Cytokinin responses counterpoint auxin signaling during rhizobial infection

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The transcriptomics approach to study gene expression in root hairs from M. truncatula has shed light on the developmental events during rhizobial infection and the underlying hormone responses. This approach revealed the induction of several cyclins and an aurora kinase which suggests that the cell-division machinery plays a role in rhizobial infection. Changes in the cell cycle in plants are governed by hormones, in particular auxin and cytokinin. Through gene expression and genetic analyses, we have shown auxin plays a role during rhizobial infection. Here we provide further analysis of the data showing the induction of a set of cytokinin signaling components. These include genes encoding 2 cytokinin-activating enzymes, the cytokinin receptor CRE1, and 5 type-A cytokinin response regulators. We discuss the possible interactions between auxin and cytokinin signaling during the infection process. We also consider a potential role for cytokinin signaling in rhizobial attachment.

Recently, Breakspear et al.1 characterized gene expression responses prior to and during rhizobial infection and in response to Nod factors in root hairs of Medicago truncatula seedlings. This single-cell type approach offered increased specificity and dramatically enhanced sensitivity of gene detection, the latter being further enhanced by the use of the hyperinfected mutant sickle (skl). It thereby allowed detection of genes that have not otherwise been detected in gene expression studies of nodulation and also allowed the detection of more subtle transcriptional changes that would have otherwise been missed in studies using whole nodulated roots. This was particularly useful in detecting changes in cell-cycle related genes, including the induction of several cyclins, an aurora kinase, and genes involved in hormone biosynthesis and signaling. In particular it led to the discovery of an auxin response factor (ARF), ARF16a, which was specifically induced in root hairs undergoing infection. arf16a mutants showed a reduced number of rhizobial infections revealing the need for regulation of auxin responses in this process. Three alleles were reported in the original study, here we report a fourth allele, arf16a-4, and show that it has a similar phenotype as the other alleles: hypersensitivity to auxin in a root growth assay (Fig. 1A and B), and reduced number of microcolonies and elongating infection threads (Fig. 1C).

The role of auxin signaling in rhizobial infection is not known. One potential role for auxin is in the control of the cell-division machinery which has been found to be associated with infection.1 The hormone cytokinin is widely accepted to act in concert with auxin, often acting to counter or antagonize auxin signaling to establish important signaling fields in different developmental contexts.2 To further explore this proposition we have reanalyzed the data set from Breakspear et al.1 to consider cytokinin responses.

Several components of cytokinin signaling were found to respond to rhizobia and/or Nod factors in root hairs (Table 1). Five Type-A cytokinin response regulators were found to be increased: MtRRA2, MtRRA3 (previously MtRR8),3 MtRRA4 (previously MtRR4),4 MtRRA8, and MtRRA10 (previously MtRR11).5 The gene names are according to Heyl et al.,6 personal communication M. Brault and F.
Frugier. The increased expression seen with MtRR48 matches well with an early report that showed that the promoter of the Arabidopsis ortholog ARR5 was expressed in infected root hairs of M. truncatula.7 Type-A response regulators, which act as a central part of a 2-component signaling pathway, are induced by cytokinin8,9 and have been shown to act as negative regulators of cytokinin signaling.10 Notably, no type-B response regulators, which mediate the downstream effects of auxin signaling, were found to be induced. This is similar to cytokinin treatments, which induce type A response regulators, but not type B (discussed in D’Agostino et al. 2000).9 Along with these, the cytokinin receptor gene CRE1, was also induced (Table 1). Mutants for LHK1 (the Lotus japonicus ortholog of CRE1) have a strong delay in the onset of cell divisions, and nodules that do form are misshapen11,12 and CRE1-knockdown roots form fewer nodules.4 Consistent with these results, promoter-GUS analysis of LHK1 in L. japonicus revealed expression in root hairs associated with infection sites.12 We also found that 2 members of the LONELY GUY gene family which encode an enzyme required for cytokinin-activation were also upregulated (Table 1), further suggesting that levels of active cytokinin may be increasing during infection. Interestingly, like CRE1, these genes LOG1 and LOG2 are also induced in nodulation and are required for nodule organogenesis.13 Together these data suggest that cytokinin is being activated in root hairs during the onset of infection and is being perceived through CRE1 to regulate cytokinin signaling.

Cytokinin as a Counterpoint to Auxin

The interactions between auxin and cytokinin signaling have been relatively well-studied, and have mainly been found to be antagonistic. For instance, studies of the Arabidopsis meristem have shown that domains of auxin and cytokinin signaling are mutually exclusive.14 The large number of RRAs induced and the apparent absence of increased expression of RRBs may indicate that although cytokinin

Figure 1. Auxin response and nodulation phenotype of M. truncatula arf16a-4 mutant. (A) and (B) The inhibition of primary root growth by 10μM indole acetic acid in the wild type (R108) and arf16a-4 (NF4811). The picture (A) and histograms (B) show plants 14 d after germination. (C) Quantification of different stages of infection and development of nodule primordia in the wild type and arf16a-4 mutants 7 dpi with S. melloti. Infection events and nodule primordia were scored 7 dpi with S. melloti 1021 carrying pXGLD4 (LacZ) after LacZ staining. IT, fully elongated infection thread in root hair; eIT, elongating infection thread in root hair; MC, microcolony; rIT, ramified infection thread in cortex; NP, nodule primordium. Bar = SE. Significant (Student’s t-test) differences between the wild type and mutant are marked with asterisks (**P < 0.01).
signaling is active, auxin signaling outcomes prevail in cells undergoing infection.

One possible explanation for the activation of cytokinin signaling during infection may be to antagonize auxin signaling. But how might this work in the case of nodulation? The basis of cytokinin-auxin interactions is not completely understood, but ethylene, which is known to crossstalk with both cytokinin and auxin, and is a major regulator of nodulation, may be part of the mechanism. One major outcome of cytokinin signaling is stabilization of the ACS5 enzyme mediating the rate limiting step of ethylene biosynthesis.\(^1\) Our data showed that ethylene signaling represses infection-related gene expression,\(^3\) which is presumably due to interference with Nod factor signaling.\(^4\) Therefore one role of cytokinin signaling may be to generate ethylene to limit infections, consistent with the observation that the \(lhk1\) mutant has greatly increased numbers of infection threads.\(^1,11\) In further agreement with this hypothesis, increased expression of \(CRE1\) and \(LOG1\) in response to rhizobial inoculation was only observed in \(skl\) (Table 1).

In addition, ethylene may in turn influence auxin. Indeed, low levels of ethylene have been shown to promote auxin biosynthesis through \textit{WEAK ETHYLENE INSENSITIVE1} (\textit{WEI1}) and \textit{WEI2}.\(^17\) However, the \textit{Medicago} orthologues of \textit{WEI1} and \textit{WEI2} are not regulated following rhizobial inoculation or Nod factor treatment (not shown), and since the ethylene insensitive mutant \(skl\) becomes hyperinfected, it seems unlikely that ethylene induction of auxin biosynthesis is required for infection. Considering this, the interaction between cytokinin and auxin during infection, if it does occur, may be more direct. Cytokinin and auxin signaling mutants are needed to help address this question.

**Cytokinin as a Regulator of Bacterial Attachment**

Recently cytokinin has been implicated in bacterial attachment. \textit{Agrobacterium tumefaciens} produces cytokinins, which were shown to activate the \textit{Arabidopsis} Type-A cytokinin response regulator \textit{ARR3}\(^18,19\) which was associated with decreased expression of the \textit{Myb Family Transcription Factor 1} (\textit{MTF1}). Mutant \(mtf1\) plants were shown to have increased \textit{A. tumefaciens} attachment and improved transformation efficiency. The authors further demonstrated that mutating the cytokinin receptors \textit{CRE1} and \textit{AHK3} increased the expression of \textit{MTF1} and reduced the transformation efficiency. Notably we find that the \textit{Medicago} ortholog of \textit{MTF1} is repressed in the hyperinfected \(skl\) mutant at the onset of infection (Table 1). This presents an unexpected role through which cytokinin may act during the early stages of the symbiosis, and may add another layer of complexity to cytokinin's role in nodulation.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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