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Research Paper

Cytokinin induces expansin gene expression in *Melilotus alba* Desr. wild-type and the non-nodulating, non-mycorrhizal (Nod$^-$Myc$^-$) mutant *Masym3*

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Abbreviations: BAP, 6-Benzylaminopurine; ENOD, early nodulin; EXP, expansin; Has, hair swelling; Myc, mycorrhizal; NAA, α-naphthalene acetic acid; Nod, nodulating; TZS, trans-zeatin synthase

Key words: cell wall, *MaENOD40*, non-nodulation, non-mycorrhizal mutant, trans-zeatin synthase

We previously showed that applying 10^{-6} M of the cytokinin 6-benzylaminopurine (BAP) to uninoculated roots of *Masym3*, a Nod$^-$Myc$^-$ *Melilotus alba* (Desr.) mutant, resulted in the accumulation of *MaENOD40* transcripts to levels similar to BAP-treated wild-type roots. In contrast, inoculation with a Nod$^-$ *S. meliloti* mutant expressing the *trans*-zeatin synthase gene of *Agrobacterium tumefaciens* (Nod$^-$/pTZS$^+$) did not induce *MaENOD40* transcript accumulation in either wild-type or *Masym3* roots. However, *Masym3* root hairs became swollen at their tips following inoculation with wild-type or Nod$^-$/pTZS$^+$ rhizobia. Because root hair distention and elongation are often correlated with increased expansin activity, we investigated whether BAP treatment or inoculation with Nod$^-$/pTZS$^+$ *S. meliloti* upregulated the expression of expansin mRNAs. We first determined that treating wild-type roots with 10^{-5} or 10^{-6} M BAP resulted in greater *MaEXPA1* transcript accumulation than treating roots with comparable concentrations of the auxin NAA. When *Masym3* roots were treated with 10^{-6} M BAP, *MaEXPA* mRNAs accumulated to levels comparable to wild-type roots. We then showed that *MaEXPA1* mRNAs accumulated in wild-type *M. alba* roots in response to the Nod$^-$/pTZS$^+$ *S. meliloti* strain. *Masym3* mutant roots inoculated with Nod$^-$/pTZS$^+$ rhizobia were also upregulated for *MaEXPA1* expression.

Introduction

Root nodule development requires cell divisions to be initiated in root cortical cells soon after perception of the rhizobial symbiont at the root surface. However, even prior to contact between the rhizobial cell and its host, the plant's root hairs swell, deform and if the proper symbiont is recognized, develop the characteristic shepherd's crook morphology where the rhizobia become entrapped and go on to initiate an infection thread.[1] This change in root hair morphology is dependent on cell wall loosening and reconfiguration. Plant cells are bounded by cell walls consisting of an extensive network of cellulose microfibrils embedded in a matrix of complex polysaccharides. A number of proteins are also included in the matrix. Upon cell enlargement or expansion, cell walls loosen to accommodate the increase in cell volume brought about by the intake of water into the vacuoles. The cell wall loosening process is mediated by a number of proteins, including xyloglucan endotransglycosylases/hydrolases, endo-(1,4)-β-D-glucanses and expansins.[2]

Expansin proteins are made up of two major domains: an N-terminal catalytic domain with similarity to a comparable domain of endoglucanases in glycoside hydrolase family-45 (GH45), and a C-terminal domain that is related to a group of grass-pollen allergens.[3] Neither the exact mechanism of action nor their targets are known, but expansins trigger cell wall loosening both in vitro and in vivo and within minutes of application. Although their N-terminal domains are similar to the family-45 endoglucanases, expansins have not been demonstrated to have endoglucanase activity. Rather, they are thought to affect the stability of hydrogen bonds between cellulosic microfibrils and the surrounding polysaccharide matrix. Consequently, expansins act in a pH-dependent manner.

Expansins are subdivided into four subfamilies: EXPA (formerly known as α expansin), EXPB (formerly known as β expansins), EXLA (expansin-like A) and EXLB (expansin-like B).[4] The latter two DNA sequences diverge significantly from those of the EXPA and EXPB expansins and their function is still unknown. Expansins are categorized based on a variety of amino acid sequence motifs including conserved cysteine residues with characteristic spacing and conserved flanking sequences, HFD motifs, or tryptophan series in characteristic positions in the protein backbone.[5] One defining characteristic of EXPA expansins is the presence of N-linked glycosylation motifs.[6,7] Expansins often comprise a large multi-gene family of which there are 26 distinct members of EXPAs and 5

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EXPBs in Arabidopsis alone.[8] In rice, an astonishing 80 ORFs that encode expansin or expansin-like proteins have been detected in the genome.[9,10]

The large number of family members may relate to the different tissues or developmental processes where expansins are required. For example, they are induced under various environmental conditions, such as flooding, drought, high salinity and abscission brought about by stress as well as by several plant hormones including auxins, gibberellins, ethylene, brassinosteroids and cytokinins.[11-23] Expansins have been shown to play a role in fruit ripening, hypocotyl and petiole expansion, internodal elongation and leaf and root development.[14,17,18,24,25] They may also be involved in root hair initiation and/or expansion.[11,26,27] *GmEXPA1* (formerly known as *GmEXP1*), a soybean expansin, was localized to the root elongation zone in soybean plant cell walls,[28] a region where differentiation of the epidermal cells into root hair cells is initiated. Two expansin genes in Arabidopsis were also found to be upregulated in the region where root hairs develop. [11] Furthermore, immunolocalization studies show that expansins are present in the root elongation zone in maize[29] and at the tips of still elongating maize root hairs.[30]

We cloned and identified in *Melilotus alba* Desr. an *EXPA* gene, which was originally designated *MaEXP1*[31] and is now changed to *MaEXPA1* to conform to the expansin nomenclature.[4] Based on DNA gel-blot analysis, only one DNA fragment hybridized after high stringency washes to the *MaEXP1* coding region, suggesting that a single copy of the gene exists in *M. alba*.[31] A similar situation was observed for soybean where gene-specific probes for *GmEXPA1* and *GmEXPA2* identified only a strong single band.[28] MaEXPA1's deduced amino acid sequence, except for the signal peptide, is very similar to other published legume expansins. *MaEXPA1* is highly expressed in root nodules, and at lower levels in stems and roots. In nodules, transcripts for *MaEXPA1* were localized to the meristem, young nodule cortex cells, the invasion zone, and interzone II/III.[31] A protein that cross-reacted with CsEXPA1 fraction S1[32] was most strongly localized to the expanding cell walls of the invasion zone of the nodule. *MaEXPA1* transcripts were also detected by Whole-mount In Situ Hybridization (WISH) methods in the root elongation zone, particularly in the epidermal cells.[31] Inoculation of *M. alba* roots with *Sinorhizobium meliloti*, its compatible symbiont, enhanced *MaEXPA1* expression as early as 5 hours post-inoculation (hpi). This increased level of expression compared to the uninoculated roots was maintained for 24 to 72 hours.

In an analysis of the cytokinin responsiveness of a non-nodulating (Nod-), non-mycorrhizal (Myc-) *M. alba* mutant, *Masym3,* we observed that this mutant, which underwent root hair tip swellings (Has+) upon inoculation with Rm1021, exhibited the same response to a strain that was Nod- (Δ*nodD1ABC*) and pTZS+ (carrying a trans-zeatin synthase gene from *Agrobacterium tumefaciens*[33]).[34] In this report, we ask the question whether the Has+ phenotype is correlated with an upregulation of *MaEXPA1* in *Masym3* root tissues. Because we had earlier found that the plant hormone cytokinin upregulates genes, such as *MaENOD40*, which serve as early markers for nodule development in both wild-type and *Masym3* root tissues, we proposed that cytokinin is a signal for inducing gene expression that is downstream of Nod Factor perception.[34] We also address the question as to whether expansin gene expression can serve as an additional marker for studying the nodulation pathway.

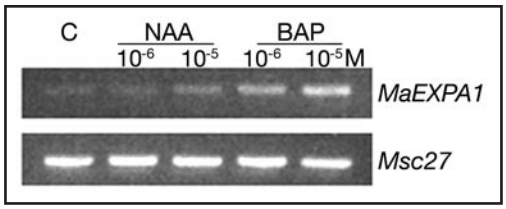

Figure 1. The effects of phytohormones, auxin (NAA) and cytokinin (BAP), on *MaEXPA1* expression in wild-type sweetclover. Roots were treated with 10^{-5} or 10^{-6} M of each hormone. C is the untreated control. *Msc27* was used as a loading control.

Results

Previously, we showed that both wild-type and the *Masym3* mutant root hairs responded, not only to *S. meliloti* inoculation, but also to inoculation with a Nod-/pTZS+ strain.[31] Wild-type roots exhibited root hair deformation and hair curling in response to Rm1021 inoculation whereas *Masym3* showed only a Has+ phenotype. Surprisingly, both wild-type and *Masym3* root hairs were Has+ following inoculation with the Nod-/pTZS+ strain. Because pTZS encodes trans-zeatin synthase, this result suggested that cytokinin could mimic *S. meliloti* inoculation in eliciting the upregulation of *MaEXPA1* expression in white sweetclover.

To test this hypothesis, we first examined whether an auxin, α-naphthalene acetic acid (NAA), which upregulates the expression of a number of expansin genes, or a cytokinin, supplied as 6-benzylaminopurine (BAP), could induce *MaEXPA1* expression. Reverse transcription-PCR demonstrated that NAA induced *MaEXPA1* gene expression, but only at the higher concentration applied and then to a very low level. In contrast, BAP induced a greater accumulation of *MaEXPA1* transcripts at both 10^{-5} and 10^{-6} M (Fig. 1).

We next utilized RT-PCR to determine whether BAP could induce *MaEXPA1* gene expression in both the wild-type and *sym3* mutant *Melilotus* roots. In our first experiment, we used the same conditions for the RT-PCR as in Figure 1. A basal level of expression was observed in RNA samples obtained from roots grown in nitrogen-depleted or nitrogen-supplemented media (Fig. 2A). Treatment with 10^{-6} M BAP resulted in an increase in *MaEXPA1* RNA accumulation in both wild-type and *Masym3* roots compared to the -N and +N controls in three biological replicates. However, RT-PCR analysis did not detect *MaEXPA1* product above the level of the -N and +N controls in either Rm1021- or Nod-/pTZS+-inoculated roots, contrary to what was expected. For example, Siciliano et al.,[37] using microarray analysis, detected the expression of *EXP*-like genes in the *dmi3* mutant of *Medicago truncatula* inoculated with the mycorrhizal fungus *Gigaspora margarita*. Like *Masym3*, *Mtdmi3* is both Nod- and Myc-., but the *dmi3* mutation results from the loss of function of the Ca^{2+}/calmodulin receptor kinase DMI3;[38] we do not know the identity of the *Masym3* gene. Also, *MaEXPA1* expression was expected in response to Nod-/pTZS+ rhizobia because this strain produces cytokinin,[33] and *MaEXPA1* is upregulated by BAP (Figs. 1 and 2). Furthermore, both wild-type and *Masym3* root hairs swelled in response to the Nod-/pTZS+ strain.[31]

We changed the RT-PCR conditions (see Materials and Methods) to see if this affected the level of transcript detection. As already shown in Figure 2A, we observed an increase in *MaEXPA1* transcript accumulation in BAP-treated wild-type and *Masym3* mutant

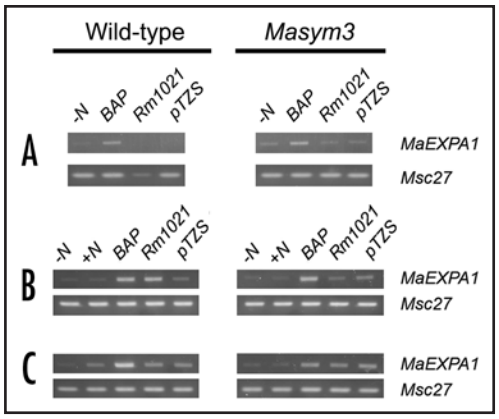

Figure 2. Reverse transcription PCR of *MaEXPA1*. Wild-type (U389) and *Masym3* (BT70) mutant roots were grown in -N (nitrogen-deficient), +N (nitrogen-supplemented), BAP treatment (cytokinin), Rm1021- (wild-type *S. meliloti*), and Nod-pTZS⁺-inoculated (Δ*nodD1ABC* mutant *S. meliloti* carrying the trans-zeatin synthase gene) conditions. *Msc27* primers were used to amplify an orthologous *M. alba* sequence, which was utilized to monitor DNA levels. Three different biological replicates are shown. Each biological replicate was repeated at least twice.

roots in two biological replicates, based on the density of the PCR product compared to the -N and +N controls (Fig. 2B and C). In addition, we detected elevated *MaEXPA1* transcript accumulation in Rm1021-inoculated wild-type and *Masym3* roots, and observed an upregulation of *MaEXPA1* in both wild-type and *Masym3* roots inoculated with the Nod⁻/pTZS⁺ strain (Fig. 2B and C).

We employed WISH analysis to examine *MaEXPA1* transcript accumulation in *S. meliloti* roots collected and fixed at the same time that the roots were harvested for RT-PCR. The tips of both the primary (data not shown) and lateral uninoculated roots grown with or without nitrogen (Fig. 3A and B) or after the various treatments (Fig. 3C–L) were blue, demonstrating that the root tip is a major area of *MaEXPA1* expression. Also, some of the root hairs stained blue, but only a slight difference in the color of the total root system was observed for +N versus -N-grown roots (data not shown). Generally, roots grown without N had color restricted to the root tips whereas some color was apparent in the vascular system in the +N-grown root (Fig. 3A and B).

The extent that the blue color continued into the root elongation zone could be correlated with the experimental treatment. Rm1021-inoculated wild-type roots were very blue with significantly more color extending into the root elongation zone (Fig. 3C). In an off-median longitudinal section of a root, *MaEXPA1* transcripts (indicated by a magenta color under phase contrast optics) were localized to cells of the root cap, to the meristem, and to the epidermis (Fig. 3D). In the wild-type roots, the color reaction was closely associated with the nodules (Fig. 3E and F), whereas for the *Masym3* roots, blue color was associated with lateral root tips, with some blue color extending into the root elongation zone (Fig. 3G). BAP-treated roots for both the wild-type and mutant plants were also blue due to the accumulation of transcripts in root hair cells (Fig. 3H–J). A root tip from a Nod⁻/pTZS⁺-inoculated root is shown in Figure 3K; a localization similar to the BAP-treated roots is observed. A root tip from a *Masym3* root that had been inoculated with a Nod⁻ *S. meliloti* lacking pTZS exhibited very little blue color in comparison (Fig. 3L).

Taken together, these results show that *MaEXPA1* expression is upregulated by cytokinin and based on its expression in the non-nodulating mutant *Masym3*, suggest that *MaEXPA1* induction is Nod-factor independent.

Discussion

Masym3 and wild-type *M. alba* root hairs respond to the Nod⁻/pTZS⁺ strain by swelling and deforming, respectively.[31] Based on this result, we hypothesized that *Masym3* roots were likely to respond to cytokinin by showing an upregulation of genes involved early on in the nodulation pathway. We further inferred that morphological changes in the root hairs required loosening of their cell walls, and that expansins were most likely to be involved in this process.

In an effort to analyze the regulation of *MaEXPA1* gene expression in *Masym3* mutant roots, we found by reverse transcription-PCR that this gene is upregulated in response to cytokinin treatment. We also concluded from these experiments that *MaEXPA1* induction might be Nod-factor independent because expression of this gene is elevated in *Masym3*, which, in spite of being a non-nodulating mutant, exhibits a Has⁺ phenotype in response to inoculation with a Nod⁻/pTZS⁺ strain. Moreover, although root hairs treated with Nod factor bear a strong resemblance to expansin-treated roots, a crude Nod factor preparation does not cause a change in cell wall extensibility in a functional assay (D.J. Cosgrove, pers. comm.).

We observed an increase in *MaEXPA1* expression in response to BAP treatment for Rm1021-inoculated *Masym3* roots, which may be explained by the fact that *S. meliloti* is reported to synthesize cytokinins. Culture filtrates have been shown to have cytokinin activity, but the compounds have not been identified.[39] Certain photosynthetic bradyrhizobia that lack *nod* genes synthesize purine derivatives that trigger nodulation in *Aeschynomene*,[40] strongly implicating rhizobial-produced cytokinins in nodule organogenesis. Alternatively, other bacterial metabolites, including volatile emissions, may alter a plant's hormonal status leading to expansin and even *ENOD* gene expression.[41,42]

Cytokinins are known to induce *Rhizobium*-independent changes, such as the induction of early nodulins (*ENOD40*,[43] *ENOD12A*[44]) in legume roots. We postulated that during nodule formation, rhizobia might stimulate in their host the production of endogenous cytokinin that induces the expression of the *ENOD* genes.[43] Because *Masym3* is a mutant defective in the early stages of nodulation, possibly the Nod factor signal transduction stages, we reasoned that the downstream elements leading to nodule development should be intact and potentially triggered by exogenous cytokinin. One of these early events is likely to be the induction of genes such as those for expansins, which are required for cell wall remodeling. We have shown in this study that this indeed is the case because addition of BAP stimulates *MaEXPA1* expression in both wild-type and mutant roots. Also, *MaEXPA1* transcripts accumulated in wild-type and *Masym3* roots inoculated with the Nod⁻/pTZS⁺ strain (Fig. 2B and C).

Several other expansins are regulated by cytokinins. One of these is *GmCIM1*, a β-expansin gene of soybean, which is regulated synergistically by both auxin and cytokinin, and is inducible by cytokinin alone.[6,45] Interestingly, other expansin genes (*TvEXPA2, TvEXPA3, SaEXPA1, SaEXPA2*) that are cytokinin upregulated have been identified from parasitic plants where cytokinin application elicits the initial stages of haustorial development—radial swelling of root cortical cells and epidermal cell proliferation, developmental events

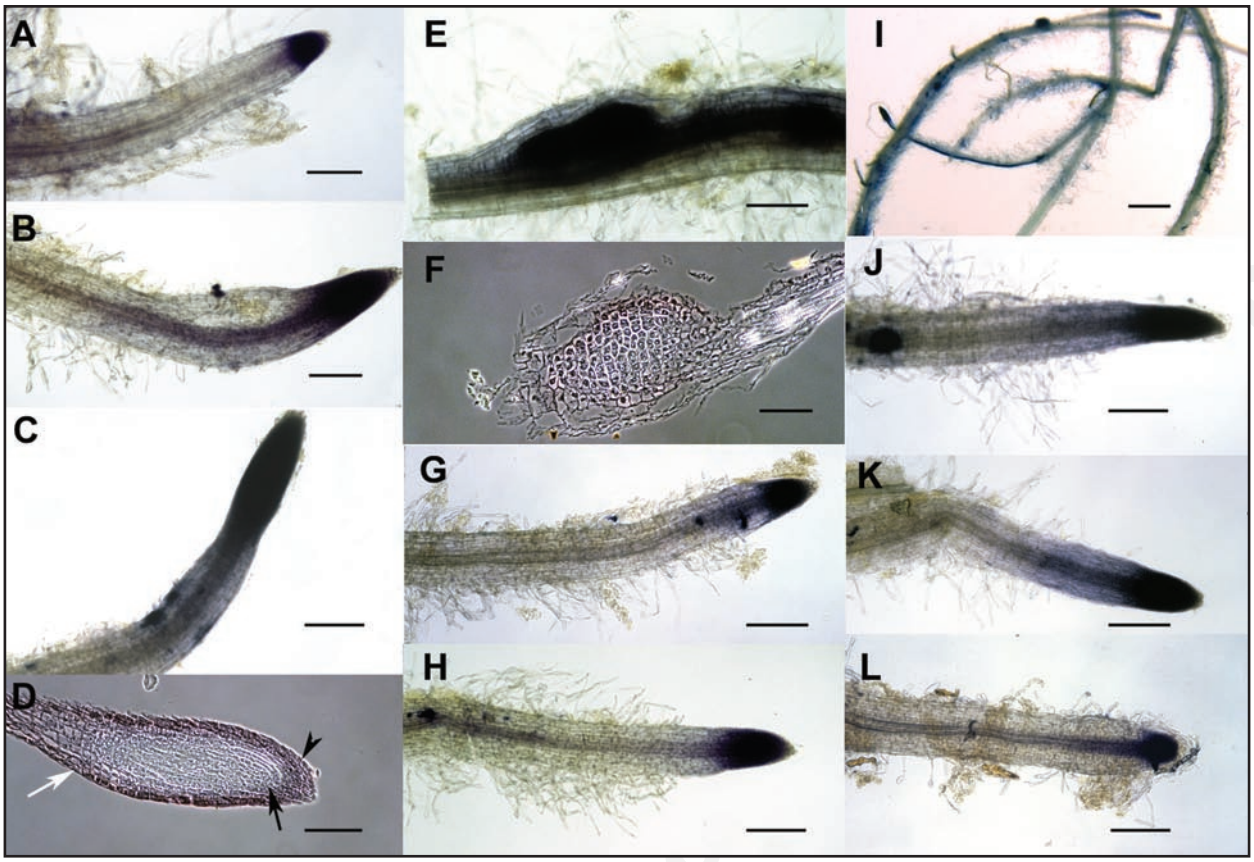

Figure 3. Whole mount in situ hybridization (WISH) of wild-type (U389) and *Masym3* mutant roots grown under different conditions. Unless indicated otherwise, only lateral roots are illustrated and the magnification bars represent 40 µm. At least five different lateral roots were examined for each treatment. (A) Wild-type root grown under conditions of N deficiency. Blue color is restricted to the tip. (B) Wild-type root grown in +N medium. Blue color is in the tip and extends into the vascular system. (C) Wild-type root inoculated with Rm1021. Both the root tip and the root elongation zone are dark-blue. (D) Off-median longitudinal section showing localization of *MaEXPA1* transcripts in the root cap (arrowhead), root meristem (black arrow), and epidermis (white arrow). Under phase contrast optics, the blue color appears magenta. (E) Young nodule showing overall blue color indicating accumulation of *MaEXPA1* transcripts. (F) Longitudinal section through a young nodule. The color (magenta) is mostly associated with the peripheral cells. (G) *Masym3* root inoculated with Rm1021. Although not as blue as the wild-type root, blue color extends into the root elongation zone. (H) BAP-treated wild-type root. Color extends into the root elongation zone and vascular system. (I) Overview of BAP-treated *Masym3* root showing accumulation of blue color in lateral root tips and root hairs. Bar = 80 µm. (J) BAP-treated *Masym3* root. Color extends into the root elongation zone. The root hairs are blue. (K) Wild-type root inoculated with Nod⁻/pTZS⁺ rhizobia. (L) *Masym3* root inoculated with Nod⁻ rhizobia. The extent of *MaEXPA1* transcript localization is comparable to the -N grown root.

that require loosening of plant cell walls.[19,23] Expansin gene expression also correlates with nematode-induced syncytial formation in Arabidopsis and tomato,[46,47] although the involvement of cytokinin on regulating this gene in the latter pathogenic interaction is yet not known. Nevertheless, cytokinins are implicated in nematode-induced root infections.[48,49] The changes in root structure brought about by parasitic plants and plant nematodes are reminiscent of the remodeling the legume root undergoes in the initial stages of nodule formation, which include cell wall elongation and expansion.

Previously, we found that 10^{-6} M BAP by itself elicits on alfalfa roots nodule primordia that express *MsENOD40*,[50] and several recent reports have elegantly demonstrated the importance of cytokinins for cortical cell division and nodule development by studying model legumes with mutations in genes encoding cytokinin receptors.[51-53] Although the Nod⁻/pTZS⁺ mutant did not elicit *MaENOD40* expression in *Masym3*,[31] *MaEXP1* was induced, strongly suggesting that expansin-mediated processes are upstream of *MaENOD40*-related development. An interesting correlation is that an *ARR5*-promter GUS construct was turned on in *Lotus japonicus* root hairs that deformed in response to *Mesorhizobium loti* inoculation.[49] *ARR5* is a cytokinin primary response gene that was originally cloned from Arabidopsis.[54] The coincidence of cytokinin responsive gene expression and *MaEXP1* expression suggests that increasing cytokinin levels in the root hair may mediate cell wall remodeling by inducing the expression of expansin genes. Expansins are likely to be one of the first steps in the transition to nodulation because root hair deformation and cell elongation are some of the earliest morphological changes to occur in response to rhizobial stimuli. Thus, monitoring the expression of expansin genes as well as hormonal response genes may serve as good markers for studying the earliest stages of nodule development, i.e., epidermal and cortical cell responses to rhizobial inoculation. In particular, it may help us understand how rhizobial inoculation leads to an increase in cytokinin concentration, a key step in the nodulation pathway.

Materials and Methods

Plant materials, bacterial strains, hormone treatments and growth conditions. For the experiments testing the effects of auxin

and cytokinin on *MaEXPA1* gene expression, white sweetclover (*Melilotus alba Desr.* U389) seeds were surface-sterilized as described earlier[35] and germinated on screens in Magenta Jars (Magenta Corp., Chicago IL), containing 1/4 strength Hoagland's medium without nitrogen. Three days after germination, the seedlings were treated with either α-napthalene acetic acid (NAA) or 6-benzylaminopurine (BAP). Hormone stocks of 10 mM were made in DMSO, and added sterilely to the culture medium at either 10^{-5} or 10^{-6} M. For the RNA used for Figure 1, entire control and treated root systems were harvested 4 days after treatment, frozen immediately in liquid nitrogen, and stored at -70°C until analysis. Three separate experiments for the control plants and for each of the phytohormone treatments were performed.

The bacterial strains were wild-type *Sinorhizobium meliloti* Rm1021, a Nod- *S. meliloti* (SL44; Δ*nodD1ABC*), and a Nod-/pTZS+ strain (SL44 carrying the trans-zeatin synthase gene of *A. tumefaciens*).[33] Prior to inoculation, *M. alba* wild-type (U389) and *Masym3* (BT70) seeds were scarified and surface-sterilized as above and then planted in autoclaved dishpans containing a 1:4 mix of perlite and vermiculite watered with ¼ strength Hoagland's minus nitrogen (for nitrogen-starved or 10^{-6} M BAP-treated or for rhizobial inoculation) or with ¼ strength Hoagland's complete medium (for nitrogen-supplemented). The planted seeds were covered with foil and placed at 4°C for 4 days. After this vernalization period, the foil-covered dishpans were moved to growth chambers at 22°C with 16-hour light/8-hour dark cycles. After 2 days, the foil was removed and plants were incubated for 12 days before inoculation or application of cytokinin.

Whole root systems were harvested 1 week and 2 weeks after rhizobial inoculation or cytokinin application for the RT-PCR and in situ hybridization experiments.

In situ hybridization. Roots were prepared for Whole-mount In Situ Hybridization (WISH) as described previously.[31] Photographs of whole roots were taken with Kodak Ektachrome Tungsten 160 film on a Zeiss Axiophot microscope using bright field optics. Selected roots were embedded into paraffin, sectioned longitudinally or transversely at 8 μm, and mounted onto glass slides. The sections were left unstained and photographed using phase contrast optics. The resulting photographs were scanned and processed with Adobe Photoshop 7.0.

RNA isolation and reverse transcription PCR. Total RNA was isolated using RNA STAT-60 (Tel-Test "B" Inc., Friendswood, Texas, USA). The RNA (1 μg) was used as a template for cDNA synthesis (Figs. 1 and 2A) after adding 0.5 mM dNTPs, 25 μg/μl oligo $d(T)_{12-18}$, 2 units/μl ribonuclease inhibitor and 10 units/μl Superscript II Reverse Transcriptase (GIBCO/BRL, Grand Island, N.Y.) at 42°C for 60 min. The reaction was inactivated by heating at 75°C for 15 min. The products were diluted and used as template for the PCR amplification, which was carried out using 1 μM of each specific primer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$ and 2.5 units *Taq* DNA polymerase (GIBCO/BRL). *Msc27*,[36] used to monitor DNA loading levels from sample to sample, primer sequences were 5'-GGAGGTTGAGGGAAAGTGG-3' and 5'-ACCAACAAAGAATTGAAGG-3', and generated a 310 nucleotide (nt) orthologous product in *M. alba*. The *MaExpA1* primer sequences were 5'-GCGCATGCGACGTTCTATGGTG-3' and 5'-CTGCCAATTCTGGCCCCAGTT-3', and generated a 537-nt product.[31] Amplification was performed using an initial 5-min cycle at 94°C, followed by 30 cycles of a 30 sec denaturation step at 94°C, a 30 sec primer annealing step at 55°C, a 90 sec elongation step at 72°C, concluding with a final 10 min at 72°C for extension. The PCR products were size-fractionated on a 1.5% agarose gel and visualized with ethidium bromide.

For the first inoculation experiment, the PCR conditions as described above were used. For the second and third experiments, 1 μg total RNA was used as template for cDNA synthesis using 0.1 mM dNTPs, 1 μM oligo-dT, 40 units of ribonuclease inhibitor, 0.02 mM DTT and 10 units Superscript II Reverse Transcriptase (GIBCO/BRL, Grand Island, NY). Reactions were incubated at 94°C for 5 min, 48°C for 60 min., and then heat-inactivated at 75°C for 15 min. The products were diluted and used as template for the PCR amplification, which was carried out using 1 μM of each specific primer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$ and 2.5 units *Taq* DNA polymerase (GIBCO/BRL) or 1 unit of Eppendorf HotMaster *Taq* DNA polymerase (Eppendorf, Westbury, NY). The *Msc27* and *MaEXPA1* primer sequences used for these experiments were as described above. Amplification cycles were performed with an initial 5 min 94°C denaturation step followed by 25 cycles of 30 sec at 94°C, 30 sec at 55°C, 90 sec. at 68°C, and a final 5 min. at 68°C. PCR products were visualized on a 1.2% agarose gel stained with ethidium bromide. Each RT-PCR experiment was repeated at least twice, using three different biological replicates.

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References

1. Lee A, Hirsch AM. Signals and responses: choreographing the complex interaction between legumes and alpha- and beta-rhizobia. Plant Signal Behav 2006; Plant Signal Behav 2006; 1:161-8.
2. Cosgrove DJ. Growth of the plant cell wall. Nat Rev Mol Cell Biol 2005; 6:850-61.
3. Cosgrove DJ. Loosening of plant cell walls by expansins. Nature 2000; 407:321-6.
4. Kende H, Bradford K, Brummell D, Cho HT, Cosgrove D, Fleming A, Gehring C, Lee Y, McQueen-Mason S, Rose J, Voeseneck LA. Nomenclature for members of the expansin superfamily of genes and proteins. Plant Mol Biol 2004; 55:311-4.
5. Cosgrove DJ, Li LC, Cho HT, Hoffmann Benning S, Moore RC, Blecker D. The growing world of expansins. Plant Cell Physiol 2002; 43:1436-44.
6. Grobe K, Poppelmann M, Becker WM, Petersen A. Properties of group I allergens from grass pollen and their relation to cathepsin B, a member of the C1 family of cysteine proteinases. Eur J Biochem 2002; 269:2083-92.
7. Downes BP, Steinbaker CR, Crowell DN. Expression and processing of a hormonally regulated β-expansin from soybean. Plant Physiol 2001; 126:244-52.
8. Li Y, Darley CP, Ongaro V, Fleming A, Schipper O, Baldauf SL, McQueen Mason SJ. Plant expansins are a complex multigene family with an ancient evolutionary origin. Plant Physiol 2002; 128:854-64.
9. Lee Y, Choi D, Kende H. Expansins: ever-expanding numbers and functions. Curr Opin Plant Biol 2001; 4:527-32.
10. Lee Y, Kende H. Expression of alpha-expansin and expansin-like genes in deepwater rice. Plant Physiol 2002; 130:1396-405.
11. Cho HT, Cosgrove DJ. Regulation of root hair initiation and expansin gene expression in Arabidopsis. Plant Cell 2002; 14:3237-53.
12. Schipper O, Schaefer D, Reski R, Fleming A. Expansins in the bryophyte *Physcomitrella patens*. Plant Mol Biol 2002; 50:789-802.
13. Wu Y, Meeley RB, Cosgrove DJ. Analysis and expression of the α-expansin and β-expansin gene families in maize. Plant Physiol 2001; 126:222-32.

14. Catala C, Rose J KC, Bennett AB. Auxin-regulated genes encoding cell wall-modifying proteins are expressed during early tomato fruit growth. Plant Physiol 2000; 122:527-34.
15. Cho HT, Kende H. Expression of expansin genes is correlated with growth in deepwater rice. Plant Cell 1997; 9:1661-71.
16. Hutchison KW, Singer PB, McInnis S, Diaz Sala C, Greenwood MS. Expansins are conserved in conifers and expressed in hypocotyls in response to exogenous auxin. Plant Physiol 1999; 120:827-32.
17. Kim JH, Cho HT, Kende H. α-Expansins in the semiaquatic ferns *Marsilea quadrifolia* and *Regnellidium diphyllum*: evolutionary aspects and physiological role in rachis elongation. Planta 2000; 212:85-92.
18. Lee Y, Kende H. Expression of beta-expansins is correlated with internodal elongation in deepwater rice. Plant Physiol 2001; 127:645-54.
19. O'Malley RC, Lynn DG. Expansin message regulation in parasitic angiosperms: Marking time in development. Plant Cell 2000; 12:1455-66.
20. Sanchez MA, Mateos I, Labrador E, Dopico B. Brassinolides and IAA induce the transcription of four α-expansin genes related to development in *Cicer arietinum*. Plant Physiol Biochem 2004; 42:709-16.
21. Vreeburg RAM, Benschop JJ, Peeters AJM, Colmer TD, Ammerlaan AHM, Staal M, Elzenga T M, Staals RHJ, Darley CP, McQueen-Mason SJ, Voesenek LA. Ethylene regulates fast apoplastic acidification and expansin A transcription during submergence-induced petiole elongation in *Rumex palustris*. Plant J 2005; 43:597-610.
22. Vriezen WH, De Graaf B, Mariani C, Voesenek LA. Submergence induces expansin gene expression in flooding-tolerant *Rumex palustris* and not in flooding-intolerant *R. acetosa*. Planta 2000; 210:956-63.
23. Wrobel RL, Yoder JI. Differential RNA expression of α-expansin gene family members in the parasitic angiosperm *Triphysaria versicolor* (Scrophulariaceae). Gene 2001; 266:85-93.
24. McQueen Mason S, Durachko DM, Cosgrove DJ. Two endogenous proteins that induce cell wall extension in plants. Plant Cell 1992; 4:1425-33.
25. Rose JKC, Lee HH and Bennett AB. Expression of a divergent expansin gene is fruit-specific and ripening-regulated. Proc Natl Acad Sci USA 1997; 94:5955-60.
26. Kim DW, Lee SH, Choi SB, Won SK, Heo YK, Cho M, Park YI, Cho HT. Functional conservation of a root hair cell-specific cis-element in angiosperms with different root hair distribution patterns. Plant Cell 2006; 18:2958-70.
27. Kwasniewski M, Szarejko I. Molecular cloning and characterization of beta-expansin gene related to root hair formation in barley. Plant Physiol 2006; 141:1149-58.
28. Lee DK, Ahn JH, Song SK, Choi YD, Lee JS. Expression of an expansin gene is correlated with root elongation in soybean. Plant Physiol 2003; 131:985-97.
29. Zhang N, Hasenstein KH. Distribution of expansins in graviresponding maize roots. Plant Cell Physiol 2000; 41:1305-12.
30. Baluska F, Salaj J, Mathur J, Braun M, Jasper F, Samaj J, Chua NH, Barlow PW, Volkmann D. Root hair formation: F-actin-dependent tip growth is initiated by local assembly of profilin-supported F-actin meshworks accumulated within expansin-enriched bulges. Devel Biol 2000; 227:618-32.
31. Giordano W, Hirsch AM. The expression of *MaEXP1*, a *Melilotus alba* expansin gene, is upregulated during the sweetclover-*Sinorhizobium meliloti* interaction. Mol Plant-Microbe Interact 2004; 17:613-22.
32. Li ZC, Durachko DM, Cosgrove DJ. An oat coleoptile wall protein that induces wall extension in vitro and that is antigenically related to a similar protein from cucumber hypocotyls. Planta 1993; 191:349-56.
33. Cooper JB, Long SR. Morphogenetic rescue of *Rhizobium meliloti* nodulation mutants by *trans*-zeatin secretion. Plant Cell 1994; 6:215-25.
34. Lee A, Lum MR, Hirsch AM. *ENOD40* gene expression and cytokinin responses in the *SYM* (Nod$^-$Myc$^-$) mutants of *Melilotus alba* Desr. Plant Signal Behav 2007; 2:33-42.
35. Löbler M, Hirsch AM. A gene that encodes a proline-rich nodulin with limited homology to *PsENOD12* is expressed in the invasion zone of *Rhizobium meliloti*-induced alfalfa nodules. Plant Physiol 1993; 103: 21-30.
36. Kapros T, Bogre L, Nemeth K, Bako L, Györgyey J, Wu SC, Dudits D. Differential expression of histone H3 gene variants during cell cycle and somatic embryogenesis in alfalfa. Plant Physiol 1992; 98:621-5.
37. Siciliano V, Genre A, Balestrini R, Cappellazzo G, deWitt, PJGM, Bonfante P. Transcriptome analysis of arbuscular mycorrhizal roots during development of the prepenetration apparatus. Plant Physiol 2007; 1455-66.
38. Levy J, Bres C, Geurts R, Chalhoub B, Kulikova O, Duc G, Journet EP, Ané JM, Lauber E, Bisseling T, Dénarié J, Rosenberg C, Debellé F. A putative Ca^{2+} and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. Science 2004; 303:1361-4.
39. Greene EM. Cytokinin production by microorganisms. Bot Rev 1980; 46:25-74.
40. Giraud E, Moulin L, Vallenet D, Barbe V, Cytryn E, Avarre JC, Jaubert M, Simon D, Cartieaux F, Prin Y, Bena G, Hannibal L, Fardoux J, Kojadinovic M, Vuillet L, Lajus A, Cruveiller S, Rouy Z, Mangenot S, Segurens B, Dossat C, Franck WL, Chang WS, Saunders E, Bruce D, Richardson P, Normand P, Dreyfus B, Pignol D, Stacey G, Emerich D, Verméglio A, Médigue C, Sadowsky M. Legumes symbioses: absence of *nod* genes in photosynthetic bradyrhizobia. Science 2007; 316:1307-12.
41. Nogueira EM, Vinegre F, Masuda HP, Vargas C, Muniz de Pádua VL, da Silva FR, dos Santos RV, Baldani JI, Cavacanti GF, Hemerly AS. Expression of sugarcane genes induced by inoculation with *Gluconacetobacter diazotrophicus* and *Herbaspirillum rubrisubalbicans*. Gen Mol Biol 2001; 24:199-206.
42. Zhang H, Kim MS, Krishnamachari V, Payton P, Sun Y, Grimson M, Farag M, Ryu CM, Allen R, Melo IS, Paré PW. Rhizobacterial volatile emissions regulate auxin homeostasis and cell expansion in Arabidopsis. Planta 2007; 226: 839-51.
43. Hirsch AM, Fang Y, Asad S, Kapulnik Y. The role of phytohormones in plant-microbe symbioses. Plant Soil 1997; 194:171-84.
44. Bauer P, Ratet P, Crespi MD, Schultze M, Kondorosi A. Nod factors and cytokinins induce similar cortical cell division, amyloplast deposition and *MsENOD12A* expression patterns in alfalfa roots. Plant J 1996; 10:91-105.
45. Downes BP, Crowell DN. Cytokinin regulates the expression of a soybean β-expansin gene by a post-transcriptional mechanism. Plant Mol Biol 1998; 37:437-44.
46. Gal TZ, Aussenberg ER, Burdman S, Kapulnik Y, Koltai H. Expression of a plant expansin is involved in the establishment of root knot nematode parasitism in tomato. Planta 2006; 224:155-62.
47. Wieczorek K, Golecki B, Gerdes L, Heinen P, Szakasits D, Durachko DM, Cosgrove DJ, Kreil DP, Puzio PS, Bohlmann H, and Grundler MW. Expansins are involved in the formation of nematode-induced syncytia in roots of *Arabidopsis thaliana*. Plant J 2006; 48:98-112.
48. Bird AF, Loveys BR. The involvement of cytokinins in a host-parasite relationship between the tomato (*Lycopersicon esculentum*) and a nematode (*Meloidogyne javanica*). Parasitology 1980; 80:497-505.
49. Lohar DP, Schaff JE, Laskey JG, Kieber JJ, Bilyeu KD, Bird D Mck. Cytokinins play opposite roles in lateral root formation, and nematode and Rhizobial symbioses. Plant J 2004; 38:203-14.
50. Fang Y, Hirsch AM. Studying early nodulin gene *ENOD40* expression and induction by nodulation factor and cytokinin in transgenic alfalfa. Plant Physiol 1998; 116:53-68.
51. Gonzalez Rizzo S, Crespi M, Frugier F. The *Medicago truncatula* CRE1 cytokinin receptor regulates lateral root development and early symbiotic interaction with *Sinorhizobium meliloti*. Plant Cell 2006; 18:2680-93.
52. Tirichine L, Sandal N, Madsen LH, Radutoiu S, Albrektsen AS, Sato S, Asamizu E, Tabata S, Stougaard J. A gain-of-function mutation in a cytokinin receptor triggers spontaneous root nodule organogenesis. Science 2007; 315:104-7.
53. Murray JD, Karas BJ, Sato S, Tabata S, Amyot L, Szczyglowski K. A cytokinin perception mutant colonized by *Rhizobium* in the absence of nodule organogenesis. Science 2007; 315:101-4.
54. D'Agostino IB, Deruére J, Kieber JJ. Characterization of the response of the Arabidopsis response regulator gene family to cytokinin. Plant Physiol 2000; 124:1706-17.