycelia of fungal cultures incubated for 7 days. Because the commercially available *cisZRMP* was heavily contaminated by *cisZR* (data not shown), it was not further considered in these investigations. All other supplemented CK remained stable during the period of the experiment of 7 days (Fig. 4). However, in all supernatants of fungal cultures, the concentration of the supplemented CK (i.e., Z, *cisZ*, ZR, *cisZR*, and ZRMP) had decreased, suggesting that *C. graminicola* had metabolized these compounds to some extent. The reduction of the concentration of supplemented CK was accompanied by the increase of other CK derivatives in the supernatant or mycelium. Amended *cisZ* was efficiently isomerized to Z in the mycelium, whereas Z was partially taken up into the mycelium but only poorly isomerized to *cisZ*. Amended ZR was transformed to various other CK, because Z and *cisZRMP* were identified in the supernatant, and *cisZ*, *cisZR*, and even dihydrozeatin-9-riboside (DHZR) in the mycelium. Addition of *cisZR* to the media was associated with accumulation of miscellaneous CK derivatives, including *cisZ*, *cisZRMP*, and especially, ZRMP in the supernatant and of Z and, to a much lower extent, *cisZ* in the mycelium. Supplemented ZRMP appeared to be transformed to Z and ZR as measured in the supernatant.

In addition, for one of the amended compounds, ZRMP, we recorded a time series to gain deeper insight into the succession of conversions (Fig. 5). Its concentration decreased steadily over a period of 12 days, whereas ZR was detected as an intermediate product peaking at 3 days that was further converted to Z with a maximal level at 7 days. The observed sequence of metabolic steps suggests that ZRMP was first dephosphorylated and then deribosylated to yield Z. Small

amounts of *cis*ZOG were detected after ZRMP application during the whole incubation period (data not shown).

The third approach assessed the ability of *C. graminicola* to synthesize CK de novo in minimal medium. CK were not detectable above background levels after 7 days of growth in unamended Czapek-Dox minimal medium (data not shown). Therefore, we supplemented the medium with putative substrates (dimethylallyl diphosphate [DMAPP] with AMP, ADP, and ATP) of CK biosynthetic enzymes (i.e., isopentenyltransferases) (Kakimoto 2001; Sakakibara 2006), to test whether the fungus might utilize these substances to synthesize CK. HPLC MS/MS measurements were carried out for control media lacking the fungus and for inoculated media; in case of the latter, immediately and at 3 dpi (Fig. 6). Analysis of the medium immediately after inoculation showed that conidia did not contribute to CK accumulation. Analysis of mycelium at 3 dpi revealed substantial amounts of Z, ZR, and ZRMP, indicating that synthesis has occurred. Also, other CK such as *cis*Z, *cis*ZRMP, dihydrozeatin (DHZ), and DHZR occurred in the mycelium at 3 dpi; however, at considerably lower levels compared with Z derivatives. The supernatant contained minor levels of CK.

In summary, the three approaches using in vitro cultures indicate that *C. graminicola* has the capacity to interconvert certain CK occurring naturally in maize and, furthermore, to produce CK using DMAPP as a precursor.

DISCUSSION

Infection of senescing maize leaves by the hemibiotroph *C. graminicola* evokes the formation of green islands. We show here that the application alone of individual CK onto senescing leaves caused physiological responses that corresponded to those observable after fungal infection. Quantification of CK levels exactly at fungal infection sites suggests that storage CK (*O*-glucosides) are locally converted to other CK forms. *C. graminicola* has the capacity to perform several CK interconversions in the absence of the host plant in in vitro cultures and, furthermore, to produce bioactive CK if the growth medium had been exogenously amended with precursors of CK biosynthesis.

Several physiological responses of maize have previously been noticed to occur at green islands during the infection with *C. graminicola* (Behr et al. 2010). Most of these responses are also seen after treatment of maize leaves at early stages of senescence with CK. Although all CK tested in this study were biologically active with respect to maintenance of photosynthetic activity and chlorophyll contents as determined by PAM and SPAD analyses, the impact of Z was stronger than that of *cis*Z. This corresponds to a generally reported lower activity of *cis*Z types compared with Z forms in a number of CK bioassays (Gajdosova et al. 2011; Kaminek et al. 1979). Although *cis-trans* isomerases of CK exist in plants (Bassil et al. 1993), isomerization of the exogenously applied CK was not found at such a degree in maize leaves that differences between Z and *cis*Z disappeared.

Both fungal infection and treatment with Z enhanced transcript levels of *incw1* on senescing leaves. This particular cell-wall-bound invertase gene exhibited the strongest transcriptional stimulation among 16 maize genes analyzed at green islands during the infection of senescing leaves with *C. graminicola* (Behr et al. 2010). Cell-wall-bound invertase is a key enzyme determining sink strength, and the activity of these enzymes or transcript levels of the corresponding genes have been shown to rise upon infection in some pathosystems (Wingler and Roitsch 2008). A link between invertase and CK has previously been shown in transgenic tobacco, in which

Fig. 4. In vitro conversion of individual cytokinins (CK) by *Colletotrichum graminicola*. The first designation of CK shown on top of each box refers to the CK added to the particular minimal medium, the second indicates the measured CK as determined by high-performance liquid chromatography tandem mass spectrometry. In all cases, measurements are first demonstrated for the particular CK added and further for CK derivatives showing alterations. Labels on x axis read as follows: s and m, CK analysis using the supernatant and the mycelium, respectively; C, noninoculated control; F, fungal culture; -0 and -7, analysis at the beginning and after 7 days, respectively. Asterisks indicate significant differences between sF-7 (supernatant of fungal culture at 7 days) and the corresponding noninoculated control (sC-7).

endogenously increased CK levels caused an increase in extracellular invertase activity, resulting in a delay of senescence (Balibrea Lara et al. 2004). Here, we provide evidence that such a link could be also important for the interaction of *C. graminicola* with maize, supporting the hypothesis that, at infection sites, CK stimulate a signaling cascade leading to an enhanced host invertase activity (Walters and McRoberts 2006).

Our measurements of CK at green islands demonstrate that, indeed, fungal infection leads to local changes in the concentrations of certain CK. Notably, the storage CK *cis*ZOG, which reaches the highest concentration of all individual CK in the noninfected maize leaf, decreased considerably after infection.

Concomitantly, the levels of *cis*ZR and *cis*ZRMP increased. Because concentration gradients exist for these three CK, the respective effect is stronger at green islands compared with the surrounding senescing tissue, indicating that CK metabolism is primarily affected at the infection site.

CK levels were measured previously in other pathosystems. However, in these experiments, whole infected plant organs were used, with lower analytical and spatial resolution. These experiments suggested that total amounts of CK increased in green islands evoked by biotrophs (Walters and McRoberts 2006). In contrast, our analyses found decreased total CK levels. It needs to be considered that our measurements allowed the detection of more individual CK compounds, particularly

Fig. 5. Time course of in vitro conversion of *trans*-zeatin-9-riboside-5'-monophosphate (ZRMP) by *Colletotrichum graminicola*. Data show the results of high-performance liquid chromatography tandem mass spectrometry analyses of the amended cytokinin (CK) ZRMP and its metabolites *trans*-zeatin-9-riboside (ZR) and *trans*-zeatin (Z), whose concentrations considerably changed during incubation. A noninoculated control documented putative decomposition. CK were analyzed in the supernatants at the beginning (0 days) and during incubation (3, 7, and 12 days). At the end, CK were also measured in the harvested mycelium. Asterisks indicate significant differences between the supernatant of the fungal culture and the corresponding noninoculated control.

Fig. 6. In vitro synthesis of cytokinins (CK) by *Colletotrichum graminicola*. Minimal medium was amended with a mixture of dimethylallyl diphosphate (DMAPP), with AMP, ADP, and ATP (100 nM each). Labels on x axis read as follows: s and m, CK analysis using the supernatant and the mycelium, respectively; C, noninoculated control; F, fungal culture; -0 and -3, analysis at the beginning and after 3 days, respectively. Data show the results of high-performance liquid chromatography tandem mass spectrometry analyses. Asterisks indicate significant differences between sF-3 (supernatant of the fungal culture at 3 days) and the corresponding noninoculated control (sC-3).

the *cisZ*-type CK, which prevail in maize leaves (Gajdosova et al. 2011; Veach et al. 2003). These CK were largely overlooked and not recorded in most previous reports. Furthermore, our assays focused on local infection sites, which may also explain the contrasting results. It was suggested that most of the obligate and hemibiotrophs analyzed were able to transform *trans*-zeatin-*O*-glucoside (ZOG) to the active compound Z, whereas most of the necrotrophs were not (Ashby 2000). This led to the hypothesis that corresponding enzymes secreted by the pathogen at the infection site may convert biologically inactive CK stored by the host to bioactive CK that, in turn, would stimulate the supply of carbohydrates to the infection sites harboring the pathogen. Our finding that the *cisZOG* level decreased at the infection site reinforces this hypothesis. The conversion of *cisZOG* does not necessarily result from fungal enzymatic activity but could also be mediated by a host enzyme induced by infection. Therefore, it is important to note that the CK measurements using fungal in vitro cultures showed that *C. graminicola* does, indeed, have the capacity to transform *cisZOG* to *cisZ*. However, *cisZ* remained at relatively steady-state levels at infection sites despite the conversion of *cisZOG*. In this context, it needs to be considered that, in infected leaves, CK levels are influenced by de novo synthesis, import and export from other organs, interconversion, transient and terminal inactivation, as well as perception and signal transduction, all of which could theoretically be mediated by both the host and the pathogen. Because terminal and irreversible inactivation is catalyzed by CK oxidase/dehydrogenase (CKX), we attempted to determine its activity in extracts obtained from excised tissue samples of green islands. However, CKX activity was undetectable in this limited amount of material using a highly sensitive radioisotope assay with tritiated CK as substrates (data not shown). Furthermore, several findings indicate that not only the free bases, which exhibited relatively constant levels at infection sites in our experiments, have biological activity as typically reported, but also other forms such as CK ribosides (Dolezal et al. 2007; Gajdosova et al. 2011; Holub et al. 1998).

Our results underline the possibility that the *cisZ*-type CK may play an as-yet not-well-understood role in the interaction between fungi with their hosts. This hypothesis is supported by a recent study on CK levels in the roots of *Arabidopsis thaliana* after colonization with the mycorrhizal basidiomycete *Piriformospora indica* (Vadassery et al. 2008). Major changes in concentrations were observed only for the *cis* isomers, especially for *cisZR*, which also exhibited the highest level of any CK after infection, as well as for *cisZROG* and *cisZRMP*. Although the *cisZ* concentration appeared somewhat increased in infected roots, compared with the other substances, it remained at relatively low levels (Vadassery et al. 2008). In another study supporting our findings, several CK were analyzed in the pathogenic interaction between the basidiomycete *Ustilago maydis* and maize (Bruce et al. 2011). Because some of the CK that we found at higher concentration in maize (e.g., *cisZOG*) were not resolved in that study, we can only partially compare the results. In *U. maydis*, major increases were observed for the concentrations of *cisZR*, *cisZRMP*, N^6 -(Δ^2 -isopentenyl)adenine-9-riboside-5'-monophosphate (iPRMP), and *cisZ* at a later stage of infection, whereas the *trans* isomers were not affected. Therefore, despite the different types of interactions and the different taxonomic positions of the plant and fungal partners involved, the most prominent effects in *cisZ*-type CK were similar. However, both the study on *P. indica* (Vadassery et al. 2008) and ours on *C. graminicola* found higher bioactivity for the *trans* isomers in several assays. It is interesting to note that *C. graminicola* converts in vitro *cisZ* to Z but does not catalyze the reverse reaction. In maize, three

CK receptors have been reported and two of them have a higher affinity for *cisZ* than for Z (Lomin et al. 2011). It remains open whether both isomers may have specific and distinct functions in planta.

Our feeding experiments using individual CK uncovered a range of additional CK conversions that the fungus is able to perform in vitro. In addition to the isomerization of *cisZ*, most notable are the conversions of ZR to *cisZRMP*, *cisZR* to ZRMP, and ZRMP to ZR and Z. Based on the data obtained in this study, we propose a model for CK metabolism of *C. graminicola* (Fig. 7). In this model, we have included only reactions supported by our experimental data but did not exclude the possibility that additional conversions may also occur. We infer only reactions leading directly from the respective exogenously amended substances to the experimentally observed compounds, except the supposed conversion of *cisZRMP* to ZRMP. This hypothesized isomerization explains why the addition of *cisZR* leads to the appearance of considerable amounts of ZRMP. Alternatively, longer reaction sequences explaining this result would be *cisZR* $\rightarrow$ ZR $\rightarrow$ ZRMP or *cisZR* $\rightarrow$ *cisZ* $\rightarrow$ Z $\rightarrow$ ZR $\rightarrow$ ZRMP. A central position within the model is taken by ZR that appears to be transformable to several other CK and that even can be synthesized by the fungus (discussed below). The conversions proposed by our model have been shown to occur in plants (Sakakibara 2006), suggesting that a fungal plant pathogen performs a rather complex CK metabolism that adopts some features also seen in plants.

Beyond the ability to interconvert CK, *C. graminicola* also has some capacity for CK biosynthesis. The addition of DMAPP, with AMP, ADP, and ATP, to minimal medium led to synthesis of considerable amounts of a series of CK (i.e., ZRMP, ZR, and Z), indicating that isopentenyltransferase (IPT) activity exists in this fungus. In plants, DMAPP is a substrate for IPT that catalyze the first step in the synthesis of isoprenoid CK. Although, in certain plant-pathogenic fungi, CK biosynthesis has been proposed (Ashby 2000), IPT activities have not been formally proven. In in vitro growing mycelium of *P. indica*, mainly N^6 -(Δ^2 -isopentenyl)adenine (iP), *cisZRMP*, and *cisZR* were detected, and lower amounts of N^6 -(Δ^2 -isopentenyl)adenine-9-riboside (iPR), *cisZROG*, and *cisZ* (Vadassery et al. 2008). Likewise, *U. maydis* produced considerable amounts of *cisZRMP* and *cisZR* as well as iPRMP and iPR in vitro (Bruce et al. 2011). Because both studies used rich media containing yeast extract, casein hydrolysate, or peptone, it is unclear whether a complete de novo synthesis of CK occurred or whether these fungi utilized supplemented compounds to generate them. Our results suggest that, during the infection process, *C. graminicola* may not only manipulate CK metabolism in the host by interconverting preexisting CK but also utilize DMAPP to synthesize CK.

In summary, our study provides a comprehensive view of fungal CK metabolism in a fungus–plant pathosystem. Using high spatial and analytical resolution, we have shown that, during infection, fungal CK metabolism is interconnected with that of the host, which adds an additional layer of complexity to the interaction between a hemibiotroph and its host plant. Future work needs to elucidate the contribution of individual CK to the establishment of a successful infection.

MATERIALS AND METHODS

Plant and fungal cultivation.

Maize (*Z. mays* ‘Mikado’) (KWS Saat AG, Einbeck, Germany) and wild-type strain *C. graminicola* (Ces.) G. W. Wilson M2 were used for this study. Conditions for cultivation of the fungus and maize in the greenhouse as well as in environmentally controlled growth chambers had been described (Behr et

al. 2010). For inoculation of 28-day-old maize plants with *C. graminicola*, conidia were sprayed onto the entire plant at a concentration of $1 \times 10^6 \text{ ml}^{-1}$, as reported (Behr et al. 2010).

Phenotypical effects of CK on senescence (Fig. 1A) were tested by applying appropriate substances (10 μl each) diluted in water onto the surface of detached number 5 leaves of 28-day-old plants. Photographs were taken after 3 days of incubation on wet filter paper in a glass petri dish at ambient temperature and light conditions.

The effects of individual CK on photosynthesis and transcript levels of maize genes (Fig. 1B and C) were assessed by spraying the substances (6 ml each) onto whole 28-day-old plants incubated in an environmentally controlled growth chamber under conditions described previously (Behr et al. 2010).

To evaluate the capability of the fungus to convert CK naturally occurring in maize in vitro (Fig. 3), a preculture was established by inoculation of 50 ml of complete medium (Krijger et al. 2008) with 1×10^6 of conidia. The mycelium obtained after 24 h of growth at 23°C and 50 rpm was transferred to 50 ml of sterile distilled water, incubated for 150 min as before, then transferred to maize leaf extract medium. The latter was prepared from 28-day-old plants by cutting five green or senescing leaves into small pieces, which were subsequently autoclaved in 50 ml of sterile distilled water for 20 min at 121°C . The resulting supernatant was filter sterilized through a $0.2\text{-}\mu\text{m}$ filter and transferred to a 200-ml Erlenmeyer

flask. Incubation of the culture continued under the same conditions as described above for 7 days. The mycelium was then harvested on filter paper and the spent medium was filter sterilized through a $0.2\text{-}\mu\text{m}$ filter and stored at -20°C .

To assess the capability of the fungus to convert commercially available CK or to utilize precursors for CK synthesis in vitro (Figs. 4 to 6), 50 ml of Czapek-Dox minimal-medium (0.2% NaNO_3 , 0.1% K_2HPO_4 , 0.05% $\text{MgSO}_4 \times 7 \text{ H}_2\text{O}$, 0.05% KCl , 0.001% FeSO_4 , and 5 mM Sucrose) in 200-ml Erlenmeyer flasks was inoculated with 1×10^6 conidia. The medium was amended with either individual CK (OChemIm Ltd., Olomouc, Czech Republic) or with a mixture of putative substrates of isopentenyltransferases, which are involved in CK biosynthesis (i.e., DMAPP, AMP, ADP, and ATP) (all Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), each at a concentration of 100 nM. Incubation was at 23°C and 50 rpm. Harvest proceeded as given above.

PAM chlorophyll fluorometry and SPAD analyses.

The impact of CK treatment on photosynthesis and chlorophyll contents in maize leaves was assessed nondestructively by PAM chlorophyll fluorometry and SPAD analysis, respectively. For both, whole plants were treated and incubated as outlined above. Each day, three plants were taken from the growth chamber and PAM and SPAD analyses were performed on 10 randomly chosen areas in the middle of leaf number 3. For each variant, all 10 measurements were used to calculate

Fig. 7. Proposed model illustrating cytokinin (CK) conversions by *Colletotrichum graminicola* in vitro. Straight arrows indicate reactions deduced from data. The broken arrow indicates a presumed conversion to explain the emergence of *trans*-zeatin-9-riboside-5'-monophosphate (ZRMP) after feeding of *cis*-zeatin-9-riboside (*cisZR*).

arithmetic means and standard deviations. We used two hand-held devices: a MINI-PAM Photosynthesis Yield Analyzer (Heinz Walz GmbH, Effeltrich, Germany) and a SPAD-502 Chlorophyll Meter (Konica Minolta Sensing Europe B.V., München, Germany). The Mini-PAM instrument assesses the effective quantum yield of photochemical energy conversion in photosynthesis by irradiation with pulse-modulated light and measuring of the chlorophyll fluorescence. The SPAD-502 instrument measures the ratio of the transmitted light at 920 nm, which is not absorbed by chlorophyll, to that at 650 nm, which is absorbed. This ratio is used as a measure of the chlorophyll content of the leaf.

Isolation of RNA and qRT-PCR.

Four days after spraying of 28-day-old whole maize plants with 100 μ M Z or *cis*Z, leaves were harvested 10 ± 1 h after beginning of the light period, immediately frozen in liquid nitrogen, and stored at -80°C until RNA preparation was done as reported (Krijger et al. 2008). For each variant analyzed, RNA was extracted from three pools, each comprising whole leaves (numbers 3 and 5, respectively) from six plants. These pools originated from independent experiments performed on successive weeks. Variants representing mock-inoculated controls were also included. qRT-PCR followed published protocols and primers (Behr et al. 2010), except that the Power SYBR Green RNA-to-CT 1-step kit (Applied Biosystems GmbH) was used.

CK determination.

To measure CK in infected plants at a high spatial resolution (Fig. 2), hundreds of tissue samples carrying green islands on leaf number 3 were excised at 4 dpi with a cannula (1 mm in diameter, custom produced at the Fraunhofer Institute for Mechanics of Materials, Polymer Applications, Biological Materials and Interfaces, Halle, Germany), pooled, and frozen in liquid nitrogen. Tissues that surrounded and excluded the green islands were excised with a cork borer (5 mm in diameter).

The analysis of profiles and concentrations of individual CK in both tissue samples (including fungal mycelia) and culture media was performed by HPLC MS/MS. The deeply frozen tissues (equivalent to 0.62 to 2.71 g of fresh weight [FW] for plant tissue and 0.02 to 0.2 g of FW for fungal mycelium) were homogenized in liquid nitrogen, extracted overnight with 10 ml of methanol/water/formic acid (15:4:1 [vol/vol/vol], pH 2.5, -20°C), and purified by dual-mode solid-phase extraction (Dobrev and Kaminek 2002). The deeply frozen media (equivalent to 1 or 2 ml) were thawed, acidified (after thorough shaking) to pH 2.5 by formic acid (98% [vol/vol]), and subjected to the same purification procedure as mentioned above using Sep-Pak Plus tC18 cartridges (Waters Corp., Milford, MA, U.S.A.) and Oasis MCX 6-cm³ (150 mg) extraction cartridges (cation exchange and reverse-phase) (Waters Corp.). Separated CK nucleotides were determined as corresponding nucleosides following their dephosphorylation by calf-intestine alkaline phosphatase (Sigma-Aldrich Chemie GmbH).

Detection and quantitation of CK employed a HPLC MS/MS system consisting of an HTS-Pal auto-sampler with a cooled sample stack (CTC Analytics AG, Zwingen Switzerland), a quaternary HPLC pump Rheos 2200 (Flux Instruments AG, Reinach, Switzerland), a DeltaChrom CTC 100 column oven (Watrex Prague Ltd., Prague), and a TSQ Quantum Ultra AM triple-quadrupole high-resolution mass spectrometer (Thermo Electron Corp., San Jose, CA, U.S.A.) equipped with an electrospray interface. Dried extracts were redissolved in 100 μ l of diluted acetonitrile (10% in water) and filtered by 0.2- μ m Micro-Spin filters (W. R. Grace & Co., Columbia, MD, U.S.A.). A 5- μ l aliquot was injected on a C18

HPLC column Synergy Hydro-RP, 250 by 2 mm, 4 μ m (Phenomenex Inc., Torrance, CA, U.S.A.) and analyzed using a ternary gradient elution (water/acetonitrile/0.01% acetic acid) starting at 8% of acetonitrile. The concentration of acetonitrile was increased after 5 min to 15% during 10 min and then to 50% during 11 min. The portion of 0.01% acetic acid was maintained at 35% throughout the analysis. The remainder of the sample was removed from the column by increasing the concentration of acetonitrile to 90% for 9 min. The column was equilibrated with 8% acetonitrile for 20 min before the next sample was injected. The mass spectrometer was operated in the MS/MS mode (selected reaction monitoring) with monitoring of two to four transitions for each compound. The most intensive ion was used for quantification, the others for identity confirmation. CK were quantified by the Xcalibur software (version 2.0; Thermo Fisher Scientific Inc., San Jose, CA, U.S.A.) using multilevel internal standards calibration graphs with [³H]-labeled CK used as internal standards (OlChemIm Ltd.). The quantitative data were expressed as picomoles per sample. The calibration allowed for quantification at a range of 0.1 to 500 pmol/sample. In total, 26 different CK were analyzed, although concentrations of some of them were below the detection limit. CK were abbreviated as proposed earlier (Kaminek et al. 2000). Statistical analyses employed the unpaired *t* test; differences at *P* < 0.05 were considered significant.

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