The Role of Auxins and Cytokinins in the Mutualistic Interaction Between *Arabidopsis* and *Piriformospora indica*

Jyothilakshmi Vadassery,¹ Claudia Ritter,¹ Yvonne Venus,¹ Iris Camehl,¹ Ajit Varma,² Bationa Shahollari,¹ Ondřej Novák,³ Miroslav Strnad,³ Jutta Ludwig-Müller,⁴ and Ralf Oelmüller¹

¹Friedrich-Schiller-Universität Jena, Institut für Allgemeine Botanik und Pflanzenphysiologie, Dornburger Str. 159, 07743 Jena, Germany; ²Amity Institute of Herbal and Microbial Studies, Sector 125, Noida 201303, UP, India; ³Laboratory of Growth Regulators, Palacky University & Institute of Experimental Botany AS CR, Slechtitelu 11, 783 71 Olomouc, Czech Republic; ⁴Institut für Botanik, Technische Universität Dresden, Zellescher Weg 20b, 01062 Dresden, Germany

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Arabidopsis growth and reproduction are stimulated by the endophytic fungus *Piriformospora indica*. The fungus produces low amounts of auxins, but the auxin levels and the expression of auxin-regulated genes are not altered in colonized roots. Also, mutants with reduced auxin levels (*ilr1-1*, *nit1-3*, *tf12*, *cyp79 b2b3*) respond to *P. indica*. However, the fungus rescues the dwarf phenotype of the auxin overproducer *sur1-1* by converting free auxin into conjugates, which also results in the downregulation of the auxin-induced *IAA6* and the upregulation of the *P. indica*-induced *LRR1* gene. The fungus produces relatively high levels of cytokinins, and the cytokinin levels are higher in colonized roots compared with the uncolonized controls. *trans*-Zeatin cytokinin biosynthesis and the CRE1/AHK2 receptor combination are crucial for *P. indica*-mediated growth stimulation, while mutants lacking *cis*-zeatin, impaired in other cytokinin receptor combinations, or containing reduced cytokinin levels respond to the fungus. Since root colonization is not affected in the cytokinin mutants, we propose that cytokinins are required for *P. indica*-induced growth promotion. Finally, a comparative analysis of the phytohormone mutants allows the conclusion that the response to *P. indica* is independent of the architecture and size of the roots.

Additional keywords: plant-microbe interaction, Sebacinaceae.

The endophytic fungus *Piriformospora indica*, a member of the Sebacinaceae family, colonizes the roots of many plant species including mono- and dicots and mosses, many of which are of forestry, agricultural, horticultural, and medicinal importance (Barazani et al. 2005; Glen et al. 2002; Kaldorf et al. 2005; Oelmüller et al. 2005; Peškan-Berghöfer et al. 2004; Pham et al. 2004; Sahay and Varma 1999; Selosse et al. 2002a and b; Shahollari et al. 2005; 2007; Sherameti et al. 2005, 2008a and b; Urban et al. 2003; Varma et al. 1999, 2001; Waller et al. 2005; Weiss et al. 2004). Root colonization is accompanied by the promotion of growth and higher seed yield, and the plants are more resistant to various biotic and

abiotic stresses (Oelmüller et al. 2004; Rai et al. 2004; Sherameti et al. 2008a and b; Varma et al. 2001; Waller et al. 2005). The apparent lack of species specificity suggests that this symbiosis is based on general recognition and signaling processes. We use *Arabidopsis thaliana* L. as a host for *P. indica* to understand the molecular basis of this interaction. Here, we investigate whether auxins and cytokinins, two phytohormones that are involved in mycorrhizal association (Barker and Tagu 2000; Ludwig-Müller 2000) and many forms of other plant-microbe interactions (Gonzalez-Rizzo et al. 2006; Ludwig-Müller 1999; Murray et al. 2007; Siemens et al. 2006; Tirichine et al. 2007) are also involved in the beneficial interactions of plants (*Arabidopsis* and tobacco) with *P. indica*.

Auxins play an important role in root growth, tropism, apical dominance, and plant senescence and are involved in cell expansion, cell division, and cell differentiation (Davies 2004). In particular, auxins profoundly influence root morphology by inhibiting root elongation, increasing lateral root production, and inducing adventitious roots (Fukaki et al. 2007; Ludwig-Müller et al. 2005; Rahman et al. 2007). Plants use several tryptophan-dependent and a tryptophan-independent pathway to synthesize auxins (Woodward and Bartel 2005). Auxin levels are also controlled by either conjugation, hydrolysis, or both and by metabolism (Seidel et al. 2006). The hormone rapidly and transiently induces the accumulation of at least three families of transcripts: SMALL-AUXIN-UP RNAs (Gil et al. 1994; Walker and Key 1982), GH3-related transcripts and AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) family members (Guilfoyle and Hagen 2007). The *GH3* gene family codes for proteins that repress the auxin signal by inactivating the hormone through conjugation (Staswick et al. 2005). Furthermore, the SCF^{TIR1} E3 ubiquitin ligase complex controls auxin signaling via the toll interleukin 1 receptor family, because it transcriptionally controls degradation of auxin-inducible Aux/IAA repressors (Quint and Gray 2006; Tan et al. 2007).

Cytokinins are a group of N⁶-substituted adenine derivatives that affect cell division, vascular development, sink/source relationships, apical dominance, stress tolerance and leaf senescence (Binns 1994; Mok and Mok 2001; Kakimoto 2003; van Rhijn et al. 1997; Werner et al. 2001). Two classes of isopentenyl transferases (IPT) catalyze the first steps in cytokinin biosynthesis (Kakimoto 2001; Miyawaki et al. 2006; Sakakibara 2006; Takei et al. 2001). ATP and ADP IPT are responsible for isopentenyladenine and *trans*-type zeatin synthesis, and tRNA IPT are required for *cis*-type zeatin synthesis (Miyawaki et al. 2006).

Corresponding author: Ralf Oelmüller; E-mail: b7oera@uni-jena.de; Telephone: ++49-3641-949231; Fax: ++49-3641-949232.

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trans-Isomers are physiologically active in plants, while *cis*-zeatins (cZ) have been less studied, although they also appear to be active (Suttle and Banowitz 2000; Yonekura-Sakakibara et al. 2004). Cytokinin oxidase and dehydrogenases cleave a side chain (Houba-Herlin et al. 1999; Morris et al. 1999; Werner et al. 2006), and a number of enzymes catalyze the conjugation of cytokinins to sugar moieties (Martin et al. 1999a and b, 2001). Models for the cytokinin signaling pathways include the two-component systems (Lohrmann and Harter 2002), in which the cytokinin receptors CRE1, AHK2, and AHK3 activate type A response regulators (RR). Further downstream signaling requires histidine phosphotransfer proteins and type B RR, which upregulate some of the type A RR (Hwang and Sheen 2001). Loss-of-function mutants of cytokinin receptors, mainly studied for CRE1, cause a reduced cytokinin sensitivity (Inoue et al. 2001; Ueguchi et al. 2001), although all receptors have partially redundant functions (Higuchi et al. 2004). The AHK2 and AHK3 receptor combination, for example, plays important roles in quantitative control of organs and growth, with opposite regulatory functions in roots and shoots (Riefler et al. 2006). More recently, the central role of cytokinins for rhizobia-induced nodule formation in legumes has been demonstrated for *Medicago truncatula* and *Lotus japonicus* (Gonzalez-Rizzo et al. 2006; Murray et al. 2007; Tirichine et al. 2007).

Neither auxins nor cytokinins are able to replace *P. indica* in inducing growth stimulation. We present evidence that the fungus reduces the free auxin level in the roots of the auxin over-producer *sur1-1* and, thus, stimulates growth and rescues the dwarf phenotype. Furthermore, substantial amounts of *cis*-cytokinins accumulate in *P. indica*-colonized *Arabidopsis* roots; however, *trans*-zeatin (tZ) produced by the plant is essential for growth promotion. Finally, the double-receptor combination CRE1 AHK2 is necessary for full responsiveness to *P. indica*.

RESULTS

Auxin and cytokinin levels in *P. indica* cultures.

Full-scan mass spectrometry reveals that *P. indica* hyphae grown in liquid medium produce free IAA (325 pmol per gram

of dry weight) and relatively high levels of cytokinins (403 pmol per gram of dry weight) (Fig. 1). We find high levels of isopentenyladenine (iP), an active cytokinin form, cZ, cZ riboside (cZR), and its nucleotide cZ riboside-5'-monophosphate (cZRMP). The high levels of the latter two compounds suggest fast synthesis or turnover. The ratio between cZ and tZ is comparable to those in other organisms (Fig. 1).

Auxin and cytokinins

in *P. indica*-colonized *Arabidopsis* roots.

Under conditions that promote the growth of *Arabidopsis* seedlings by the fungus, no significant differences in free and conjugated auxin levels can be detected in uncolonized and colonized roots (Fig. 2A). As expected, younger roots contain higher auxin levels. Interestingly, the level of conjugated auxins increases with increasing age in infected roots, indicating that the fungus may convert some IAA into inactive compounds. In contrast, the cytokinin levels in colonized roots differ from the control (Fig. 2B). While the amounts of tZ, its riboside, and glycoside are comparable, those of the *cis* forms are 2 (cZ), 6 (cZR), or 10 times (cZ riboside-*O*-glycoside [cZROG]) higher in colonized roots. The amount of cZRMP which is high in fungal hyphae (Fig. 1), is much lower in colonized roots (Fig. 2B), suggesting that it is converted into non-phosphorylated isoforms in symbiotic roots. Finally, iP, the most abundant cytokinin in the hyphae (Fig. 1), is at the detection limit in colonized and uncolonized roots (Fig. 2B). It appears that cZ cytokinins of fungal origin are metabolized in colonized roots.

Auxin and *P. indica*.

The *mas* promoter does not respond to *P. indica* in tobacco seedlings, although the fungus promotes growth. Some of the best-responding host plants for *P. indica* are *Nicotiana* spp. (*N. tabacum* [Oelmüller et al. 2005; Sherameti et al. 2005] and *N. attenuata* [Barazani et al. 2005]). Under light-limiting conditions (100 $\mu\text{mol s}^{-1} \text{m}^{-2}$), a huge difference in growth can be detected between colonized and uncolonized seedlings (Supplementary Fig. S1; Sherameti et al. 2005), and flower devel-

Fig. 1. Endogenous levels of different cytokinins in *Piriformospora indica*, cultivated in liquid media. tZ = *trans*-zeatin; cZ = *cis*-zeatin; tZR = *trans*-zeatin riboside; cZR = *cis*-zeatin riboside; iP = isopentenyladenine; iPR = isopentenyladenosine; tZOG = *trans*-zeatin-*O*-glucoside; cZOG = *cis*-zeatin-*O*-glucoside; tZROG = *trans*-zeatin riboside-*O*-glucoside; cZROG = *cis*-zeatin riboside-*O*-glucoside; DHZOG = dihydrozeatin-*O*-glucoside; tZRMP = *trans*-zeatin riboside-5'-monophosphate; cZRMP = *cis*-zeatin riboside-5'-monophosphate; iPRMP = isopentenyladenosine-5'-monophosphate.

opment started approximately 5 weeks earlier in colonized plants. Analysis of 66 plants revealed that colonized plants produce $85 \pm 7\%$ more seeds. A similar result is obtained with another member of the Sebacinaceae family, *Sebacina vermifera* (Barazani et al. 2005; $81 \pm 9\%$ more seeds). However, β -glucuronidase (GUS) levels in *P. indica*-colonized lines with an *uidA* gene expressed under the control of the auxin-inducible mannopin synthase (*mas*) promoter are not increased, either at the seedling level or in adult plants (Table 1). IAA- or 2,4-D-treated seedlings (0.2 to 50 $\mu\text{g/ml}$) contain 20- and 35-fold higher GUS levels but do not grow taller than controls (Table 1). Thus, auxin levels of either fungal or plant origin do not increase when *P. indica* promotes growth and seed production in tobacco.

The DR5-GUS reporter system and the vast majority of auxin-responsive genes do not respond to *P. indica* in *Arabidopsis*, although the fungus promotes growth. An auxin-responsive element containing a TGTCTC sequence and an adjacent or overlapping coupling element was identified in the auxin-responsive *GH3* promoter from soybean (Liu et al. 1994; Ulmasov et al. 1995). The TGTCTC element alone is

Table 1. β -glucuronidase (GUS) activity in 20-day-old roots and shoots of tobacco seedlings with a *mas*-promoter::*uidA* gene construct

Material	Treatment ^a	GUS activity (nmol/mg per min)	
		Roots ^b	Shoots/leaves ^b
Seedling	LL, control	0.77 ± 0.03	0.98 ± 0.08
	LL + <i>P. indica</i>	0.83 ± 0.06	1.21 ± 0.14
	HL, control	0.98 ± 0.06	1.13 ± 0.11
	HL + <i>P. indica</i>	1.02 ± 0.09	1.09 ± 0.10
	LL + IAA	24.36 ± 2.51	27.47 ± 2.74
	LL + 2,4-D	34.15 ± 2.85	49.87 ± 5.17
Plants	LL, control	4.14 ± 0.34	5.32 ± 0.61
	LL + <i>P. indica</i>	3.92 ± 0.84	5.97 ± 0.92

^aSeedlings were grown in the absence or presence of *P. indica* in either low white light (LL, 100 $\mu\text{mol s}^{-1} \text{m}^{-2}$) or high white light (HL, 600-750 $\mu\text{mol s}^{-1} \text{m}^{-2}$).

^bBased on 42 experiments with seven independent primary transformants each, errors represent standard error. Uncolonized seedlings were also treated with indole-3-acetic acid or 2,4-D (25 $\mu\text{g/ml}$). The GUS levels for adult plants ($n = 66$, either grown in the presence or absence of *Piriformospora indica*) were determined after 120 days of growth in a temperature- and light-controlled growth chamber, before flowering.

Fig. 2. A, Endogenous levels of free (left) and conjugated (right) indole-3-acetic acid in *Arabidopsis* roots, which were cocultivated with *Piriformospora indica* for 7, 10, or 14 days (black bars). Open bars represent the uncolonized control roots of the same age. dai = days after inoculation. Based on three independent experiments, error bars represent standard error. **B,** Endogenous levels of different cytokinins in *Arabidopsis* roots, which were cocultivated with *P. indica* for 10 days (black bars) and control roots of the same age (white bars). tZ = *trans*-zeatin; cZ = *cis*-zeatin; tZR = *trans*-zeatin riboside; cZR = *cis*-zeatin riboside; tZ9G = *trans*-zeatin-9-glucoside; cZ9G = *cis*-zeatin-9-glucoside; DHZR = dihydrozeatin riboside; DHZ9G = dihydrozeatin-9-glucoside; iP = isopentenyladenine; iPR = isopentenyladenosine; iP9G = isopentenyladenine-9-glucoside; tZOG = *trans*-zeatin-*O*-glucoside; cZOG = *cis*-zeatin-*O*-glucoside; tZRMP = *trans*-zeatin riboside-5'-monophosphate; cZRMP = *cis*-zeatin riboside-5'-monophosphate; iPRMP = isopentenyladenosine-5'-monophosphate; BAPRMP = benzylaminopurin riboside-5'-monophosphate. Based on three independent experiments, error bars represent standard error.

sufficient to confer auxin responsiveness to reporter genes in transgenic *Arabidopsis* (Ulmasov et al. 1995, 1997). IAA and 2,4-D in concentrations from 0.2 to 50 μg per plate stimulate GUS levels between two- (0.2 μg) and 25-fold (10 μg) in roots and shoots of *DR5::uidA* lines, respectively (data not shown), while cocultivation of these seedlings with *P. indica* does not result in higher GUS levels (Table 2). Growth promotion, in contrast, is only observed in the presence of the fungus.

Table 2. β -glucuronidase (GUS) activities in the shoots and roots of *Arabidopsis* seedlings with the *DR5-GUS* reporter gene system^a

Material	Treatment	GUS activity (nmol/mg per min)	
		Roots	Shoots/leaves
Seedlings	6 dai, control	24.3 $\pm$ 3.3	27.4 $\pm$ 4.1
	6 dai + <i>P. indica</i>	26.1 $\pm$ 5.1	29.2 $\pm$ 3.5
	10 dai control	22.9 $\pm$ 4.1	21.5 $\pm$ 2.9
	10 dai + <i>P. indica</i>	26.4 $\pm$ 4.0	29.2 $\pm$ 3.5
	14 dai, control	28.7 $\pm$ 4.4	78.2 $\pm$ 5.3
	14 dai + <i>P. indica</i>	26.4 $\pm$ 4.0	81.2 $\pm$ 6.5
Plants	5 weeks, control	58.8 $\pm$ 6.1	199.2 $\pm$ 12.3
On soil	5 weeks, + <i>P. indica</i>	61.8 $\pm$ 5.3	191.2 $\pm$ 11.1

^a GUS activities 6, 10, and 12 days after inoculation (dai) with *Piriformospora indica*. Controls (– *P. indica*) were mock-treated. Based on 36 experiments, bars represent standard error. The GUS levels for adult plants were determined 5 weeks after transfer to soil and growth in a temperature- and light-controlled growth chamber, before flowering.

Growth of seedlings in media with up to 10 μg of IAA has no effect on growth, while auxin concentrations >10 μg of auxin start to inhibit the growth of the seedlings. Thus, similar to tobacco, it is unlikely that *P. indica*-mediated growth promotion in *Arabidopsis* is caused by altered auxin levels.

Furthermore, 390 genes have been described in the literature to be either up- or downregulated at least twofold by auxin in *Arabidopsis* seedlings or shoots (Goda et al. 2004; Liscum and Reed 2002; Sawa et al. 2002; Tian et al. 2002). Analysis of our microarray data uncovered that 154 of them are significantly expressed in roots and that 91% of the 154 genes do not respond to the fungus (data not shown).

Arabidopsis lines with reduced auxin levels respond to *P. indica*. Next, we analyzed whether *P. indica* can stimulate growth of knockout lines impaired in different steps of auxin biosynthesis or metabolism (Fig. 3). *ilr1-1* has a lesion in the IAA amino acid hydrolase ILR1. This enzyme releases IAA from IAA-leucine and -phenylalanine conjugates (Bartel and Fink 1995). *nit1-3* has a lesion in the nitrilase, so that indole-3-acetonitrile cannot be converted to active IAA (Hillebrand et al. 1996). *tfl2* has a lesion in a chromodomain protein. Although the role of this protein in auxin metabolism is not clear, the mutant contains reduced IAA and glucosinolate levels (Ludwig-Müller et al. 1999; Bennett et al. 2005). The double mutant *cyp79 b2b3* is defective in two cytochrome P450-dependent monooxygenases that catalyze the conversion of

Fig. 3. *Arabidopsis* seedlings (wild type [WT] and transgenic lines manipulated in their auxin contents) that were grown in the presence (left) or absence (right) of *Piriformospora indica* for 10 or 15 (for *tfl2*) days.

tryptophan to indole-3-acetaldoxime. This results in reduced auxin levels in *Arabidopsis* (Zhao et al. 2002). In spite of substantial differences in the phenotypes and root architectures, all mutant seedlings are taller in the presence of *P. indica* (Fig. 3). Cocultivation of the mutant seedlings with *P. indica* for 10 days results in an increase in fresh weight (*ilr1-1*: $28 \pm 4\%$, *nit1-3*: $39 \pm 5\%$, *tfl2*: $33 \pm 4\%$, *cyp79 b2b3*: $36 \pm 5\%$), which is in the range of the wild-type control ($34 \pm 3\%$; data not shown). Thus, reduced auxin levels do not affect the growth response of *Arabidopsis* seedlings to *P. indica*.

tfl2 has a dwarf phenotype, and the poorly developed roots with only a few lateral roots and small root hairs offer few possibilities for fungal colonization. We barely observe hyphae growing in root cells and never see spores in the cells. Polymerase chain reaction (PCR) analysis for the *P. indica* gene encoding the transcription elongation factor 1 (*Pitef1*) confirms that colonized *tfl2* roots contain severely reduced amounts of fungal DNA and RNA, as compared with the wild type and the *ilr1-1* mutant that was used as a control (Fig. 4). This indicates that the extent of root colonization does not correlate with the growth response induced by *P. indica*.

The dwarf phenotype of the auxin overproducer *sur1-1* is rescued by the fungus. The *sur1-1* mutant of *Arabidopsis* lacks a P450-dependent monooxygenase, which catalyzes the N-oxidation of indole-3-acetaldoxime. This enzymatic reaction is considered to be the step directing the pathway to indole glucosinolates. The mutant accumulates indole-3-acetaldoxime that is converted to active IAA. Thus, *sur1-1* can be considered as an auxin overproducer (Mikkelsen et al. 2004); the seedlings are much smaller and their root length is approximately half the length of the wild type. Growth of *sur1-1* seedlings is strongly stimulated by the fungus, and *P. indica*-treated *sur1-1* seedlings are almost as big as wild-type seedlings cocultivated with the fungus (Fig. 3). In particular, the size, weight, and architecture of the roots (28.3 ± 0.9 cm lateral roots and 5.9 ± 0.3 cm main root) are indistinguishable from wild-type seedlings grown in the presence of the fungus (Fig. 5A). Growth stimulation of the aerial parts of the seedlings by the fungus indicates an efficient root-shoot communication (Figs. 3 and 5A).

The free auxin level in *sur1-1* roots 10 days after cocultivation is strongly reduced in *P. indica*-colonized seedlings compared with the uncolonized control (132 ± 99 vs. 338 ± 191 ng per gram of fresh weight, respectively), while the conjugated IAA level increases ($6,195 \pm 140$ vs. $3,148 \pm 925$ ng per gram

of fresh weight, respectively). As a consequence, the *IAA6* (At1g52830; Abel et al. 1995) mRNA level, which is upregulated in *sur1-1* roots and shoots compared with the wild type, is repressed in the presence of the fungus (Fig. 5B). Furthermore, the message level for *LRR1* (At5g16590), which is upregulated in response to *P. indica* in the wild type and can be used as a marker for a successful recognition of the two symbiotic partners (Shahollari et al. 2007), is much lower in the

Fig. 5. A, Fresh weight of wild-type (black) and *sur1-1* (gray) roots that were either mock-treated (dots) or cocultivated with *Piriformospora indica* (triangles). dai = days after inoculation. Bars represent standard error, based on six independent experiments. **B**, Northern analysis for *IAA6* (At1g52830) and *LRR1*. *GAPDH* was used as a loading control. Wild-type (WT), *sur1-1*, and *ilr1-1* seedlings were grown in the absence (-) or presence (+) of *P. indica* for 6 days, and RNA was isolated from roots and leaves. Hybridization occurred with *IAA6*-, *LRR1*-, and *GAPDH*-specific gene probes.

Fig. 4. Comparison of fungal translation elongation factor 1 (*cPitef1*) mRNA and genomic DNA (*gPitef1*) in roots of wild-type (WT), *tfl2*, and *ilr1-1* seedlings with the plant *actin* nucleic acids. RNA was isolated from uncolonized (0) roots and roots that were cocultivated with *Piriformospora indica* for 12 days (12). After reverse transcription, *cPitef1* (38 cycles) and *actin* (24 cycles) were amplified. (12*), genomic DNA was isolated 12 days after cocultivation of the two symbiotic partners and the DNA fragments were amplified.

sur1-1 mutant (Fig. 5B). Cocultivation of *sur1-1* roots with the fungus results in an almost 10-fold upregulation of the *LRR1* message, and the level is as high as in colonized wild-type seedlings (Fig. 5B). *ilr1-1*, which is used as a control for both genes, behaves like the wild type. Strong upregulation of the *LRR1* mRNA level is also detectable in leaves, demonstrating again the importance of root-shoot communication in *P. indica*-colonized *sur1-1* seedlings. Together, these results indicate that *P. indica* rescues the dwarf phenotype of *sur1-1* by reducing the amount and action of free auxin.

Cytokinin and *P. indica*.

Cytokinin-responsive genes respond to P. indica in Arabidopsis roots. The cytokinin-responsive *ARR5* gene is highly expressed in the base of the lateral root primordia and at the root tip during lateral root development of *Arabidopsis* (Lohar et al. 2004), and the *ARR5* mRNA level in *P. indica*-treated roots is $54 \pm 5\%$ ($n = 4$) higher than in control roots. A similar stimulation ($45 \pm 5\%$; $n = 4$) is obtained for the GUS activity

in the roots of transgenic *ARR5::uidA* lines. Although the stimulation induced by *P. indica* is low compared with the >20-fold induction by exogenously applied iP (data not shown), these results prompted us to investigate in greater detail the role of cytokinins in the interaction.

Kiba and associates (2005) presented a list of genes that are regulated in response to cytokinin in *Arabidopsis* seedlings. Approximately 20% of them are also expressed in roots, and approximately 35% showed a low (30 to 80%) response to *P. indica* (data not shown). The significance of the response of each gene has been confirmed by other means, and the data are consistent with the results obtained for *ARR5*, suggesting that cytokinin-regulated genes may play a role in the interaction between *P. indica* and *Arabidopsis*.

tZ but not cZ biosynthesis in Arabidopsis is required for P. indica-mediated growth promotion. The *atipt1 atipt3 atipt5 atipt7* quadruple mutant possesses decreased levels of iP, tZ-type cytokinins, and their metabolites and slightly increased levels of cZ (Miyawaki et al. 2006). Neither the roots nor the shoots of the *atipt1 atipt3 atipt5 atipt7* seedlings respond to *P. indica* (Fig. 6). In contrast, roots and shoots of the *atipt2 atipt9* double mutant, which contains reduced levels of iP and cZ-type cytokinins, respond to the fungus, comparable to the wild type (Fig. 6). Thus, biosynthesis of tZ- but not cZ-type cytokinins appears to be required for *P. indica*-induced growth promotion in *Arabidopsis*. Apparently, cZ isomers produced by the fungus cannot replace the missing tZ isomers in symbiotic roots of the quadruple mutant (discussed below). Furthermore, it is interesting to note that the response to *P. indica* is independent of the organ size. While the aerial parts of the quadruple mutant are relatively small and those of the double mutant comparable to the wild type, the opposite is observed for the roots (Fig. 6).

Table 3 demonstrates that cZ and tZ can activate *ARR5::uidA* gene expression in wild-type roots, although cZ is less effective than tZ. This is consistent with the results obtained by Spíchal and associates (2004). Furthermore, cZR and cZROG, an inactive stable storage form of cytokinin that can be deglycosylated and reversibly converted into cZR, are active in stimulating *ARR5::GUS* gene expression in roots. Both isomers are synthesized by the fungus and accumulate in colonized roots (Figs. 1 and 2B). One explanation why cZ-cytokinins cannot compensate for the missing tZ-cytokinins in the quadruple mutant can be that the available amount of cZ-cytokinins in colonized roots is too low or not effective enough to stimulate growth in the presence of the fungus. Furthermore, it appears that cZ cytokinins cannot be converted efficiently to tZ cytokinins.

The CRE1/AHK2 receptor combination is crucial for P. indica-mediated growth promotion in Arabidopsis. Three cytokinin receptors, CRE1, AHK2, and AHK3, have been described

Fig. 6. A. The response of the cytokinin biosynthesis mutants *atipt1 atipt3 atipt5 atipt7* and *atipt2 atipt9* to *Piriformospora indica*. Seedlings were grown in the presence (left) or absence (right) of the fungus for 10 days. To compare the architecture of the roots of the mutants and to determine the root-to-shoot ratio, we also grew the seedlings in liquid Murashige-Skoog medium for 6 days to facilitate optimal root development. Weights were 10.1 ± 3 mg for *atipt1 atipt3 atipt5 atipt7* and 16 ± 6 mg for *atipt2 atipt9*. Root-to-shoot weight ratios were 0.84 for *atipt1 atipt3 atipt5 atipt7* and 0.75 for *atipt2 atipt9*. **B.** Weight of the roots of 10-day-old wild-type (WT) seedlings and of the cytokinin biosynthesis defective mutants *atipt1 atipt3 atipt5 atipt7* and *atipt2 atipt9*, grown in the absence (black) or presence (white) of *P. indica*. Based on seven independent experiments. Bars represent standard error.

Table 3. β -glucuronidase (GUS) activities in the roots and shoots of 20-day-old *Arabidopsis* seedlings with a *ARR5*-promoter::*uidA* gene construct^a

Applied zeatin	GUS activity (pmol/g min)	
	Roots	Shoots
<i>trans</i> -Zeatin	1,0839 $\pm$ 1,126	6,267 $\pm$ 372
<i>cis</i> -Zeatin (cZ)	5,934 $\pm$ 622	5,502 $\pm$ 372
cZ riboside	7,102 $\pm$ 781	4,476 $\pm$ 239
cZ riboside- <i>O</i> -glycoside	8,189 $\pm$ 914	5,646 $\pm$ 249
Control	281 $\pm$ 12	220 $\pm$ 17

^a Seedlings were grown in the absence or presence of the indicated zeatin isomers. Seedlings were grown on Murashige-Skoog medium for 10 days and were transferred to new plates containing 4 nM of the indicated zeatin isomers for an additional 10 days before GUS assay. Based on six independent experiments.

(Kyojuka 2007). All three receptors are expressed at relatively high levels in roots, but the message levels are not regulated by *P. indica* (relative mRNA levels in roots grown in the absence [–] or presence [+] of *P. indica* for 2 days: *CRE1*, [–] set as $100 \pm 8\%$, [+] $102 \pm 7\%$; *AHK2*, [–] $66 \pm 5\%$, [+] $69 \pm 4\%$; *AHK3*, [–] $67 \pm 4\%$, [+] $59 \pm 6\%$; $n = 4$). Mutations in any of the receptor genes have no effect on the response to *P. indica* (Supplementary Fig. S2; $35 \pm 4\%$ [*cre1*], $33 \pm 4\%$ [*ahk2*], and $31 \pm 5\%$ [*ahk3*] increase in fresh weight after cocultivation with the fungus for 10 days). Therefore, double mutants for different receptor combinations are investigated (Fig. 7). The double mutant *cre1 ahk2* shows almost no growth response to *P. indica*. In particular, the roots do not respond to *P. indica*, while some response ($3.2 \pm 0.6\%$) is observed for the shoot. The other double mutants *cre1 ahk3* and *ahk2 ahk3* respond to the fungus in a manner similar to the wild type (Fig. 7). Growth of the triple mutant is too slow to obtain meaningful results. This indicates that the CRE1 AHK2 receptor combination is crucial for response to *P. indica*.

The cytokinin oxidase and dehydrogenase overproducers 35S::*AtCKX1* and 35S::*AtCKX2* show reduced cytokinin levels due to an increased cytokinin degradation. The lines have stunted and bushy shoots and their roots are bigger than the wild type (Werner et al. 2003). Growth of both overproducers is clearly promoted by *P. indica*. After 10 days of cocultivation, a $37 \pm 4\%$ increase in the fresh weight is detectable, which is comparable to the wild type ($34 \pm 3\%$). This indicates that the reduced levels of cytokinins in 35S::*CKX1* and 35S::*CKX2* and the different root architectures do not affect response to *P. indica*.

Regulation of P. indica-responsive genes in atipt1 atipt3 atipt5 atipt7 and cre1 ahk2 and colonization of their roots. The

importance of tZ biosynthesis and the CRE1 AHK2 receptor combination for the beneficial interaction between the two symbiotic partners is further supported by the observation that the message levels for *LRR1* and *germin*, two genes that are upregulated in wild-type roots in the response of *P. indica*, are not upregulated in colonized roots of the two mutants (Fig. 8). While the *LRR1* mRNA level is comparable to the level in uncolonized wild-type seedlings, that of *germin* is reduced. The *P. indica*-responsive cZ mutant *atipt2 atipt9* and the double receptor mutant *ahk2 ahk3* were used as controls. For both mutants, the mRNA levels are upregulated in the roots in response to *P. indica*, comparable to the wild type (Fig. 8). Finally, this is not caused by differences in the root colonization. A comparative PCR analysis of the *Pitief1* mRNA levels relative to the root *actin* gene (Fig. 4) shows that the ratios do not differ significantly from the wild type. If the wild-type ratio after 12 days of cocultivation is set as 1.00 ± 0.13 , that for *atipt1 atipt3 atipt5 atipt7* is 1.09 ± 0.15 and that for *cre1 ahk2* is 0.96 ± 0.17 (controls: *tfl2*: 0.08 ± 0.01 [Fig. 4], *atipt2 atipt9*: 0.94 ± 0.16 , *ahk2 ahk3*: 1.07 ± 0.10 ; data based on four independent experiments, errors are standard error).

Root and shoot architecture and growth promotion. The architecture, weight, and branching of the roots, as well as the root-to-shoot fresh-weight ratios (RSFWR) of the cytokinin mutants do not correlate with their response to *P. indica*. For instance, the phenotypes of *atipt1 atipt3 atipt5 atipt7* and *ahk2 ahk3* are similar under our growth conditions, and both mutants have smaller shoots and longer roots than the wild type (Miyawaki et al 2006; Riefler et al 2006). *Atipt1 atipt3 atipt5 atipt7* does not respond to *P. indica* (Fig. 6), while *ahk2 ahk3* does. Also, *atipt2 atipt9* has a reduced root growth, but its response to *P. indica* is comparable to that of the wild type

Fig. 7. A, Combinations of cytokinin-receptor double-mutants (*ahk2*, *ahk3*, and *cre1*) were grown in the presence (left) or absence (right) of *Piriformospora indica* for 10 days. To compare the architecture of the roots of the receptor mutants and to determine the root-to-shoot ratio, seedlings were grown in liquid Murashige-Skoog medium for 6 days to facilitate optimal root development. Weights were 11.2 ± 3 mg for *cre1 ahk2*, 19.6 ± 3 mg for *cre1 ahk3* and 7.0 ± 2.0 mg for *ahk2 ahk3*. Root-to-shoot weight ratios were 0.20 for *cre1 ahk2*, 0.28 for *cre1 ahk3* and 0.74 for *ahk2 ahk3*. **B**, Weight of the roots of wild-type (WT) seedlings as well as double knockout lines, grown in the absence (black) or presence (white) of *P. indica* for 10 days. Based on seven independent experiments. Bars represent standard error.

(Fig. 6). Furthermore, *ahk3 ahk2* and *atipt2 atip9* have RSFWR of 0.74 and 0.75, respectively, although their response to *P. indica* is comparable to that of the wild type (RSFWR = 0.23). *cre1 ahk2* has a RSFWR of 0.20, which is comparable to that of the wild type and quite different from that of *atip1 atip3 atip5 atip7* (RSFWR = 0.84); however, neither mutant responds to *P. indica*. These examples demonstrate that the size, weight, and RSFWR of the cytokinin mutants have little effect on the response to *P. indica*.

DISCUSSION

The involvement of auxin and cytokinin in plant-microbe interactions has been widely studied (Barker and Tagu 2000; Cooper and Long 1994; Fitze et al. 2005; Gonzalez-Rizzo et al. 2006; Herrmann et al. 2004; Lohar et al. 2004; Murray et al. 2007; Oldroyd 2007; Reddy et al. 2006; Sirrenberg et al. 2007; Tirichine et al. 2007); however, relatively little is known about their involvement in the interactions between plants and endophytic fungi. Here, we use well-characterized *Arabidopsis* mutants as well as molecular and biochemical tools to understand the role of these two hormones in the beneficial interaction with *P. indica*.

The role of auxin in the *Arabidopsis*–*P. indica* interaction.

Mutants with reduced auxin levels respond to *P. indica* and the auxin levels and the expression of auxin-regulated genes in *Arabidopsis* and tobacco roots do not change when the fungus promotes plant growth. The expression of auxin-responsive promoter::*uidA* constructs is also not stimulated after transfer of the seedlings to soil for several months, although beneficial effects can clearly be seen under these conditions. Although the auxin concentrations that stimulate growth can differ substantially from those that activate the expression of auxin-responsive genes, none of the auxin concentrations that were tested in our system stimulated growth and, at concentrations higher than 10 μ g per plate, even inhibited growth of *Arabidopsis* seedlings. Thus, we propose that the auxin levels in seedlings and adult plants have little or no effect on *P. indica*-mediated growth promotion and stimulation of productivity in adult plants.

In contrast, the auxin overproducer *sur1-1* responds more sensitively to *P. indica* than to the wild type, and its dwarf phenotype is almost rescued in the presence of the fungus. To our knowledge, *sur1-1* shows the strongest response of a plant to an endophytic fungus reported so far. *P. indica* reduces the free

auxin levels in the roots, and consequently, the expression of the auxin-upregulated *IAA6* mRNA level is downregulated and the *P. indica*-induced *LRR1* mRNA upregulated in both roots and shoots of colonized *sur1-1* seedlings (Fig. 5). The approximately twofold decrease in the free auxin level in colonized roots can be partially explained by the fact that these roots are approximately twice as large as the uncolonized control and thus in a different developmental stage (Fig. 5). However, the conjugated auxin levels do not change significantly in response to the fungus between days 7 and 14 in the wild type (Fig. 2A), while the level is doubled in colonized *sur1-1* roots compared with the uncolonized control. These results demonstrate that the fungus interferes with the auxin metabolism in *sur1-1*. It remains to be determined whether *P. indica* directly influences the auxin levels or whether they are manipulated by the plant as a consequence of the growth response to the fungus.

The role of cytokinins in the *Arabidopsis*–*P. indica* interaction.

Many root-associated microorganisms produce cytokinin, and plants possessing mycorrhiza accumulate high concentrations of iP-like compounds in roots and shoots. Differences between mycorrhizal and nonmycorrhizal plants in total ZR-like compounds have also been reported (Ginzberg et al. 1998; van Rhijn et al. 1997). Nod-factor signaling by rhizobia in the legume *Lotus japonicus* requires the cytokinin-receptor LHK1 for cell division and initiation of nitrogen-fixing nodule development (Murray et al. 2007; Tirichine et al. 2007). LHK1 is critical for nodule organogenesis, but bacterial entry through infection thread formation is independent of cytokinin signaling. Several lines of evidence support the idea that cytokinins also play a role in the beneficial interaction between *P. indica* and *Arabidopsis*. i) Cytokinin- but not auxin-responsive genes are differentially regulated in *P. indica*-colonized *Arabidopsis* roots. ii) The CRE1/AHK2 receptor combination is crucial for the response to *P. indica*. Riefler and associates (2006) also analyzed cytokinin receptor mutants and found that the *ahk2 ahk3* double mutant develops a strongly enhanced root system through faster growth of the primary root and more branching. The dry weight of these roots increases more than twofold as compared with the wild type, while the enhancement of the other double-mutant combinations was in the order of 20%. The response of the *ahk2 ahk3* mutant to *P. indica* may be explained by the larger root morphology, although we do not see a direct correlation between root size and the growth response to *P. indica* (discussed below). The absence of the

Fig. 8. Change of the mRNA levels for *LRR1* (black) and *germin* (white) in the presence of *Piriformospora indica* in *Arabidopsis* roots. Wild-type (WT), *cre1 ahk2*, and *atipt1 atipt3 atipt5 atipt7* seedlings were cocultivated with or without *P. indica* for 6 days, and the amount of *LRR1* and *germin* mRNA was determined by real-time polymerase chain reaction relative to the *GAPDH* control. The ratios shown are the mRNA levels in colonized roots to uncolonized control. Bars represent standard error.

response of the *cre1 ahk2* mutant to the fungus defines a new role of this receptor combination. However, besides CRE1 and AHK2, other receptors must exist in the root that perceive signals from *P. indica*. For instance, *P. indica* induces a rapid increase in the intracellular calcium level in root cells, and this response occurs also in the *cre1 ahk2* mutant (data not shown). iii) *P. indica* itself produces iP and cZ-type cytokinins, and elevated levels of these isomers accumulate in colonized roots. Although both isomers are able to stimulate *ARR5* gene expression in the roots, we could not demonstrate a specific effect of cZ isomers on growth promotion. However, our data clearly demonstrate that tZ is required for the beneficial interaction. This suggests that cZ cytokinins are either not or not efficiently converted to tZ cytokinins, although a *cis-trans* isomerase has been identified in *Phaseolus* spp. (Bassil et al. 1993). Alternatively, the available amount of cZ of either fungal or plant origin in the quadruple mutant is too low or not effective enough to stimulate growth in the symbiosis. It is known that cZ is less effective than tZ in activating the cytokinin receptors, both in yeast and in planta (Inoue et al. 2001; Spichal et al. 2004), which is consistent with our data on *ARR5* regulation in roots (Table 3). Another reason for the lack of a response to cZ can be that enzymatic activities deriving from one of the four inactivated genes in the quadruple mutant, *atipt1*, *atipt3*, *atipt5*, or *atipt7*, may be required for the beneficial interaction. Finally, the dwarf phenotype of the aerial part of the quadruple mutant may be responsible for the lack of the response, although this appears to be unlikely, since the aerial parts of the *ahk2 ahk3* receptor mutant have a similar phenotype and respond to the fungus. Furthermore, the roots of the quadruple mutant resemble those of the wild type, although their growth is not stimulated by *P. indica*.

Auxin and cytokinin action and ratio in the *Arabidopsis*–*P. indica* interaction.

Colonization of the *sur1-1*, *cre1 ahk2* and quadruple mutant roots is comparable to that of the wild type, suggesting that recognition of the two symbiotic partners is independent of the phytohormones. We propose that downstream processes such as initiation of either cell division, elongation, or both may require the phytohormones. For instance, *P. indica*-induced growth stimulation of *sur1-1* roots is accompanied by an increase in the DNA content (data not shown), which supports the idea that the fungus induces cell division in the mutant. Thus, it is not surprising that the levels of the growth-regulating phytohormones are altered by the fungus. It can also be important to manipulate the auxin to cytokinin ratio, in particular in *sur1-1*, when the ratio does not support growth stimulation. One possibility can be the conversion of free auxin into inactive conjugates. Since both root and shoot growth is promoted by *P. indica*, it is likely that the fungus exerts different effects on the phytohormones or their ratios, or both, in the two organs.

P. indica-mediated growth promotion is independent of the root architecture.

Many of the mutants analyzed in this study have root architectures, lengths, or root to shoot ratios, which differ substantially from the wild type. For instance, *ahk2 ahk3* has long roots relative to the aerial parts (Riefler et al. 2006; Fig. 7), the 35S::CKX1 and 35S::CKX2 lines have stunted and bushy roots (Werner et al. 2003), the *sur1-1* roots are approximately half as long as the wild type (Fig. 5), and *tfl2*, in addition to its dwarf phenotype, with fewer lateral roots and shorter root hairs, contains a greatly reduced root surface (Bennett et al. 2005). All of these mutants respond to *P. indica*, suggesting that the root size and architecture have little effect on the growth response

induced by the fungus. This is further supported by the comparative analysis of the cytokinin receptor double mutants. The *ahk2 ahk3* mutant has an increased root system, while that of the other combinations are comparable to the wild type. However, *cer1 ahk2* fails to respond to *P. indica*, while the other mutant combinations do. Analysis of *tfl2* also demonstrates that the reduced root colonization does not lead to a reduced growth response to the fungus. Taken together, it appears that the size and architecture of the roots and its colonization does not correlate with the growth response induced by *P. indica*.

MATERIALS AND METHODS

Seed material, growth, and cocultivation experiments.

Wild-type, mutant, or transgenic *Arabidopsis thaliana* or *Nicotiana tabacum* seeds were surface-sterilized and cocultivated with *P. indica*, as described in Sherameti and associates (2008a and b). For cocultivation, 7-day-old *A. thaliana* and 14-day-old *Nicotiana tabacum* seedlings were used. After surface-sterilization, the seeds were placed on petri dishes containing Murashige-Skoog nutrient medium (Murashige and Skoog 1962). After cold treatment at 4°C for 48 h, plates were incubated for 7 days at 22°C under continuous illumination (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) to allow growth of the seedlings without *P. indica*. *P. indica* was cultured as described previously (Verma et al. 1998; Peřkan-Berghöfer et al. 2004) on Aspergillus minimal medium (Kaldorf et al. 2005). Nine days after the beginning of the experiments, *A. thaliana* seedlings were transferred to nylon disks (mesh size 70 μm) and were placed on top of a modified PNM culture medium (5 mM KNO₃, 2 mM MgSO₄, 2 mM Ca(NO₃)₂, 0.01 μM FeSO₄, 70 μM H₃BO₃, 14 μM MnCl₂, 0.5 μM CuSO₄, 1 μM ZnSO₄, 0.2 μM Na₂MoO₄, 0.01 μM CoCl₂, 10.5 g of agar per liter, pH 5.6) in petri dishes. One seedling was used per dish. After 24 h, fungal plugs of 5 mm in diameter were placed at a distance of 1 cm from the roots. The uninfected control plants received the same plugs without the fungus. The petri dishes were incubated at 22°C under continuous illumination from the side (80 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and were normally assayed 10 days after cocultivation, if not otherwise stated. Fresh weights were determined directly after seedlings (roots and shoots separately) were harvested. One experiment consisted of 80 infected and 80 uninfected seedlings, and this was repeated four times, if no other information is given. The following mutants and transgenic lines were used for the experiments: tobacco plants containing the *mas* promoter fused to *uidA* (Comai et al. 1990), *Arabidopsis: ilr1-1* (Bartel and Fink 1995), *nit1-3* (Hillebrand et al. 1996), *sur1-1* (Mikkelsen et al. 2004), *cyp79 b2b3* (Zhao et al. 2002), *tfl2* (Bennett et al. 2005), *cry1* (Moon et al. 2003), 35S-AtCKX1, and 35S-AtCKX2 (Werner et al. 2003), *cre1*, *ahk2*, *ahk3*, *ahk2 cre1* double, *ahk3 cre1* double, *ahk2 ahk3* double mutants (Nishimura et al. 2004), *atipt1 atipt3 atipt5 atipt7* quadruple mutant, *atipt2 atipt9* double mutant (Miyawaki et al. 2006).

GUS activity.

For GUS expression studies, transgenic lines with the auxin-responsive DR5 promoter (Ulmasov et al. 1995, 1997) and the cytokinin-responsive *ARR5* promoter (Brandstatter and Kiefer 1998) were used. GUS assays were described earlier (Sherameti et al. 2002). The system of reference was an equal amount of fresh weight.

Reverse transcription (RT) and real time PCR and Northern analyses.

Real-time quantitative RT-PCR was described in Pfalz and associates (2006). The following primer pairs were used: *ARR5*: GAGCTGACTACGTTGTTGAG and GGAGACAATG

TCAAGGTCGG; *germin* (At5g38910): CTTCCGACCCAAG CTCTCTTCAG and CAAGACCTCGGAGGCACGTGGGT; the mRNA for a *P. indica*-inducible leucine-rich repeat transmembrane protein called LRR1 (Shahollari et al. 2007): CGGCGAGTTTGATCTTGATGG and CTCAGGAACCACG ACATCTCTC. *Piriformospora indica* was monitored with a primer pair for the *elongation factor 1* (*Pitef1*; Büttehörn et al. 2000): ACCGTCTTGGGGTTGTATCC and CGTCGCTGTC AACAAGATG. The gene for the cytosolic glyceraldehyde-3-phosphate dehydrogenase (At3g04120) served as control: GAGCTGACTACGTTGTTGAG and GGAGACAATGTCAA GGTCCG.

For Northern blot analyses, gene-specific primers were designed to amplify cDNA fragments from our cDNA library (Sherameti et al. 2004). The primers were designed such that they amplify the entire coding region including four nucleotides up- and downstream of the genes. Northern analysis was performed with gel-purified, labeled PCR products (Shahollari et al. 2007).

Microarrays.

Arabidopsis seedlings, grown as described above, were co-cultivated (or mock-treated with an agar plug without the fungus) with *P. indica* for 2, 3, and 6 days. RNA was extracted from 70 mg of root materials with the RNeasy plant mini kit, followed by an On-Column DNase treatment (Qiagen, Hilden, Germany). Microarray hybridization was performed with the *Arabidopsis* genome array ATH1 from Affymetrix, and the data were analyzed with the GCOS1.4 software. Three independent RNAs per timepoint were used for the hybridizations, and means of the three signals were taken for further analysis. The data were screened for those genes that have been reported to be regulated by auxins (Goda et al. 2004; Liscum and Reed 2002; Sawa et al. 2002; Tian et al. 2002) or cytokinins (Kiba et al. 2005). Those genes that showed a significant expression in roots according to the GCOS1.4 software were further analyzed for their response to *P. indica*. A gene was considered to be regulated by phytohormones and *P. indica*, if it responds to the fungus at all three timepoints.

IAA analysis.

Approximately 1.5 g of fungal material was used for auxin analysis from cultures of *P. indica* grown 4 weeks in liquid medium in the dark. For the analysis of IAA in roots, 100 µg of root material was used, and all experiments were performed in triplicates, and results given are averages of the three assays. Free IAA was extracted using isopropanol; H₂O (70:30, vol/vol), the resulting extract, was centrifuged, and then, the supernatant was evaporated to the aqueous phase, using a rotary evaporator (Büchi Labortechnik GmbH, Essen, Germany). The aqueous phase was brought to pH 7 and was extracted with ethyl acetate, and the resulting organic phase was removed. The remaining aqueous phase was brought to pH 3 and was again extracted twice with ethyl acetate. The organic fractions were pooled and evaporated to dryness, were resuspended in methanol and centrifuged for 10 min at 13,000 × g, and the supernatant was again evaporated to dryness. The sample was taken up in 100 µl of ethyl acetate and was subsequently methylated with freshly prepared diazomethane (Cohen 1984). Gas chromatographic-mass spectrometry analysis was carried out on a Varian Saturn 2100 ion-trap mass spectrometer, using electron impact ionization at 70 eV, connected to a Varian CP-3900 gas chromatograph equipped with a CP-8400 autosampler (Varian, Darmstadt, Germany). From a 50-µl sample in ethyl acetate, 2.5 µl were injected in the splitless mode (splitter opening 1:100 after 1 min) onto a Phenomenex ZB-5 column (Aschaffenburg, Germany), 30 m × 0.25 mm × 0.25 µm,

using helium carrier gas at the rate of 1 ml min⁻¹. Injector temperature was 250°C, and the temperature program was 60°C for 1 min, followed by an increase of 25°C per min to 180°C, 5°C per min to 250°C, 25°C per min to 280°C, and then, 5 min isothermally at 280°C. Transfer line temperature was 280°C, and the trap temperature was 200°C. IAA was identified by its mass spectrum, monitoring the molecular ion with m/z 189 and the quinolinium ion with m/z 130. For the determination of endogenous free IAA levels, approximately 100 mg of fresh weight was extracted as described above, but 100 ng of ¹³C₆-IAA (Cambridge Isotope Laboratories, Andover, MA, U.S.A.) was added as internal standard. For total IAA analysis, the supernatant of a 100-mg fresh-weight equivalent sample was brought to the aqueous phase and was then hydrolyzed with 7 M NaOH at 100°C under N₂ for 3 h. The hydrolysate was filtered, the pH brought to 2.5, and the auxins were extracted twice with equal volumes of ethyl acetate. The organic phase was evaporated, and the extract was methylated as described above. Free and total IAA were calculated according to the principles of isotope dilution (Cohen et al. 1986) in the µSIS mode for higher sensitivity (Varian manual; Wells and Huston 1995), using the ions at m/z 130 and 136 deriving from endogenous and ¹³C₆-IAA, respectively. The amount of conjugated IAA was calculated by subtraction of the values for free IAA from those of total IAA.

Cytokinin quantification.

For quantitative analysis, the separation method was modified as follows (Novák et al. 2003). Aliquots of freeze-dried fungal material (100 to 500 mg) were processed, adding the following internal standards at the extraction stage in 70% cold ethanol (vol/vol): *trans*-[²H₅]zeatin, *cis*-[¹³C₃]zeatin; *trans*-[²H₅]zeatin riboside, *trans*-[²H₅]zeatin-9-glucoside, [²H₃]dihydrozeatin, [²H₃]dihydrozeatin riboside, [²H₃]dihydrozeatin-9-glucoside, [²H₇]dihydrozeatin-*O*-glucoside, [²H₇]dihydrozeatin-*O*-glucoside riboside [²H₆]isopentenyladenine, [²H₆]isopentenyladenine riboside, [²H₆]isopentenyladenine 9-glucoside, *trans*-[²H₃]zeatin-*O*-glucoside, *trans*-[²H₃]zeatin-*O*-glucoside riboside, *trans*-[²H₃]zeatin riboside-5'-monophosphate, [²H₃]dihydrozeatin riboside-5'-monophosphate, [²H₆]isopentenyladenosine-5'-monophosphate, [²H₇]benzyladenine, [²H₇]benzyladenosine, [²H₇]benzyladenine-9-glucoside, [²H₇]benzyladenosine-5'-monophosphate, [¹⁵N₄]meta-topolin, and [¹⁵N₄]ortho-topolin. (OIChemIm Ltd, Olomouc, Czech Republic). All topolins were analyzed using internal deuterium standards for [¹⁵N₄]mT and [¹⁵N₄]oT, as no other labeled standards were available.

The combined supernatants were concentrated to approximately 1.0 ml by rotary evaporation under vacuum at 35°C. The samples were diluted to 20 ml with ammonium acetate buffer (40 mM, pH 6.5). The extracts were purified using a combined DEAE-Sephadex (Sigma-Aldrich, St. Louis) (1.0 × 5.0 cm) octadecylsilica (0.5 × 1.5 cm) column and immunoaffinity chromatography (IAC), based on wide-range specific monoclonal antibodies against cytokinins (Faiss et al. 1997). This resulted in three fractions: i) the free bases and 9-glycosides (fraction B), ii) a nucleotide fraction, and iii) an *O*-glucoside fraction. The last two fractions were also purified by immunoaffinity chromatography, the second fraction after pretreatment with alkaline phosphatase and the third fraction after treatment with β-glucosidase. The metabolic eluates from the IAC columns were evaporated to dryness and were dissolved in 20 µl of the mobile phase used for quantitative analysis. The samples were subjected to high-pressure liquid chromatography on a reversed-phase column (150 mm × 2.1 mm; particle size, 5 µm) (Symmetry C₁₈; Waters, Milford, MA, U.S.A.) operated at 30°C. The components of the eluent were 15 mM ammonium formate, pH 4.0 (A), and methanol (B). They were

mixed as follows (difference to 100% is A): 0 min, 10% B, 0 to 25 min, linear gradient to 50% B, 25 to 30 min, 50% B, 30 to 35 min, 100% B, all at a flow rate of 250 μl per min. Using post-column splitting (1:1), the eluent was simultaneously introduced into a diode array detector (Waters PDA 996), and the electrospray source (source temperature, 100°C; capillary voltage, +3.0 kV; cone voltage, +20 V; desolvation temperature, 250°C) of a single-stage quadrupole mass spectrometer (ZMD 2000, Micromass, Manchester, U.K.). Nitrogen was used as both the desolvation gas (400 liter h^{-1}) and the cone gas (50 liter h^{-1}). Quantification was done by single-ion monitoring of the quasimolecular ($[\text{M}+\text{H}]^+$) ions of the plant cytokinins and the corresponding deuterated internal standards.

Statistics.

Statistics were performed by one-way analysis of variance, and the experiments were repeated four times, with values presented with standard error.

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