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⁻ 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 strain 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 mRNA 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-glucans and expansins.²

Expansins 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: EXP (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 EXP 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 EXP expansins is the presence of N-linked glycosylation motifs.⁶ Expansins often comprise a large multi-gene family of which there are 26 distinct members of EXPAs and 5...
In rice, an astonishing 80 ORFs that encode expansin or expansin-like proteins have been detected in the genome.\textsuperscript{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.\textsuperscript{11-25} Expansins have been shown to play a role in fruit ripening, hypocotyl and petiole expansion, internodal elongation and leaf and root development.\textsuperscript{14,17,18,24,25} They may also be involved in root hair initiation and/or expansion.\textsuperscript{11,26,27} *GmEXPA1* (formerly known as *GmEXP1*), a soybean expansin, was localized to the root elongation zone in soybean plant cell walls,\textsuperscript{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.\textsuperscript{31} Furthermore, immunolocalization studies show that expansins are present in the root elongation zone in maize\textsuperscript{29} and at the tips of still elongating maize root hairs.\textsuperscript{30}

We cloned and identified *Melilotus alba* Destr. an *EXP1* gene, which was originally designated *MaEXP1\textsuperscript{31}* and is now changed to *MaEXP1* to conform to the expansin nomenclature.\textsuperscript{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*.\textsuperscript{31} A similar situation was observed for soybean where gene-specific probes for *GmEXPA1* and *GmEXP2* identified only a strong single band.\textsuperscript{28} *MaEXP1*’s deduced amino acid sequence, except for the signal peptide, is very similar to other published legume expansins. *MaEXP1* is highly expressed in root nodules, and at lower levels in stems and roots. In nodules, transcripts for *MaEXP1* were localized to the meristem, young nodule cortex cells, the invasion zone, and interzone II/III.\textsuperscript{31} A protein that cross-reacted with Cs*EXP1* fraction S1\textsuperscript{32} was most strongly localized to the expanding cell walls of the invasion zone of the nodule. *MaEXP1* transcripts were also detected by Whole-mount In Situ Hybridization (WISH) methods in the root elongation zone, particularly in the epidermal cells.\textsuperscript{31} Inoculation of *M. alba* roots with *Sinorhizobium meliloti*, its compatible symbiont, enhanced *MaEXP1* 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*\textsuperscript{-}), non-mycorrhizal (*Myc*) *M. alba* mutant, *Masym3*, we observed that this mutant, which underwent root hair tip swellings (Ha’s\textsuperscript{*}+) upon inoculation with Rm1021, exhibited the same response to a strain that was *Nod*\textsuperscript{3} (*nodD1ACB*) and pTZS\textsuperscript{+} (carrying a trans-zeatin synthase gene from *Agrobacterium tumefaciens*)\textsuperscript{33,34}. In this report, we ask the question whether the Has\textsuperscript{*} phenotype is correlated with an upregulation of *MaEXP1* 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.\textsuperscript{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 *MaEXP1* expression in wild-type sweetclover. Roots were treated with 10\textsuperscript{5} or 10\textsuperscript{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*\textsuperscript{+}/pTZS\textsuperscript{+} strain.\textsuperscript{31} Wild-type roots exhibited root hair deformation and hair curling in response to Rm1021 inoculation whereas *Masym3* showed only a Has\textsuperscript{*} phenotype. Surprisingly, both wild-type and *Masym3* root hairs were Has\textsuperscript{*} following inoculation with the *Nod*\textsuperscript{+}/pTZS\textsuperscript{+} strain. Because pTZS encodes trans-zeatin synthase, this result suggested that cytokinin could mimic *S. meliloti* inoculation in eliciting the upregulation of *MaEXP1* 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 *MaEXP1* expression. Reverse transcription-PCR demonstrated that NAA induced *MaEXP1* gene expression, but only at the higher concentration applied and then to a very low level. In contrast, BAP induced a greater accumulation of *MaEXP1* transcripts at both 10\textsuperscript{5} and 10\textsuperscript{6} M (Fig. 1).

We next utilized RT-PCR to determine whether BAP could induce *MaEXP1* 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 brought about by stress as well as by several plant hormones conditions, such as flooding, drought, high salinity and abscission, expressed 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.\textsuperscript{34} We also address the question as to whether expansin gene expression can serve as an additional marker for studying the nodulation pathway.
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. 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. Certain photosynthetic Bradyrhizobium that lack nod genes synthesize purine derivatives that trigger nodulation in Aeschynomene, 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.

Cytokinins are known to induce Rhizobium-independent changes, such as the induction of early nodulins (ENOD40, 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. 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 \(\beta\)-expansin gene of soybean, which is regulated synergistically by both auxin and cytokinin, and is inducible by cytokinin alone. 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
Cytokinin induces expansin mRNAs in Nod Myc *Melilotus* that require loosening of plant cell walls.\textsuperscript{19,23} Expansin gene expression also correlates with nematode-induced syncytial formation in *Arabidopsis* and tomato,\textsuperscript{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.\textsuperscript{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*,\textsuperscript{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.\textsuperscript{51-53} Although the Nod/pTZS\textsuperscript{+} mutant did not elicit *MaENOD40* expression in *Masym3*,\textsuperscript{31} *MaEXP1* was induced, strongly suggesting that expansin-mediated processes are upstream of *MaENOD40*-related development. An interesting correlation is that an *ARR5*-promoter GUS construct was turned on in *Lotus japonicus* root hairs that deformed in response to *Mesorhizobium loti* inoculation.\textsuperscript{49} *ARR5* is a cytokinin primary response gene that was originally cloned from *Arabidopsis*.\textsuperscript{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 MaEXP1 gene expression, white sweetclover (Melilotus alba Desr. U389) seeds were surface-sterilized as described earlier 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-naphthalene acetic acid (NAA) or 6-benzylaminopurine (BAP). Hormone stocks of 10 mM were made in DMSO, and added steriley 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 strain (SL44; ΔnodD1ABC), and a Nod/pTZS strain (SL44 carrying the trans-zeatin synthase gene of A. tumefaciens). Prior to inoculation, M. alba wild-type (U389) and M. alba ΔStrain (BT70) seeds were scarified and surface-sterilized as above and then planted in autoclaved dishes containing a 1:4 mix of perlite and vermiculite watered with 1/4 strength Hoagland’s minus nitrogen (for nitrogen-starved or 10⁻⁶ M BAP-treated or for rhizobial inoculation) or with 1/4 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 dishes 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. Photographs of whole roots were taken with Kodak Ektachrome Tungsten 160 film on a Zeiss Axiohot 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, NY) 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 MgCl₂ and 2.5 units Taq DNA polymerase (GIBCO/BRL) or 1 unit of Eppendorf HotMaster Taq DNA polymerase (Eppendorf, Westbury, NY). The Msc27 and MaEXP1 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

Cytokinin induces expansin mRNAs in Nod Medicago littorea


