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Article Addendum

Signaling via Histidine-Containing Phosphotransfer Proteins in Arabidopsis

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ABSTRACT

The Arabidopsis genome encodes a number of proteins with similarity to two-component phosphorelay signaling elements, including hybrid receptor histidine kinases, two classes of response regulator proteins (type-A and type-B ARRs) and a family of six histidine-containing phosphotransfer proteins (AHPs), five of which contain a conserved His residue that is required for phosphorelay signaling. The current model for cytokinin signaling includes a multistep phosphorelay: three histidine kinases and at least five type-B ARRs have been shown to act as positive regulators of cytokinin signaling, while a number of type-A ARRs, and AHP6, act as negative regulators of the pathway. In our recent Plant Cell paper, we provided genetic evidence that at least four AHPs can act as positive regulators of cytokinin signaling, affecting responses to cytokinin in the root and the shoot. In this addendum, we discuss the role of AHPs in cytokinin signaling and speculate on their potential interactions with other signaling pathways in Arabidopsis.

CYTOKININ SIGNALING IS MEDIATED BY A HIS-ASP PHOSPHORELAY PATHWAY

A simple two-component phosphorelay consists of a sensor histidine kinase that perceives a signal, and a response regulator with a conserved receiver domain that is phosphorylated by the histidine kinase. The response regulator mediates the response to the signal. Extended versions of this pathway include further receiver domains and additional histidine-containing phosphotransfer domains that maintain His-Asp-His-Asp phosphorelay through the pathway.[1] The two-component signaling elements identified in the Arabidopsis genome are predicted to form multistep phosphorelay pathways consisting of a hybrid sensor kinase that includes a sensor, histidine kinase and receiver domain in one molecule, a histidine-containing phosphotransfer protein, and response regulator(s).[2] This is the current model for cytokinin signaling.[3-5] Previous studies have shown that three histidine kinases (AHK2, AHK3 and AHK4) are cytokinin receptors,[3,6-11] at least five type-B ARRs act as positive elements in the cytokinin signaling pathway,[5,12] and multiple type-A ARRs play a role in negative regulation of cytokinin signaling as well as other roles in plant development.[4,13,14] We took a reverse genetic approach to investigate the role of AHPs in cytokinin signaling, combining insertion mutants in the five *AHPs* that are predicted to encode functional phosphotransfer proteins to make various *ahp* mutant combinations. These mutants were then analyzed for cytokinin-responsiveness using a variety of assays.

Cytokinins affect many aspects of plant development including cell division, shoot induction, and vascular development.[15] Growth of Arabidopsis plants on cytokinin inhibits root elongation, lateral root formation and hypocotyl growth, reduces shoot chlorophyll content, and alters gene expression.[4,5] Disruption of AHPs in many of the *ahp* mutant combinations resulted in reduced sensitivity to exogenous cytokinin in these responses, which indicates that the AHPs act as positive regulators of cytokinin signaling. In addition, some *ahp* mutant combinations resulted in plants with a short primary root with reduced vascular development, increased seed size and reduced seed set—phenotypes similar to those of cytokinin receptor triple mutants.[9-11] This suggests that the AHPs mediate signaling from the cytokinin receptors that is required for normal development.

Our analysis provided evidence for a positive role for AHP1, AHP2, AHP3 and AHP5 in cytokinin signaling, supporting previous in vitro and transgenic studies that implicated AHP function in general in cytokinin signaling.[3,16,17] However, it did not provide consistent evidence for a similar role for AHP4. The effect of mutation of *AHP4* was negligible in many assays, but in some assays and mutant combinations AHP4 appeared to play a

positive role in cytokinin signaling, while in others its role appeared negative.

A sixth AHP, AHP6, does not contain the conserved histidine residue that is required for phosphorelay and acts as a negative component in the pathway, most likely via a dominant negative mechanism by interfering with phosphotransfer from the AHKs via the other AHPs to the ARRs.[18] While AHP4 does contain a His at the conserved site, it could still interfere with cytokinin signaling by a dominant negative mechanism if it retains its ability to interact with the AHKs and/or the ARRs, but is unable to receive or donate a phosphoryl group. Different splice variants of *AHP4* might encode isoforms of AHP4 that are active in, or interfere with, cytokinin-responsive phosphorelay.[19] Differential expression of these isoforms could underlie the variability in cytokinin response between the assays described. Other assays might reveal a clearer role for AHP4 in cytokinin signaling in particular tissues or times during development. Alternatively AHP4 might act primarily in another phosphorelay signaling pathway.

THE CYTOKININ-SIGNALING PATHWAY IS NOT LINEAR

Mutation of the *AHK* cytokinin receptors, the *AHP*s and the type-B *ARR*s results in broadly similar phenotypes, consistent with these proteins acting together to mediate cytokinin signaling during development.[9-11] However, a potential branch in the cytokinin pathway is indicated by the cytokinin-responsiveness of subcellular localization of the CRFs: transcription factors that mediate cytokinin response in parallel with the type-B ARRs.[20] The CRFs move to the nucleus in the presence of cytokinin. This movement requires AHK and AHP, but not ARR, function, suggesting that cytokinin signaling branches at the AHPs.[20]

Phosphorylation of the type-A ARRs, by which they are activated, via the AHPs would represent a similar branch in the current linear cytokinin signaling model.[13,21] Alternatively activation of the type-A ARRs might be achieved via direct phosphorylation by the AHKs.[22]

DO THE AHPS MEDIATE MORE THAN ONE PHOSPHORELAY PATHWAY?

AHPs could mediate additional phosphorelay pathways in Arabidopsis, as well as providing a potential point of integration with other signaling pathways through modification of AHP expression or behavior. Apart from the cytokinin receptors, the Arabidopsis genome encodes five potentially active histidine kinases: AHK1, a suggested osmosensor;[23] CKI1, required for female gametophyte development;[24,25] CKI2/AHK5, involved in response to ABA and the ethylene precursor ACC;[26] and the ethylene receptors ETR1 and ERS1.[2] ETR1 histidine kinase activity is required for seedling responses to low levels of ethylene and for growth recovery after removal of ethylene.[27,28] It is not known whether the AHPs or other two-component elements are involved in these responses.

Interactions between many Arabidopsis two-component signaling elements have been detected in yeast two-hybrid assays, and their ability to participate in phosphorelay pathways has been shown in vitro and in heterologous phosphorelay systems (for example Refs. 6-8,19,21,29,30). Such studies highlight the potential for additional phosphorelay signaling pathways in Arabidopsis, but also for non-specific signaling between components. Further phenotypic analysis of *ahp* and other two-component element mutants might shed light on whether phosphorelay signaling pathways lie downstream of ETR1 or other histidine kinases.

If phosphorelays in addition to the cytokinin signaling pathway are functional in planta, the observation that the six AHPs can affect cytokinin signaling raises the questions of whether the additional phosphorelays are mediated by AHPs and, if they are, whether and/or how a specific response to each input is achieved. The availability of AHPs and potential signaling partners in particular cell types, growth conditions, or during development could affect the amplitude and/or specificity of phosphorelay signaling. Alternatively, specificity might be maintained by additional, as yet unidentified, signaling components.

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