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Ustilago maydis Produces Cytokinins and Abscisic Acid for Potential Regulation of Tumor Formation in Maize

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Abstract The infection of maize (*Zea mays*) by the basidiomycete fungus *Ustilago maydis* leads to common smut of corn characterized by the production of tumors in susceptible aboveground plant tissues. LC-(ES)MS/MS profiles of abscisic acid (ABA) and 12 different cytokinins (CKs) were determined for infected and uninfected maize tissues over a time course following fungal exposure. Samples were taken at points corresponding to the appearance of disease symptoms. Axenic cultures of haploid and dikaryon forms of *U. maydis* were also profiled. This study confirmed the capability of *Ustilago maydis* to synthesize CKs, ABA, and auxin (IAA). It also provided evidence for the involvement of CK and ABA in the *U. maydis*-maize infection process. Significant quantities of CKs and ABA were detected from axenic cultures of *U. maydis* as was IAA. CKs and ABA levels were elevated in leaves and stems of maize after infection; notable was the high level of *cis*-zeatin 9-riboside. Variation among hormone profiles of maize tissues was observed at different time points during infection and between infections with nonpathogenic haploid and pathogenic dikaryon strains. This suggested that CKs and ABA accumulate and are likely metabolized in maize tissue infected with *U. maydis*. Because *U. maydis* produced these phytohormones at significant levels, it is possible that the fungal pathogen is a source of these compounds in infected tissue. This is the first study to confirm the production of CKs and document the production of ABA by *U. maydis*. This study also established an involvement of these phytohormones and a possible functional role for ABA in *U. maydis* infection of maize.

Keywords Cytokinin · Abscisic acid and auxin synthesis · Plant tumor physiology · Phytopathology

Abbreviations

ABA	Total abscisic acid
*cis*Z	*cis*-zeatin
*cis*Z-group	*cis*Z + *cis*ZR + *cis*ZRMP
*cis*ZR	*cis*-zeatin 9-riboside
*cis*ZRMP	*cis*-zeatin 9-riboside-5′-monophosphate
CK	cytokinin
DHZ	Dihydrozeatin
DHZ-group	DHZ + DHZR + DHZRMP
DHZR	Dihydrozeatin 9-riboside
DHZRMP	Dihydrozeatin 9-riboside-5′-monophosphate
Free bases	DHZ + IP + Z + *cis*Z
IAA	Total auxin (indole-3-acetic acid)
IP	N6-(Δ^2isopentenyl)adenine
IP-group	IP + IPR + IPRMP
IPR	N6-(Δ^2isopentenyl)adenine 9-riboside
IPRMP	N6-(Δ^2isopentenyl)adenine 9-riboside-5′-monophosphate
LC-(ES)MS/MS	Liquid chromatography-electrospray ionization-tandem mass spectrometry

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Nucleotides	DHZRMP + iPRMP + ZPRMP + *cis*ZRMP
Ribosides	DHZR + iPR + ZR + *cis*ZR
Z	*trans*-zeatin
Z-group	Z + ZR + ZRMP
ZR	*trans*-zeatin 9-riboside
ZRMP	*trans*-zeatin 9-riboside-5′-monophosphate

Introduction

Infections by fungi of the order *Ustilaginales* (the smuts) yield destructive diseases of cereals and other grass crops. *Ustilago maydis* is the model for investigating the physiological and molecular basis of smut diseases. It infects maize (*Zea mays*) causing common smut of corn, which is characterized by the production of tumors in susceptible embryonic or actively growing aboveground tissues. Little is known about the role of classic phytohormones in infection of maize by *U. maydis*. However, microbial production of phytohormones or *in planta* phytohormone modification by microbes has been documented to play a role in plant disease. Notable among these microbes is the modification of cytokinin (CK) and auxin (IAA) by *Agrobacterium*, the causal agent of crown gall disease. In addition, salicylic acid (SA), jasmonates (JA), and ethylene (ET) have been identified as having roles in plant responses to biotic stresses, and signalling pathways involving other hormones such as abscisic acid (ABA), IAA, gibberellic acid (GA), CK, brassinosteroids (BR), polyamines, and peptide hormones may be involved in directing plant responses to disease-causing agents and pests (Rodriguez-Kessler and others 2008; Bari and Jones 2009). The research presented here provides an assessment of CK and ABA production by *U. maydis* during common smut disease development.

Earlier research suggested that CK and IAA were produced by *U. maydis* and played a role in the development of smut tumors of maize (Moulton 1942; Mills and Van Staden 1978). However, these findings were not confirmed and lacked direct quantification of hormones through techniques such as mass spectrometry. Similarly, Khozina and others (1986) found that the level of CK activity as well as CK-like activity in extracts taken from aboveground organs of maize seedlings varied significantly during the haploid, dikaryon, and diploid stages of the fungal infection by *U. maydis*. They proposed that the level of CK activity rose during the pathological process of the fungal infection in host plants; although this was never confirmed with biochemical and physiochemical analyses of CKs.

It is likely that there is a link between CK and pathogenicity because CK levels in plants are altered after pathogen infection, there is an upregulation of stress and defence-related genes in CK-treated seedlings, and exogenous CK application leads to changes in pathogen susceptibility (Argueso and others 2009). There is also evidence of crosstalk between CK and defence response pathways (Igari and others 2008). However, the sources and species of CKs involved in pathogenesis and plant–pathogen interactions are uncertain (Argueso and others 2009). Recently, CK profiling was completed for the biotrophic actinomycete *Rhodococcus fascians* by Pertry and others (2008). The synthesis of CKs by *R. fascians* is known to be important for its pathogenicity, and profiling identified isopentenyladenine, *trans*-zeatin, and *cis*-zeatin and their 2-methylthio (2MeS)-derivates (Pertry and others 2008). Each of these CKs exhibited typical CK responses in bioassays and, as a mixture, the bacterial CKs showed clear synergistic effects (Pertry and others 2008). However, the specific 2MeScZ and *cis*Z CKs accumulated in *Arabidopsis* tissue because they were poor substrates of the apoplastic CK oxidase/dehyrogenase enzymes which breakdown CKs. Therefore, it is likely that these forms contribute to the continuous stimulation of tissue proliferation that occurs in leaf galls after infection with these bacteria (Pertry and others 2008).

The biosynthesis and release of IAA from *U. maydis* during host tumor formation was reported recently by Reineke and others (2008). This confirmed a much earlier suggestion that *U. maydis* was capable of synthesizing IAA (Moulton 1942). However, Reineke and others (2008) concluded that the IAA produced by the fungus was not important for triggering host tumor formation, although fungal IAA could contribute to the overall IAA levels in infected tissue. The impact of *U. maydis* biosynthesis and release of IAA on its host's physiological state remains to be determined.

Curiously, ABA may also play a functional role in *U. maydis*-maize disease development because other microbial species that interact with plants are capable of ABA production and the compound is a good candidate for the activity in the pathology (Marumo and others 1982; Veselov and others 2003; Siewers and others 2004). In addition, the phytopathogenic fungi *Cercospora rosicola*, *C. beticola*, and *Botrytis cinerea* are capable of ABA biosynthesis, and ABA production by these fungi is thought to be involved in interactions with respective host plants (Marumo and others 1982; Schmidt and others 2008), including the negative regulation of plant defence against biotrophs and necrotrophs (Bari and Jones 2009).

U. maydis infects developing tissue, and the observed changes in carbohydrate metabolism suggest that this infection arrests sink-to-source transitions. This favours the

maintenance of sink metabolism in host cells rather than transforming existing source leaf tissue into sink tissue. There is evidence that fungal invertase assures carbohydrate supply during the biotrophic growth phase and that it is indispensable for pathogenesis (Horst and others 2008). This type of physiological response strongly suggests a role for hormones like CKs in the disease process. CKs, along with IAA, ABA, ethylene, and brassinosteroids, are known to regulate source/sink relations through cell cycle control and the upregulation of sugar-metabolizing enzymes (Roitsch and Ehneb 2000).

In the present study we examined the potential involvement of phytohormones in *U. maydis* infection of maize. We determined the profile of 12 different CKs and ABA for infected and control tissues during a time course following infection. The time points chosen corresponded to the appearance of symptoms at different infection stages. To examine whether the fungus itself was capable of producing CKs, ABA, and IAA, profiles of axenic cultures of the saprophytic haploid and pathogenic dikaryon forms of *U. maydis* were generated and compared to each other as well as to profiles from plant tissues exposed to *U. maydis*. This exposure included haploid saprophytic "infections" which produced few symptoms, and dikaryon infections which induced the classic disease symptoms. All of these profiles were analyzed by liquid chromatography–electrospray tandem mass spectrometry (LC-(ES)MS/MS). Comparisons among the profiles provided insight regarding the involvement and role of these hormones in *U. maydis* physiology, host interaction, and pathogenesis.

Material and Methods

Strains and Growth Conditions

Ustilago maydis strains 518(*a2 b2*) and 521(*a1 b1*), originally obtained from Sally Leong (University of Wisconsin at Madison), were used for all studies. *U. maydis* strains were grown at 28°C in YEPS Gold medium composed of (g l^{-1}) yeast extract (10.0), peptone (20.0), and sucrose (20.0) or in double complete medium (DCM) composed of (g l^{-1}) ammonium acetate (3.0), casamino acids (10.0), yeast extract (20.0), activated charcoal (10.0) at pH 7.0 with 62.5 ml salt solution composed of (g l^{-1}) K_2HPO_4 (16.0), Na_2SO_4 (4.0), KCl (8.0) $MgSO_4{\cdot}7H_2O$ (2.0), $CaCl_2{\cdot}2H_2O$ with 8 ml trace element solution composed of (ng l^{-1}) H_3BO_3 (60.0), $MnCl_2{\cdot}H_2O$ (220.0), $ZnCl_2$ (400.0), $Na_2MoO_4{\cdot}2H_2O$ (40.0), $FeCl_3$ (100.0), and $CuSO_4{\cdot}5H_2O$ (626.0). Solid medium for plates contained 20 g l^{-1} agar. Mating of compatible strains was performed on DCM plates at 28°C for 4 days.

Plant Material and Growth Conditions

Fungicide-treated seeds of a hybrid sweet corn (*Zea mays* cv. Golden Bantum) highly susceptible to *U. maydis* (Snetselaar and Mims 1993) were used for all infection assays. Twenty-one seeds were sown per 6.5-in. round pot containing moistened soil (Fafard Agromix, growth optimum, professional high-performance growth medium, with vermiculite, Saint-Bonaventure, Quebec) at 2.5-cm spacing, covered with 2.5 cm of soil, watered, covered with clear plastic, and placed in a greenhouse at 21.5–24.5°C for 3 days to germinate. Then the plants were uncovered and watered as needed for 14 days and then watered every day with 28:14:14 $N{:}P_2O_5{:}K_2O$ fertilizer at 0.22 g/l.

For the pathogenic infection of 7-day-old maize plants, sporidia were grown in 400 ml of YEPs Gold media, to an OD_{600} between 0.5 and 2.0 determined using a Spectra$_{max}$ PLUS spectrophotometer (Molecular Devices, Sunnyvale, CA). Prior to inoculation, compatible 518 and 521 sporidia cultures were brought to equivalent cell densities with autoclaved H_2O. Volumes representing the equivalent number of 518 and 521 cells were combined and used for inoculation of maize seedlings at a rate of 3 ml of the mixed haploid cell suspension per pot. Inoculation was completed with the injection of the stem, via a disposable syringe with a 20G1, sterile, single-use needle, with the sporidia suspension until the suspension was visually noted at the base of the seedlings' first leaves.

For the study of the facultative saprophytic infection, maize seedlings were inoculated similar to the pathogenic infection but with a single *U. maydis* strain, 521. Sporidia were grown in 400 ml of YEPS Gold to an OD_{600} of 1.7.

In addition to the pots of maize plants infected with pathogenic and facultative saprophytic cultures, six pots of maize seeds were sown 7 days prior to any *U. maydis* infection for use as controls. Of these, three were left untreated and three were inoculated with 3 ml of autoclaved dH_2O. The dH_2O injections were completed in the same way as *U. maydis* was inoculated into the maize seedlings to control for the wounding effect of inoculation.

Characterization of the Disease Time Course

A time course describing the occurrence of characteristic disease symptoms during maize infection with *U. maydis* was established (Fig. 1). Disease symptoms over the time course of infection were rated and classified, based upon the presence of symptoms, as follows: no symptoms, chlorosis, anthocyanin, leaf galls, small stem galls, large stem galls, or plant death.

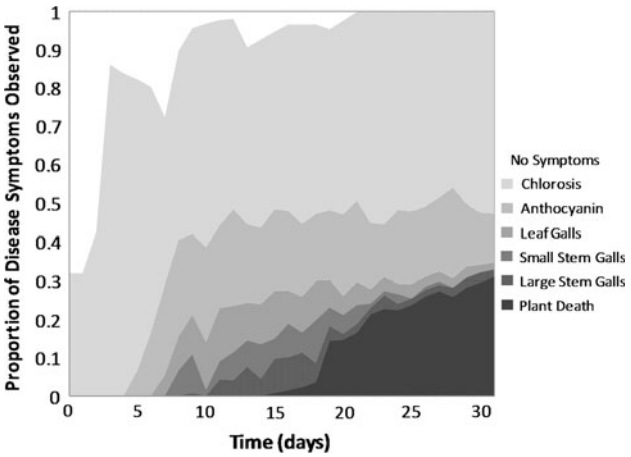

Fig. 1 Proportion of disease symptoms observed over a period of time after *Ustilago maydis* infection of 7-day-old maize (*Zea mays*) seedlings. Day 0 represents the date of infection of 7-day-old seedlings with a cross of 521 and 518 strains of *U. maydis*. $n = 80$ plants with seven disease classes recorded as pathogenesis proceeds

Sample Collection

Tissues collected for CK and ABA analysis were organized as described in Table 1. Hormone profiles were generated from control, water-injected, and 518 × 521- and 521-injected plants at 0, 1, 5, 6, and 14 days post inoculation (dpi). Both nontreated "control" plants and "water-injected" plants were included in this study to control for any possible hormonal effect incurred from wounding the plants by injection. All plant tissues were collected in the same manner across the treatments and dates sampled, and the collections were composed of tissues taken from at least three plants equalling 2 g and replicated four times. Because phytohormone concentrations are thought to be subject to daily variations caused by circadian rhythm, all plant samples were collected at approximately the 11:00 hour (McClung and others 2002). Plant tissues were frozen at −20°C for storage until hormone extraction.

Haploid fungal cultures were sampled from DCM-agar plates after 3 days of growth, and dikaryon cultures were sampled from DCM-agar plates at 4 days of growth. Cells were scraped off media for collection under sterile conditions, frozen at −80°C for storage, and freeze-dried under vacuum at −40°C in a FreeZone 4.5 freeze drier (Labconco, Kansas City, MO, USA) for hormone extraction. All fungal samples contained 2 g of tissue and each was collected from four replicates, except for the day 0 control which had eight replicates.

Table 1 Maize tissues profiled for CK and ABA analysis

Plant tissue	Days post inoculation (dpi)	Sample name	*n*	*U. maydis* development or disease symptoms
Seedlings	0	Control	8	–
Leaves	1	Control	4	–
		Water injected	4	–
		518 × 521 injected	4	Haploid fusion, appressorium formation, dikaryon entry into host surfaces (Snetselaar and Mims 1993).
		518 injected	4	Budding morphology, no host surface entry (Snetselaar and Mims 1993)
Leaves	5	Control	4	–
		Water injected	4	–
		518 × 521 injected	4	Pretumor
		518 injected	4	Unknown abiotic or biotic effect
Leaves	6	Control	4	–
		Water injected	4	–
		518 × 521 injected	4	First day of tumor appearance
		518 injected	4	Unknown abiotic or biotic effect
Leaves	14	Control	4	–
		Water injected	4	–
		518 × 521 injected	4	Late-day tumors
		518 injected	4	Unknown abiotic or biotic effect
Stems	14	Control	4	–
		Water injected	4	–
		518 × 521 injected	4	Late-day tumors
		518 injected	4	Unknown abiotic or biotic effect

Hormone Extraction and Purification from Plant Tissues and Fungal Cultures

Phytohormone extraction and purification from plant tissues was performed according to procedures adapted from Dobrev and Kaminek (2002), as modified by Quesnelle and Emery (2007) to prevent enzyme activity that causes CK nucleotide degradation and CK isomerisation. Briefly, the fresh weight of each sample was recorded, samples were homogenized in ice cold Bieleski extraction solvent, and internal standards were added for eventual quantification of endogenous hormones by isotope dilution. The following standards were added: 50 ng each of [$^2H_6$]iP, [$^2H_6$][9R]iP, *trans*-[$^2H_5$]Z, [$^2H_3$]DZ, *trans*-[$^2H_5$][9R]Z, [$^2H_3$][9R]DZ, [$^2H_6$][9RMP]iP, and [$^2H_6$][9RMP]DZ (OlChemim, Czech Republic); 200 ng of [$^2H_4$] ABA (NRC-PBI, Saskatoon, Canada); and 200 ng of isotopic IAA (indole-3-acetic acid), [$^{13}C_6$] IAA (Cambridge Isotopes, Andover, MA, USA).

Samples were re-extracted twice and the supernants were dried, reconstituted in 1.0 M HCOOH (pH $<$ 1.4), and purified using Oasis MCX SPE (Waters, Mississauga, ON). ABA and IAA were eluted first with MeOH followed by CK nucleotides with 0.35 M NH_4OH, and, lastly, CK free bases and CK ribosides with 0.35 M NH_4OH in 60% (v/v) CH_3OH. For stability during analysis, CK nucleotides, separated from CK ribosides earlier by MCX treatment, were converted to nucleosides by overnight incubation with approximately 3 units of alkaline phosphatase (P 4252; Sigma-Aldrich, Oakville, Ontario) in 0.1 M ethanolamine–HCl (pH 10.4) at 37°C (Emery and others 2000). CK nucleotides were dried, reconstituted in Millipore-H_2O, and purified with C_{18} SPE (500 mg, AccuBOND ODS; Fisher Scientific, Mississauga, ON). CK ribosides were eluted with CH_3OH:Millipore-H_2O (80:20 v/v).

Phytohormone extraction from *U. maydis* cultures was performed as described above for plant tissues with the exception that instead of being ground for homogenization, freeze-dried cell suspensions were vortexed in Bieleski extraction solvents in 50-ml tubes with 3 ml of acid-washed glass beads (Sigma 68772-5016) for 10-15 min to lyse the cells.

Analysis of Phytohormones via LC-(ES)MS/MS

Cytokinins were separated and analyzed according to the conditions outlined in Quesnelle and Emery (2007) using a Waters 2680 Alliance HPLC system (Waters, Milford, MA, USA) linked to a Quattro-LC triple quadrupole mass spectrometer (Micromass, Altrincham, UK) equipped with a Z-electrospray ionization source (ESI) and a Genesis C18 reversed-phase column (4 µm, 150 × 2.1 mm; Jones Chromatography, Foster City, CA, USA). CK elution was done with an increasing gradient of acetonitrile (A) and 0.1% acetic acid in Millipore-H_2O (C) at a flow rate of 0.2 ml min^{-1}. Initial conditions were 12% A:88% C and changed on a linear gradient to 70% A:30% C in 11 min, then to 99% A:1.0 C in 13 min, and at 16 min returned to initial conditions for 4 min. CKs were quantified using the isotopic dilution assay method as described in Quesnelle and Emery (2007). CK nucleotides were quantified based upon the addition of 50 ng of deuterated [$^2H_4$] CK nucleotides. *cis*-Isomers of ZR and [9RMP]Z were quantified based on the recovery of deuterated standards of the corresponding *trans* compounds. Abscisic acid (ABA) and auxin (indole-3-acetic acid, IAA) were quantified in a manner similar to that used for CKs, but using conditions specific to each, obtained by multiple reaction monitoring (MRM) of the mother (parent) ion and the appropriate daughter (product) ion as in Ross and others (2004) for ABA and upon measurements in the (173.87 $>$ 129.92) ion channel for IAA. Using a minimum signal-to-noise ratio of 3, detection limits for the cytokinins ranged from 5 to 60 fmol. Detection limits of IAA and ABA were 1 and 2 pmol, respectively.

An attempt was made to quantify auxin (IAA); however, it was not possible because of low recovery of the internal standards, possibly caused by matrix effects. Therefore, for fungal samples, IAA was semiquantified based on a linear regression from a LC-(ES)MS/MS response curve generated with 100, 10, 1, and 0.1 ppm exogenous IAA. The same approach was not possible for plant material because the amounts of endogenous IAA were too low.

Statistical Analysis

Data were analyzed using a one-way analysis of variance (ANOVA) with a Bonferroni test of all pairs of columns. An ANOVA was performed for each time point (1, 5, 6, 14 dpi). The control, water-injected, 518 × 521-infected, and 521-infected groups were included in each comparison to determine significant differences at each time point. Unless otherwise noted, all statements made about statistical significance are at the $p = 0.05$ level.

Results

Cytokinin, Abscisic Acid, and Auxin Profiles from *Ustilago maydis*

Haploid and dikaryon cells from *U. maydis* were monitored by LC-(ES)MS/MS for four free-base cytokinins (iP, Z, *cis*Z, DHZ) and their corresponding riboside (ZR, *cis*ZR, DHZR, iPR) and nucleotide forms (ZRMP, *cis*ZRMP, DHZRMP,

iPRMP) (Table 2), as well as ABA and IAA (Fig. 2). It was clear that both haploids and the dikaryon *Ustilago* cells produced ABA, IAA, and CKs (Fig. 2). Total CK produced by *U. maydis* averaged 656.82 pmol/g fresh weight (FW) (SE = ± 59.77, $n = 12$), which was quite high compared to the maize tissues of this study which averaged 22.53 pmol/g FW (SE = ±2.17, $n = 88$). Six different forms of CKs were consistently produced by *U. maydis* cultures and five of these were either *cis*-isomers or iP forms, including iP, iPR, iPRMP, *cis*ZR, and *cis*ZRMP. Among these, *cis*ZR was the most predominant (Table 2). The sixth CK, DHZ, was detected at much lower levels (Table 2). There were no *trans*-CKs. Thus, appreciable amounts of ribosides and nucleotides were present in *U. maydis* cells, whereas the amounts of free-base forms (iP and DHZ) were much lower (Fig. 3). The *U. maydis* haploid sporidia strain 521 was found to produce significantly more CK nucleotide forms than the haploid sporidia strain 518 and dikaryon cells ($p < 0.04$) (Fig. 3).

A significant quantity of ABA was produced by *U. maydis*, averaging 313.61 pmol/g FW (SE = ± 130.53, $n = 12$), compared to plant tissues sampled in this study, which averaged 46.64 pmol/g FW (SE = ± 9.39, $n = 88$). Dikaryon cells produced considerable amounts of ABA but this level was not significantly different ($p > 0.05$) from that of the haploid cells (Fig. 2). A minor amount of IAA was produced by *U. maydis* [50.23 pmol/g FW (SE = ±20.31, $n = 12$)] (Fig. 2), but the concentrations of the two haploids and dikaryon were not statistically different.

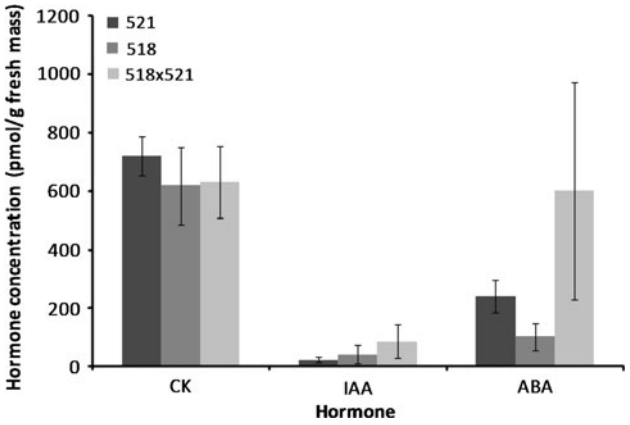

Fig. 2 Quantities of total CK, IAA, and ABA in axenic haploid (521, 518) and dikayron (518 × 521) cells. $n = 4$ with standard error. There were no significant differences among strains within hormone groups

ABA and Cytokinin Profiles in Maize Infected with Pathogenic and Facultative Saprophytic *Ustilago maydis*

Maize plants injected with pathogenic dikaryons (518 × 521), "facultative" saprophytic (521) haploid cells, and water as well as uninjected plants were monitored by LC-(ES)MS/MS for ABA and CKs, including four free-base CKs (iP, Z, *cis*Z, DHZ) and their corresponding riboside (ZR, *cis*ZR, DHZR, iPR) and nucleotide forms (ZRMP, *cis*ZRMP, DHZRMP, iPRMP), during a time course following infection with *U. maydis* and corresponding to the appearance of disease symptoms at different infection stages. We were not able to detect endogenous IAA because of poor recoveries.

Patterns in ABA accumulation showed a clear consistent increase of ABA concentration with time only in leaves that were infected with the dikaryon (Fig. 4). At days 1 and 5 none of the treatments were significantly different; however, by the time of tumor appearance (6 dpi), ABA was significantly more concentrated (Fig. 4) in the dikaryon-infected tissue. This ABA accumulation was even more pronounced by 14 dpi (Fig. 4). In stems, the situation was very similar to leaves, that is, there was a marked accumulation of ABA in the dikaryon-infected tissues.

Table 2 Cytokinin forms (pmol/g fresh mass) in axenic haploid and dikaryon *Ustilago maydis* cells cultured on double complete media

Cytokinin form	Haploid strain 521	Haploid strain 518	Dikaryon 518 × 521
Z	0	0	0
ZR	0	0	0
ZRMP	0	0	0
cisZ	0	0	0
cisZR	294.73 ± 41.20	331.35 ± 119.88	276.72 ± 60.76
cisZRMP	130.39 ± 17.78	92.91 ± 16.39	124.62 ± 25.01
DHZ	4.68 ± 1.23	3.94 ± 0.92	27.25 ± 22.43
DHZR	0	0	0
DHZRMP	0	0	0
iP	19.37 ± 4.84	13 ± 4.40	34.06 ± 4.90
iPR	117.87 ± 26.11	73.14 ± 7.90	85.57 ± 22.01
IPRMP	153.46 ± 19.16	103.85 ± 1.25	83.57 ± 8.37

$n = 4$, ± = standard error

There were no significant differences among strains for any of the cytokinin forms

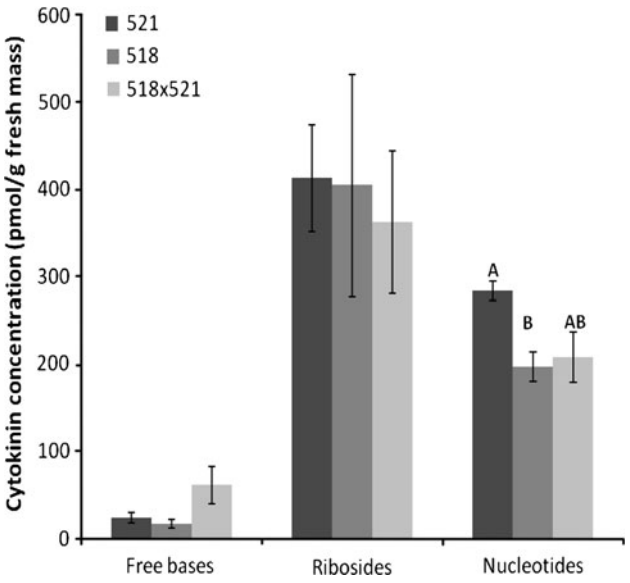

Fig. 3 Quantities of free bases, riboside, and nucelotide CKs in axenic haploid (518, 521) and dikaryon (518 × 521) cells. $n = 4$ with standard error. Different letters indicate significant differences at $p < 0.05$. The absence of letters indicates no significant differences

It was more than tenfold higher than for any other treatment, including plants infected with the haploid culture, which showed no change from control levels (Fig. 4).

All 12 forms of CKs that were studied were present to some degree in the profiles generated (Table 3). However, the predominant forms were the same five forms as for the *Ustilago* cultures described above: free-base iP, the ribosides, iPR, and *cis*ZR and their nucleotide precursors *cis*ZRMP and iPRMP (Tables 2 and 3).

With respect to concentrations, total CKs revealed some patterns similar to those of ABA (Table 3). For example, total CK was significantly higher in the dikaryon-infected plant material at the first day of tumor appearance (6 dpi) and in stems with tumors (14 dpi) compared to control tissues (Table 3). At 6 dpi the haploid-infected tissue also had significantly more concentrated total CK than controls (Table 3). However, in the case of 14-dpi stems, the situation was much clearer and the dikaryon infection effect was approximately tenfold greater than all other treatments, including the haploid infection (Table 3).

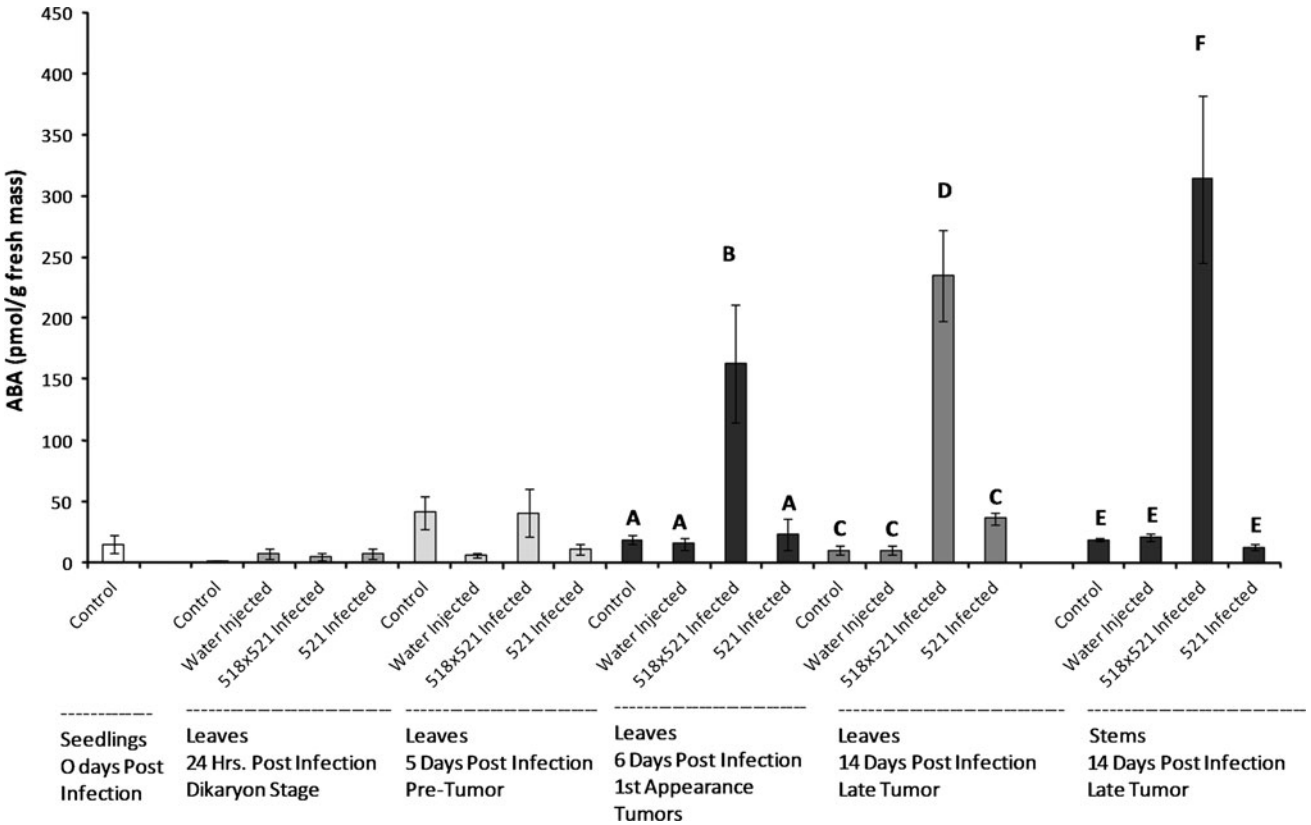

Fig. 4 Quantities of ABA (pmol/g FW) in maize leaves and stems infected with dikaryon (518 × 521) and haploid (521) *U. maydis* compared to controls tissues sampled at 0, 1, 5, 6, and 14 days post infection. Day 0 represents the date of inoculation of 7-day-old seedlings. $n = 4$ samples composed of tissue taken from at least three plants with standard error. Different letters indicate significant differences at $p < 0.05$. The absence of letters indicates no significant differences

Table 3 Time course of 12 CKs in maize exposed to *U. maydis* compared to controls

Cytokinin form	Control Seedlings	Control Leaves	Water injected	518 × 521 infected dikaryon stage	521 infected
Seedlings: 0 dpi, $n = 8$ leaves, 24 h post inoculation, $n = 4$					
Z	1.46	0.00	0.00	0.00	0.00
ZR	2.37 ± 0.60	0.00	0.74 ± 0.74	0.00	0.00
ZRMP	0.95 ± 0.33	0.07 ± 0.07	0.00	0.00	0.00
cisZ	7.02 ± 4.06	0.00	0.00	0.00	0.00
cisZR	2.39 ± 0.86	2.02 ± 0.22	1.64 ± 0.22	4.71 ± 1.46	2.59 ± 0.62
cisZRMP	4.79 ± 1.88	1.19 ± 0.46	1.40 ± 0.63	3.94 ± 2.16	2.85 ± 0.69
DHZ	0.62 ± 0.19	0.79 ± 0.79^{A}	1.55 ± 0.52	3.44 ± 0.54^{B}	2.58 ± 0.21
DHZR	1.65 ± 1.16	0.27 ± 0.17	0.14 ± 0.14	0.00	0.00
DHZRMP	0.00	0.00	0.00	0.00	0.00
iP	0.57 ± 0.10	0.40 ± 0.09^{A}	0.30 ± 0.10	0.84 ± 0.15^{B}	0.58 ± 0.07^{B}
iPR	12.23 ± 3.49	5.28 ± 0.79^{A}	5.33 ± 0.32^{A}	9.23 ± 0.99^{B}	6.71 ± 0.89
IPRMP	4.23 ± 0.61	3.91 ± 0.66	3.99 ± 0.59	4.12 ± 0.38	3.72 ± 0.95
Free bases	9.67 ± 3.92	1.19 ± 0.88^{A}	1.84 ± 0.51^{AB}	4.28 ± 0.65^{B}	3.16 ± 0.22^{AB}
Ribosides	18.64 ± 4.95	7.56 ± 0.83^{A}	7.86 ± 1.28^{A}	13.94 ± 2.01^{B}	9.30 ± 0.59^{AB}
Nucleotides	9.97 ± 1.28	5.18 ± 0.86	5.39 ± 1.10	8.07 ± 2.05	6.57 ± 0.82
Total CK	38.28 ± 7.43	13.93 ± 2.14^{A}	15.09 ± 1.59^{AB}	26.28 ± 4.47^{B}	19.03 ± 1.47^{AB}

Cytokinin form	Control	Water injected	518 × 521 infected pretumor	521 infected
Leaves: 5 dpi, $n = 4$				
Z	0.00	0.00	0.00	0.00
ZR	2.13 ± 1.31	0.00	0.00	0.00
ZRMP	0.00	0.00	0.00	0.00
cisZ	0.00	11.25 ± 8.36	10.54 ± 3.91	0.00
cisZR	0.70 ± 0.70^{A}	0.97 ± 0.56^{A}	6.92 ± 0.91^{B}	3.41 ± 0.81^{A}
cisZRMP	7.11 ± 1.66^{A}	1.86 ± 0.62^{B}	4.82 ± 0.50	3.16 ± 0.73
DHZ	0.84 ± 0.37	3.40 ± 2.56	1.32 ± 0.47	6.40 ± 1.02
DHZR	0.33 ± 0.20	0.07 ± 0.07	0.55 ± 0.32	0.00
DHZRMP	0.00	0.00	0.00	0.00
iP	0.55 ± 0.07	0.68 ± 0.23	0.57 ± 0.03	0.39 ± 0.05
iPR	7.87 ± 0.47	6.06 ± 4.07	5.13 ± 1.38	2.08 ± 0.16
IPRMP	1.34 ± 0.29	0.63 ± 0.07	5.97 ± 4.81	1.50 ± 0.12
Free bases	1.39 ± 0.40	15.32 ± 8.44	12.43 ± 4.33	6.80 ± 1.00
Ribosides	11.03 ± 2.00	7.10 ± 3.76	12.60 ± 2.19	5.49 ± 0.69
Nucleotides	8.45 ± 1.87	2.49 ± 0.62	10.79 ± 4.81	4.66 ± 0.80
Total CK	20.88 ± 3.43^{AB}	24.91 ± 6.18^{AB}	35.82 ± 4.35^{A}	16.94 ± 1.06^{B}

Cytokinin form	Control	Water injected	518 × 521 Infected first-day tumors	521 infected
Leaves: 6 dpi, $n = 4$				
Z	0.00	0.00	0.00	0.00
ZR	0.00	0.00	0.00	0.00
ZRMP	0.00	0.00	0.00	0.00
cisZ	0.00	0.00	0.00	0.00
cisZR	2.84 ± 0.50^{A}	1.08 ± 0.62^{A}	11.56 ± 2.28^{B}	5.83 ± 1.38
cisZRMP	0.92 ± 0.29^{A}	1.66 ± 1.49^{A}	5.74 ± 0.72^{B}	2.69 ± 0.63
DHZ	0.51 ± 0.18^{A}	0.60 ± 0.16^{A}	1.71 ± 0.42^{A}	6.24 ± 1.65^{B}

Table 3 continued

Cytokinin form	Control	Water injected	518 × 521 Infected first-day tumors	521 infected
DHZR	0.11 ± 0.11	0.00	0.00	0.00
DHZRMP	0.00	0.00	0.00	0.00
iP	0.21 ± 0.02^{AB}	0.18 ± 0.03^{A}	0.34 ± 0.04^{BC}	0.42 ± 0.04^{C}
iPR	0.71 ± 0.03^{A}	0.65 ± 0.18^{A}	2.70 ± 0.53^{B}	2.42 ± 0.42^{B}
IPRMP	0.38 ± 0.05^{A}	0.51 ± 0.12^{A}	1.71 ± 0.20^{B}	1.22 ± 0.07^{B}
Fee bases	0.72 ± 0.20^{A}	0.78 ± 0.19^{A}	2.05 ± 0.45^{A}	6.66 ± 1.66^{B}
Ribosides	3.66 ± 0.61^{A}	1.72 ± 0.54^{A}	14.26 ± 2.75^{B}	8.25 ± 1.79^{AB}
Nucleotides	1.30 ± 0.30^{A}	2.18 ± 1.61^{A}	7.45 ± 0.64^{B}	3.92 ± 0.61^{AB}
Total CK	5.67 ± 1.02^{A}	4.67 ± 1.67^{A}	23.76 ± 2.94^{B}	18.82 ± 2.86^{B}
Cytokinin form	Control	Water injected	518 × 521 infected late-day tumors	521 infected
Leaves: 14 dpi, $n = 4$				
Z	0.00	0.00	0.00	0.00
ZR	0.36 ± 0.36	0.00	0.00	0.00
ZRMP	0.00	0.00	0.00	0.43 ± 0.43
cisZ	0.00 ^{A}	0.00 ^{A}	11.40 ± 3.13^{B}	2.27 ± 1.33^{B}
cisZR	1.71 ± 0.85	1.11 ± 0.12	7.86 ± 1.44	5.71 ± 3.83
cisZRMP	0.08 ± 0.08^{A}	0.00^{A}	5.79 ± 1.31^{B}	2.02 ± 1.02^{A}
DHZ	0.00	0.00	0.00	9.14 ± 4.57
DHZR	1.08 ± 0.56	0.00	0.00	0.00
DHZRMP	0.00	0.00	0.00	0.17 ± 0.17
iP	0.84 ± 0.25	0.37 ± 0.04	0.34 ± 0.04	0.26 ± 0.11
iPR	10.24 ± 2.10^{A}	2.84 ± 0.29^{B}	2.74 ± 0.31^{B}	0.43 ± 0.06^{B}
IPRMP	0.62 ± 0.32	0.18 ± 0.01	1.01 ± 0.15	0.61 ± 0.20
Free bases	0.84 ± 0.25	0.37 ± 0.04	11.74 ± 3.48	11.66 ± 5.11
Ribosides	13.39 ± 2.99	3.96 ± 0.30	10.60 ± 1.73	6.13 ± 3.88
Nucleotides	0.70 ± 0.40^{A}	0.18 ± 0.01^{A}	6.81 ± 1.44^{B}	3.24 ± 1.65^{AB}
Total CK	14.93 ± 3.27	4.51 ± 0.30	29.14 ± 5.29	21.03 ± 9.48
Cytokinin form	Control	Water injected	518 × 521 infected late-day tumors	521 infected
Stem: 14 dpi, $n = 4$				
Z	0.00	0.00	0.00	0.00
ZR	1.38 ± 0.81	0.00	0.00	0.00
ZRMP	0.00	0.00	0.00	0.00
cisZ	0.00	0.00	0.00	0.00
cisZR	6.08 ± 0.64^{A}	6.99 ± 1.12^{A}	46.19 ± 3.13^{B}	3.87 ± 0.55^{A}
cisZRMP	0.50 ± 0.19^{A}	0.00^{A}	34.36 ± 1.98^{B}	0.45 ± 0.18^{A}
DHZ	0.00	0.05 ± 0.05	0.00	0.31 ± 0.20
DHZR	0.00	0.01 ± 0.01	0.00	0.00
DHZRMP	0.00	0.00	0.00	0.64 ± 0.64
iP	0.15 ± 0.01^{A}	0.21 ± 0.02^{B}	0.24 ± 0.01^{B}	0.29 ± 0.01^{B}
iPR	4.18 ± 0.33^{A}	2.84 ± 0.47	4.90 ± 0.83^{A}	0.71 ± 0.19^{B}
IPRMP	0.43 ± 0.04^{A}	0.60 ± 0.15^{A}	8.14 ± 2.20^{B}	0.26 ± 0.13^{A}
Free bases	0.15 ± 0.01^{A}	0.26 ± 0.06^{AB}	0.24 ± 0.01^{AB}	0.60 ± 0.19^{B}
Ribosides	11.64 ± 0.14^{A}	9.85 ± 1.56^{A}	51.09 ± 3.35^{B}	4.58 ± 0.73^{A}

Table 3 continued

Cytokinin form	Control	Water injected	518 × 521 infected late-day tumors	521 infected
Nucleotides	0.93 ± 0.19^{A}	0.60 ± 0.15^{A}	42.50 ± 1.63^{B}	1.35 ± 0.55^{A}
Total CK	12.72 ± 0.17^{A}	10.71 ± 1.54^{AB}	93.82 ± 2.07^{C}	6.52 ± 0.80^{B}

Quantities of 12 CKs (pmol/g FW) in maize leaves and stems at 0, 1, 5, 6, and 14 days post infection with dikaryon (518 × 521) and haploid (521) *U. maydis* cultures compared to respective control and water-injected tissues. $n = 4$, $\pm =$ standard error. Superscript A, B, C mean value is significantly different at $p < 0.05$. The absence of letters indicates no significant differences

The analysis of specific groups of CKs revealed similar trends for CK ribosides and CK nucleotides (Table 3). The concentration of both groups was significantly increased, relative to all other treatments, in 518 × 521 infections at 14-dpi stem tumors. Both CK ribosides and CK nucleotides were also elevated in the dikaryon-infected 6-dpi leaves; however, the same magnitude of increase was observed in the haploid-infected tissue. Haploid and dikaryon infections caused significant CK nucleotide increases in 14-dpi leaf tissue. Total free-base CKs were generally low and did not show any consistent pattern that could be accounted for by dikaryon infection (Table 3). The haploid infection caused a small increase in 6-dpi leaves and 14-dpi stems.

The patterns of the individual CKs, *cis*ZR and *cis*ZRMP, were similar to that of ABA. For example, *cis*ZR showed clear increases over controls in pathogenically infected tissue in pretumor leaves (5 dpi), leaves on the first day of tumor appearance (6 dpi), leaves with tumors (14 dpi), and stems with tumors (14 dpi) ($p \leq 0.005$) (Table 3). In the cases of 5-dpi leaves and 14-dpi stems, these increases were also significantly higher than levels seen in the haploid-infected tissue. Likewise, following dikaryon infection, *cis*ZRMP was more concentrated compared to controls and haploid infections in 6- ($p \leq 0.04$) and 14-dpi ($p \leq 0.002$) leaves. Furthermore, in 14-dpi stems dikaryon infection caused a massive *cis*ZRMP accumulation, whereas the controls and haploid-infected tissue had only trace levels [that is, <1 pmol/g FW ($p \leq 0.04$)]. Consistent with pathogenic infection causing *cis*-CK accumulation, *cis*Z was detected in one experimental situation: in 14-dpi leaves. It was detected only in *Ustilago*-infected leaves and was fivefold higher after the dikaryon infection compared to haploid infection.

Discussion

Cytokinin, Abscisic Acid, and Auxin Production by *Ustilago maydis*

Through monitoring with LC-(ES)MS/MS, this study clearly demonstrated that *Ustilago maydis* is capable of producing significant quantities of CK, ABA, and IAA. These phytohormones are produced in this fungus at levels that are substantially higher than those measured in the plant tissues. This substantiates early literature that proposed that *U. maydis* was capable of synthesizing and releasing CK and IAA (Moulton 1942; Mills and Van Staden 1978; Martinez and others 1997; Sosa-Morales and others 1997; Reineke and others 2008). Moreover, this study is the first account of the synthesis and release of ABA by *U. maydis*. Other phytopathogenic fungi, including *Cercospora rosicola*, *C. cruenta*, *C. pini-densiflorare*, and *Botrytis cinerea*, are also known to synthesize ABA (Nambara and Marion-Poll 2005).

In terms of the CKs, the predominance of *cis*Z-group and iP-group CKs in *U. maydis* suggested activity of the IPT (iPRMP, iPR, iP) and tRNA-IPT (*cis*ZRMP–*cis*ZR) CK biosynthesis pathways (Sakakibara 2006). The most prominent form was *cis*ZR, whereas other ribosides and nucleotides occurred in lower amounts. Only two free bases, iP and DHZ, were detected and these were at lower levels than the other CKs. The absence of *trans*-CK appears to be a signature in the *U. maydis* CK profile. The presence of appreciable amounts of ribosides and nucleotides in *U. maydis* cells along with lesser amounts of free base forms suggested some metabolism of CKs in *U. maydis* cultures. The difference in the amount of CK nucleotide production or accumulation between the two haploid sporidia strains 518 and 521 and dikaryon (518 × 521) cells may reflect a difference in hormone production between these strains and cell types. CK production was different between two mating types of the hemibiotrophic fungus *Pyrenoeziza brassica* in a range of media tests (Murphey and others 1997). It is possible that the difference in CK nucleotide release between the two strains of compatible *U. maydis* seen herein reflects a mating-type-specific effect on hormone production. Also, two of four replicate samples had extremely elevated ABA production compared to other dikaryon replicate samples and haploid culture samples. This may reflect variation in the percentage of haploid cells that have fused to create the dikaryon or that there are microenvironmental cues for dikaryon *U. maydis* cultures to produce significantly more ABA.

Identification and Quantification of CK and ABA in Maize Tissue Infected with *U. maydis*

Comparisons of controls and *Ustilago*-exposed maize clearly showed that ABA levels were the most strongly impacted by infection. The data implicate ABA in the disease process because ABA was significantly elevated in infected leaves on the first day of tumor appearance (6 dpi), in stems with tumors (14 dpi), and in leaves with tumors (14 dpi). Moreover, the ABA accumulation intensified with time post infection and it occurred only in the pathogenic dikaryon-exposed plants. Because the 521 haploid-infected plants never showed any such accumulations, it appears that ABA is involved with pathogenesis, perhaps in the physiology of either the development of tumors or the maintenance of tumor growth. ABA may also play a role in the evasion of host defences during the infection process. ABA has been reported to be both a positive and a negative regulator of the defence response in various plant pathosystems, and its role may depend on the specific plant–pathogen interaction (Mauch-Mani and Mauch 2005; Yasuda and others 2008; Bari and Jones 2009). Other possible roles of ABA include the osmoprotection of maize tumors, similar to that of *Agrobacterium* (crown gall)-induced plant tumors (Aloni and Ullrich 2008), or the maintenance of sink status in infected host cells along with CK (Brenner and Cheikh 1995; Radchuk and others 2006; Horst and others 2008).

Work conducted many years ago suggested that CK and IAA hormones are produced by *Ustilago* and played a role in the smut tumors of maize (Moulton 1942; Mills and Van Staden 1978; Khozina and others 1986). However, no one to date has documented the biochemical identities and quantities of these hormones with the exception of IAA (Reineke and others 2008), although a functional role for IAA, if any, during this disease process remains unknown (Reineke and others (2008)). While we also found that *Ustilago* produces IAA, we were unable to resolve IAA concentrations during the infection process.

On the other hand, the results of this study demonstrated that infected and control maize tissue contained a dynamic pool of CKs, including 12 forms, and that there was a predominance of *cis*-isomers. This is consistent with previous maize CK profiling work by Veach and others (2003) that identified substantial amounts of *cis*-isomers in maize tissues. Specifically, the consistent presence of *cis*ZR and *cis*ZRMP, as well as iP-group CKs iP, iPR, and iPRMP, in all maize profiles suggests that to some extent, both the known IPT and tRNA-IPT CK synthesis pathways are active in maize. However, activity is likely strongly in favour of the tRNA-IPT CK synthesis. Interestingly, this type of profile is strongly matched by that of the *U. maydis* cultures. In terms of affecting plant growth, the similar profiles would likely make maize quite responsive to *Ustilago*-produced CK. Similar to the new idea that *Rhodococcus fascians* pathology is based on the local and persistent secretion of an array of bacterial CKs (Pertry and others 2008), *U. maydis* may secrete an array of CKs that closely match those found in host maize tissue which allows this fungal pathogen to biochemically interact with and "shape the plant" to its own advantage.

In general, it is thought that CKs and other hormones may have specific activity in different tissues at different time points during development (Srivastava 2002). The CKs in maize in this study were found to accumulate in pathogenically infected leaves on the first day of tumor appearance (6 dpi) and in stems with tumors (14 dpi) compared to control tissues. Overall, the pattern was similar to that of ABA accumulation and it likewise suggested that CK is involved in the physiology of either tumor development or tumor maintenance in these tissues. Different forms of CKs appear to be involved in the infection process in different tissues at different time points during the *U. maydis*–maize disease course. For instance, in pathogenetically infected leaves, free base CKs and CK ribosides accumulated on the first day of tumor appearance (6 dpi). Furthermore, the free base CK *cis*Z accumulated only in leaves with tumors (14 dpi); whereas CK ribosides and CK nucleotides began to accumulate at 6 dpi and both accumulated highest in stems with tumors (14 dpi). This fluctuation of CK forms suggested that CKs are metabolized during the disease time course and that the metabolism of CKs is likely involved in symptom development during *U. maydis* infection of maize. At the individual CK level, *cis*ZR and its immediate precursor *cis*ZRMP appeared to be involved in the disease process because they were detected at increased levels in pathogenically infected tissue relative to the controls at all stages of infection, including pretumor leaves (5 dpi), leaves on the first day of tumor appearance (6 dpi), leaves with tumors (14 dpi), and stems with tumors (14 dpi). *cis*ZR is bioactive in pea embryogenesis (Quesnelle and Emery 2007), and this particular CK form may also be active during the infection of corn with *U. maydis*. Recently, *cis*Z was indicated to have a particularly important role in the pathology of infection with the disease agent *R. fascians* (Pertry and others 2008).

Thus, the evidence presented here demonstrates that differential CKs are likely involved in the physiology of *U. maydis* disease symptoms. Their most likely function is to increase sink strength of infected areas for the production of tumors. For example, studies of CK deficiency strongly support a role for CK in regulating shoot sink strength (Werner and others 2008). Werner and others (2008) showed that reduced CK content via increased cytokinin oxidase activity was associated with reduced

soluble sugars and decreased invertase activities and ATP content. CKs are known for enhancing sink strength via sugar metabolism by promoting invertase and hexose transporters to degrade sucrose, so it is possible that CKs are also involved in enhancing or maintaining *U. maydis* tumor physiology via sugar sensing (Ehneb and Roitsch 1997; Weber and others 1997; Truernet and others 1996; Emery and others 2000; Horst and others 2008).

Ustilago maydis as the Source of CK and ABA Accumulation in Infected Maize Tissue during Tumor Formation?

From the evidence presented herein it is proposed that a source of the CK and ABA accumulated during corn smut disease development is *U. maydis* release of these compounds. Because there is no known response to these molecules by *U. maydis* itself, it is likely that the production of these compounds is related to the host plant's physiological response for which they have known effects. However, it is acknowledged that the elevated CK and ABA levels in infected tissue may be of plant or fungal origin. The increased production of these hormones may also reflect a maize response to *U. maydis* infection.

The hormone levels profiled for maize infected with *U. maydis* closely matched the signature of ABA, *cis*Z-group, and iP-group CK compounds produced by *U. maydis* in axenic cultures. This suggests that *U. maydis* hormone production is involved in the production of disease symptoms observed on the plant and has a role in this plant–fungus interaction. A comparison between the hormone profiles of pathogenically and saprophytically *U. maydis*-infected maize revealed that ABA is not elevated in saprophytically infected tissue (haploid infection). In contrast, ABA was dramatically elevated in pathogenically infected tissue. Also, although CK and *cis*ZR were dramatically elevated in late stem tumors, the concentrations of these CKs were variable in comparison to respective controls and "diseased" tissues at early time points. This suggested that elevated ABA levels in infected maize are directly linked to *U. maydis* pathogenic development, whereas the link between elevated CK levels in infected maize and pathogenesis was less definitive.

CKs may also play a role in plant defence or the evasion of host defence by the pathogen during infection. At the time of writing, one putative tRNA isopentenyltransferase gene is predicted in the *U. maydis* genome; however, Bolker and others (2008) suggest that it has no biological significance. In light of the data presented here, this bears further examination. CKs produced and released by *U. maydis* may be linked to pathogenesis through the formation or maintenance of tumors, or they may function in host plant evasion or plant growth promotion.

The phytohormone profiles observed during the *U. maydis*–maize interaction may be regulated by molecules present or released by either the host plant or fungus. It has been suggested that the level of CKs produced by phytopathogens *R. fascians* and *Erwinia herbicola* are insufficient to produce the symptoms observed in plants and that molecules released during pathogenesis stimulate phytohormone gene expression in the plant (Crespi and others 1992). Walbot and Skibbe (2010) showed that injections of MeJ and BR in maize tissue caused chlorotic and necrotic patterns in leaves identical to patterns with *U. maydis* injection and that pretreatment with these two compounds prevented *U. maydis*-induced tumorigenesis. Although plant metabolism and other factors may affect the uptake and transport of injected hormones at the site of action, it is interesting that this work suggests that for symptomatology *U. maydis* likely induces the local production of one or both of the MeJ and BR hormones in maize tissue (Walbot and Skibbe 2010). Also, with maize mutants with hormonal or growth abnormalities, Walbot and Skibbe (2010) established that tumor formation induced by *U. maydis* is modulated by host physiology and genotype.

The research outcome of the present study, with direct identification of phytohormones with mass spectrometry, suggests that CKs and ABA accumulate and are likely metabolized in *U. maydis*-infected maize tissue. It also establishes that *U. maydis* produces these phytohormones at significant levels and it is likely that the fungal pathogen is a source of these compounds in infected tissue. However, future work on signal transduction and the expression of ABA and CK metabolism genes by the maize plant during *U. maydis* infection will help to elucidate the origin and role of these hormones during corn smut disease development. It will also be productive to examine the biosynthesis of these hormones by *U. maydis*.

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