

The *Arabidopsis* *HP6* gene is expressed in *Medicago truncatula* lateral roots and root nodule primordia

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Expression patterns of orthologous genes can be similar between distantly related species, suggesting that developmental programs can be conserved between organisms. Here, we show that the promoter of *AHP6*, a gene which is involved in *Arabidopsis* lateral root development, also drives the expression of the reporter *GUS* gene in lateral roots of *Medicago truncatula* suggesting that similar regulatory elements are involved in lateral root organogenesis in these species. Interestingly, the *AHP6* promoter was able to drive *GUS* expression in root nodules and nodule primordia, structures that are absent in *Arabidopsis*. We found two *AHP6* orthologous genes in the *M. truncatula* genome and we speculate that these putative cytokinin inhibitors may play a role during lateral root and nodule development in this species.

Phytohormones are important regulators of plant development that function to coordinate a plant's responses to the environment and regulate growth. Cytokinin, a class of adenine-derived signaling molecules, has long been recognized to be involved in a myriad of processes throughout a plant's life (e.g., senescence, meristem function, vascular development, shoot phyllotaxis). The model for cytokinin signal transduction pathway proposed in the plant-model *Arabidopsis* consists on a phosphorelay system which begins with cytokinin sensing and initiation of signaling by histidine kinases (HK) receptors, followed by transference of the phosphoryl group to histidine phosphotransfer proteins (HPts) and their nuclear translocation to activate or inhibit downstream partners, the response regulators (RRs).¹ In addition *Arabidopsis* contains a "pseudo-HPt." This differs from the HPts as the conserved histidine residue required to accept the incoming phosphoryl group from the receptors is replaced by an asparagine. As a result, *AHP6* functions as a cytokinin signaling inhibitor. This effect was first characterized during root vascular development,² however we recently showed that *AHP6* also acts as a cytokinin repressor during early stages of *Arabidopsis* lateral root development. *AHP6* is expressed at the initial stages of lateral root development and it is required to establish the correct pattern of cell divisions. We further proposed that *AHP6* might modulate auxin response by affecting the localization of PIN1, an auxin efflux carrier.³

To examine the role of cytokinin signaling during the formation of newly emerged organs in other species, we examined the model legume *Medicago truncatula*. Like *Arabidopsis*, legumes have branched roots with newly formed organs (lateral roots) rising along the primary root. However, legumes also have the

capacity to form root nodules, where nitrogen fixation occurs through a symbiotic association with *Rhizobium*. In *M. truncatula*, it was shown that rhizobial infection activates both a receptor *HK* gene, *MtCRE1*, which is orthologous to the *Arabidopsis* *HK4* (*AHK4*) and two cytokinin signaling RR, *MtRR1* and *MtRR4*.⁴ *MtCRE1* and *MtRR4* are expressed in mature nodules suggesting an additional role for cytokinin at this stage.⁵ Moreover, in a study that searched for novel regulators of nodulation in legumes, two other cytokinin signaling *RRs* were identified in *M. truncatula*, *MtRR9* and *MtRR11*.⁶

One way to explore conservation of gene function between different species is to analyze the expression pattern of orthologous genes. If the expression pattern is similar, it suggests that gene function might be conserved.⁷ To gain insights into whether pseudo HPts may function in *M. truncatula*, we performed an heterologous transcriptional fusion, i.e., we transformed transiently *M. truncatula* roots with the *Arabidopsis* *HP6* promoter fused to the β -glucuronidase reporter gene (*AHP6pro::GUS*).² The promoter was activated in *M. truncatula* roots. Its vascular expression slightly deviates from the endogenous *Arabidopsis* expression² (Fig. 1A and B) but for lateral root formation it shows a similar expression pattern³ (Fig. 1C–F). Next, we searched for putative *AHP6* orthologs in the *M. truncatula* genome and we found seven HPt-related genes. In their predicted amino acid sequence, two of them contained a polymorphism in the conserved phospho-accepting histidine (similar to *AHP6*). We named these atypical HPts, *MtHP6* and *MtHP7* (Fig. 2A). *AHP6* is an auxin responsive gene and its expression is dependent on a series of auxin response elements (AuxREs) within the promoter.⁸ To further explore

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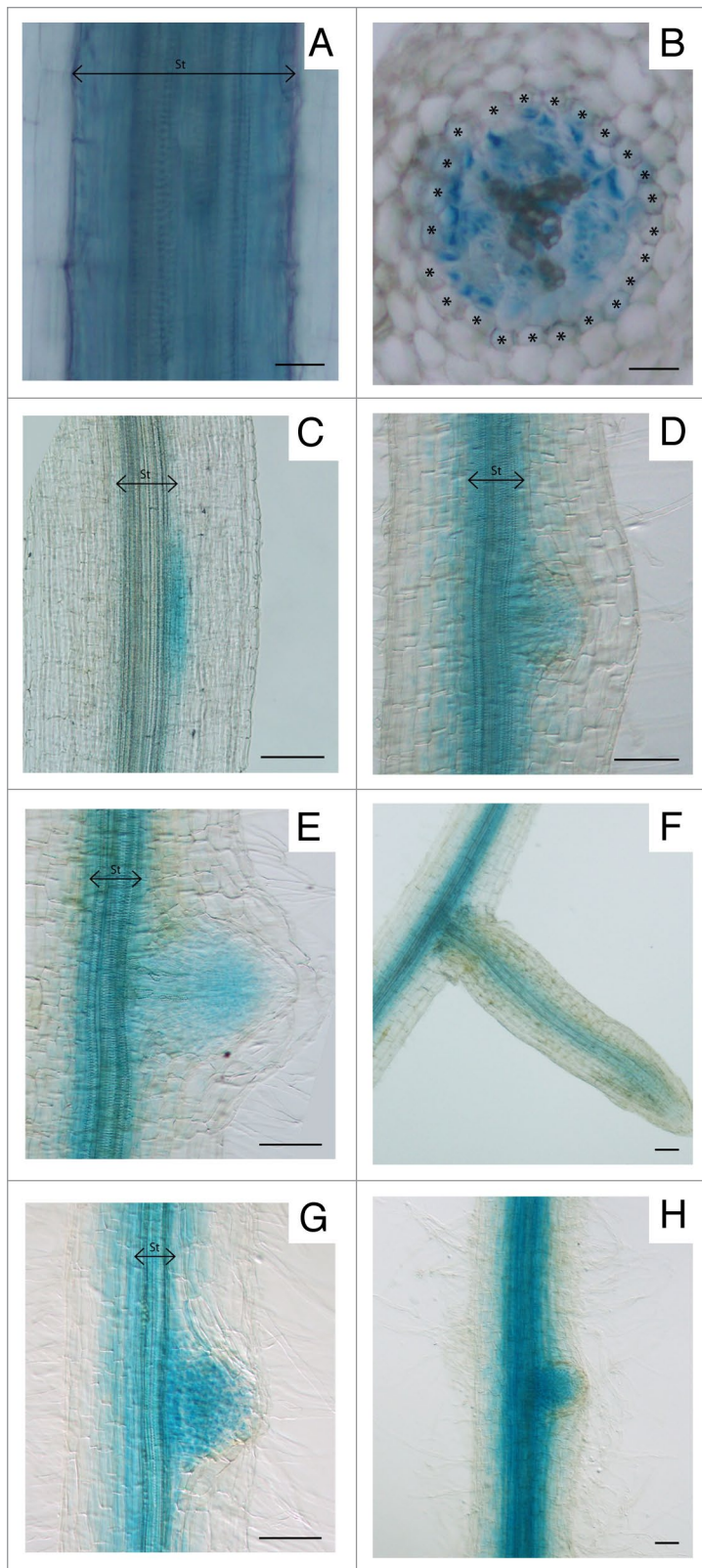


Figure 1. The *Arabidopsis* *HP6pro::GUS* is expressed during development of both *Medicago truncatula* root-derived lateral organs: lateral roots and nodules. For plant transformation we used *Agrobacterium rhizogenes*-mediated hairy root transformation.¹² Hairy roots collected 48 d and 7 d post inoculation with *Sinorhizobium medicae* were observed under an Olympus optical microscope (SZ × 1) and images were obtained using an Olympus digital camera (DP21). Histochemical staining for GUS activity was performed as previously described.¹³ (A) Longitudinal section of a *M. truncatula* root highlighting the stele (vascular cylinder + pericycle). (B) Cross-section of *M. truncatula* root highlighting the stele: the endodermis cell layer surrounds the stele and it is marked with *; (C) initial stage of lateral root primordium; (D) intermediate stage of lateral root primordium; (E) emerging lateral root; (F) lateral root; (G) root nodule primordia; (H) root nodule. St, stele; bars in (A and B) 25 μm; bars in (C–H) 100 μm.

genes. Together these results suggest that these *AHP6* orthologs may be expressed similarly to *AHP6*. As the conversion of the phospho-accepting residue has previously been shown to confer a negative effect on cytokinin signaling, it is likely that the *M. truncatula* “pseudo HPts” would have similar effects and may have similar functions during lateral root developmental programs in these two species.

Interestingly, the *AHP6* promoter was able to drive *GUS* expression in root nodule primordia and nodules (Fig. 1G and H). The finding that a cis-regulatory sequence of a non-nodulating plant is able to drive the expression of a reporter gene in root nodules of *M. truncatula* suggests that regulatory pathways might be shared between both root-derived lateral organs. This idea is further supported by the existence of legume mutants affected in genes with dual function in nodule and lateral root development, such as *latd* (*lateral root organ-defective*)⁹ showing an arrest of both types of root lateral organs or *har1* (*hypernodulation and aberrant root 1*),¹⁰ which forms an increased number of nodules and lateral roots. Also, in a study investigating microRNA (miRNA) involvement in the regulation of root lateral organogenesis, it was shown that the overexpression of *MtMIR166a* precursor leads to a decrease in both lateral roots and nodule formation.¹¹ Furthermore, an RNAi construct targeting A-type cytokinin *RRs* (that act as negative regulators of cytokinin signaling) lead to decrease numbers of lateral root and root nodules.⁶

In conclusion, although a nodule is not considered a modified lateral root because of its different ontogeny; similarities exist in the formation of both organs and there is gathering evidence that part of the genetic programs involved in lateral root development have been recruited for nodule development. Future work will focus on the study of expression and function of *MtHP6* and *MtHP7* during lateral root and root nodule development in *M. truncatula*. The presence of these putative cytokinin inhibitors may limit the number of cells responding to this phytohormone and may thereby help to define and sharpen cell differentiation boundaries during root-derived lateral organ development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

the possibility that *MtHP6* and *MtHP7* show auxin responsive gene expression similar to *AHP6*, we screened their cis-regulatory regions for AuxREs (TGCT motif). We found 10 occurrences in the *MtHP6* and 18 in the *MtHP7* upstream region (Fig. 2B), suggesting that these genes are also putative direct auxin responsive

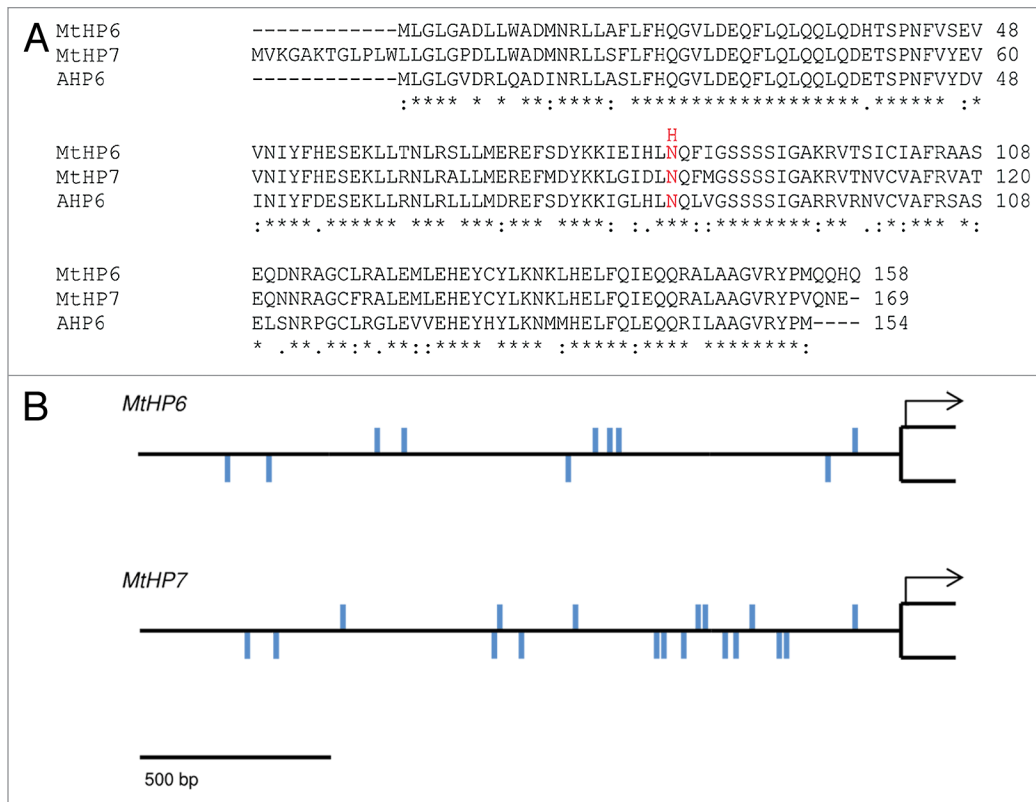


Figure 2. (A) Amino acid sequence alignment of AHP6 (Q9SSC9.2) and the two *Medicago truncatula* histidine phosphotransfer proteins (HPTs) containing the polymorphism in the conserved histidine residue (red H above), MtHP6 (XP_003597907.1) and MtHP7 (XP_003604376.1). The histidine was replaced by an asparagine (N in red). Residues fully conserved (*); highly conserved (;); poorly conserved (.); not conserved (). The amino acid sequences were obtained from NCBI databases and aligned by CLUSTAL W (1.8). **(B)** A schematic representation of the 2 kbp upstream of *MTHP6* and *MTHP7*. TGCT motifs (auxin response element) on sense and antisense strands as blue bars.

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