Final report for DE-FG02-05ER15669 “Role of nuclear shuttling in cytokinin signaling”

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DOE/Office of Science Program Office: Energy Biosciences Program

This grant was focused on an analysis of the nuclear movement of one of the components of cytokinin signaling. Cytokinins are N^6 -substituted adenine derivatives that play a role in almost all aspects of plant growth and development, including shoot and root growth, vascular and photomorphogenic development, and leaf senescence. A model for cytokinin perception and signaling has emerged that is similar to bacterial two-component phosphorelays. In this model, binding of cytokinin to the extracellular domain of the Arabidopsis histidine kinase (AHKs) receptors induces autophosphorylation within the intracellular histidine-kinase domain. The phosphoryl group is subsequently transferred to cytosolic Arabidopsis histidine phosphotransfer proteins (AHPs), which have been suggested to translocate to the nucleus in response to cytokinin treatment, where they then transfer the phosphoryl group to nuclear-localized response regulators (Type-A and Type-B ARR).

Prior studies in Arabidopsis mesophyll protoplasts suggested that AHP-GFP fusion proteins are mainly localized to the cytoplasm, but rapidly translocate into the nucleus in response to cytokinin treatment^{1,2}. To determine the subcellular localization of these proteins in planta, we analyzed transgenic Arabidopsis plants harboring either a ProAHP2:AHP2-GFP (Figure 1) or a ProAHP5:AHP5-GFP transgene. We observed that AHP2-GFP fluorescence was distributed throughout the cells distal to the meristematic zone (early transition zone) of the root in plants harboring a ProAHP2:AHP2-GFP transgene (Figure 1a,b). A high concentration of GFP fluorescence was observed within the nuclei; however GFP signal was also clearly visible in the cytoplasm (Figure 1k). Application of trans-zeatin did not modify this localization pattern (Figure 1e,f,l). Similar results were observed in the meristematic zone in the absence (Figures 1c,d) and presence of cytokinin (Figures 1g,h), root hair cells (data not shown), and epidermal cells of the leaf (Figure 1i,j). **These results indicate that AHPs are localized to both the nucleus and cytosol in planta and that this localization is unchanged in response to cytokinin treatment.**

Figure 1. Representative confocal micrographs of serial z-sections from roots of five-day old plants expressing *AHP2-GFP* from its native promoter.

(a) and (b) Transition zone of an untreated (control) root.

(c) and (d) Meristematic zone of an untreated (control) root.

(e) and (f) Transition zone of a root treated with 1 μ M *trans*-zeatin.

(g) and (h) Meristematic zone of a root treated with 1 μ M *trans*-zeatin.

(i) and (j) Abaxial surface of an untreated (control) leaf.

(k) and (l) High-magnification micrographs of roots displayed in (a) and (e) showing that AHP2-GFP is localized to both the nucleus and cytosol. Dashed line outlines a single cell.

(a), (c), (e), (g), (i), (j), (k), and (l) show GFP fluorescence only, while (b), (f), (d), and (h) show propidium iodide counterstained cell walls overlaid on GFP fluorescence signals. c, cytosol; n, nucleus. Scale bars = 20 μ m.

Because our *in planta* observations of AHP-GFP subcellular localization contradict the initial observations of cytokinin-induced AHP-GFP relocalization in protoplasts, we re-examined these original observations and quantified the results. To this end, we analyzed Arabidopsis mesophyll protoplasts transfected with various Pro35S:AHP-GFP constructs. We grew our plants in conditions as similar as possible to those used in the original observations, used similar constructs, and a similar transfection protocol (Hwang and Sheen, 2001; Yoo et al., 2007). Both in the absence of exogenous cytokinin, AHP1-GFP, AHP2-GFP, and AHP5-GFP are located in both the nucleus and the cytosol of most cells. However, across the population of protoplasts, we did observe a subset of cells with primarily nuclear or primarily cytoplasmic AHP localization. Cytokinin treatment had no obvious effect on the subcellular distribution of AHP-GFP across an entire population of protoplast. Although the subcellular localization of AHP-GFP fusion proteins did not appear to change in response to cytokinin treatment across entire populations of

cells, it is possible that within a cell, the relative amount of nuclear-localized AHP increases upon cytokinin treatment. To address this question, we analyzed the percentage of AHP-GFP fluorescence that was localized to the nucleus before and after cytokinin treatment in cells that showed both nuclear- and cytosolic-localized AHP-GFP fluorescence. The relative level of nuclear-localized fluorescent signal from multiple AHP-GFP fusion proteins did not appreciably change in response to treatment with cytokinin. **These results indicate that AHPs are localized to both the nucleus and cytosol in mesophyll protoplasts, and this localization is unchanged in response to cytokinin treatment either on a whole cell population level, or within an individual cell.**

Our observations suggest that AHP-GFP fusion proteins are localized to both the nucleus and cytoplasm, despite activation of cytokinin signal transduction via application of exogenous cytokinin. During cytokinin signaling, phosphoryl groups are transferred from an aspartate residue on the activated cytokinin receptors (AHKs) to a histidine residue on the AHP proteins³. To test whether phosphorylation of AHP2 might be required for its proper localization, we examined the subcellular localization of a phospho-insensitive version of AHP2-GFP. We mutated the conserved histidine (H82)⁴ residue to an alanine (A) residue in AHP2-GFP and examined the subcellular localization of the resulting fusion protein. This mutant fusion protein, AHP2H82A-GFP, exhibits the same subcellular localization as the wild-type AHP2-GFP fusion protein (Fig. 2) and displayed a similar ratio of nuclear:cytoplasmic localized fluorescent signal as the wild-type AHP2-GFP. **These results suggest that the phosphorylation status of the AHPs does not alter their intracellular localization.**

Figure 2. Analysis of the distribution of AHP2-GFP and AHP2^{H82A}-GFP in various cytokinin mutants.

Bar graph showing quantification of the subcellular distribution of AHP2-GFP and AHP2^{H82A}-GFP in mesophyll protoplasts made from various cytokinin signaling mutants. Yellow bars show the percentage of cells with predominantly cytoplasmic localization, red bars show the percentage of cells with nuclear and cytoplasmic localization, blue bars show the percentage of cells with predominantly nuclear localization. Values are the mean of three independent experiments, error bars indicate SEM.

Prior reports have shown that AHPs can homo- and hetero-dimerize in yeast-two-hybrid assays⁵. This suggests that AHP2H82A-GFP may have “piggybacked” into the nucleus in the above assays by dimerizing with an endogenous, wild-type AHP2 protein. To confirm the yeast-two-hybrid assays, we tested whether AHP2 can homo-dimerize using bimolecular fluorescence complementation.

Both a wild-type and phospho-insensitive version of AHP2 (AHP2 and AHP2H82A, respectively) were fused at their C-termini to both the N-terminal and C-terminal halves of the YFP coding region (YFPn and YFPc, respectively). Combinations of these proteins as well as either half of YFP alone were tested for interaction in Arabidopsis mesophyll protoplasts by assaying for YFP fluorescence using confocal microscopy. If the two proteins interact when co-expressed in the same cell, the two complementary halves of YFP are brought together, resulting in fluorescence. As shown in Figure 3b, both AHP2-YFPn and AHP2H82A-YFPn interact with

AHP2-YFPc in the nucleus and cytosol. In contrast, YFPn alone did not interact with AHP2-YFPc, suggesting that the interaction was not due to affinity between the YFP halves. Reciprocal experiments showed the same pattern of interactions. **Combined with previous yeast-two-hybrid assays, these results strongly suggest that AHP proteins are able to homo-dimerize in vivo.**

Figure 3. BiFC analysis of AHP2 homodimerization. Images are confocal scanning laser micrographs of protoplasts, top row shows YFP-fluorescence signal in green, bottom row shows overlay of YFP-fluorescence in green and chlorophyll auto fluorescence in red. Scale bar is 25 μ m.

It is possible that activation of the cytokinin signal transduction pathway results in phosphorylation of AHP2 at a non-conserved phospho-acceptor, which may in turn cause AHP2 to become localized to the nucleus. To address this, we analyzed the subcellular distribution of AHP2-GFP in mesophyll protoplasts isolated from *ahk2,3* or *ahk3,4* mutant plants. AHK2, AHK3, and AHK4 encode the cytokinin receptors of Arabidopsis; AHK2 and AHK3 are expressed in all tissues, whereas AHK4 is predominantly expressed in the root^{6,7}. Therefore, leaf mesophyll protoplasts isolated from *ahk2,3* plants are strongly insensitive to cytokinin signaling through the canonical two-component signaling pathway^{6,7}. As in wild-type cells, AHP2-GFP is distributed in both the nucleus and cytoplasm of the *ahk2,3* and *ahk3,4* protoplasts (Figure 3a). **Taken together, these results strongly suggest that AHP2 localization is not regulated by changes in the phosphorylation state of its conserved histidine or by activation of the cytokinin signaling pathway through the known cytokinin receptors.**

Our results suggested that AHP proteins are actively transported into and out of the nucleus in a cytokinin-independent manner. Thus, we attempted to identify sequences within the AHP2 protein that were required for proper active transport by generating a series of N- and C-terminal deletions of AHP2. All of the C-terminal deletions resulted in fusion proteins that were predominantly localized to the cytosol (Figure 4). These results suggest that active transport of AHP2 is complex, and may involve multiple protein-protein interaction domains.

Figure 4. Analysis of the distribution of various AHP2 deletions fused to GFP.

(a) Bar graph showing quantification of the subcellular distribution of AHP2 deletions. Yellow bars show the percentage of cells with predominantly cytoplasmic localization, red bars show the percentage of cells with nuclear and cytoplasmic localization, blue bars show the percentage of cells with predominantly nuclear localization. Values are the mean of three independent experiments, error bars indicate SEM. (j) Schematic of AHP2 protein and location of deletion endpoints analyzed in this study. Green box indicates histidine-containing phosphotransfer domain, star indicates location of the conserved histidine phospho-acceptor residue (H82).

One postdoctoral fellow, Jayson Punwani, was supported by this award.

Publications supported by this award

Punwani, J.A., Hutchison, C.E., Schaller, G.E., Kieber, J.J. (2010) The subcellular distribution of the Arabidopsis Histidine Phosphotransfer Proteins is independent of cytokinin signaling. **Plant J.** 62:473-82

References

1. Hwang, I and Sheen, J (2001) Two-component circuitry in *Arabidopsis* signal transduction. *Nature* **413**: 383-389.
2. Yamada, H, Koizumi, N, Nakamichi, N, Kiba, T, Yamashino, T, and Mizuno, T (2004) Rapid response of Arabidopsis T87 cultured cells to cytokinin through His-to-Asp phosphorelay signal transduction. *Biosci. Biotechnol. Biochem.* **68**: 1966-1976.
3. Mähönen, AP, et al. (2006) Cytokinins regulate a bidirectional phosphorelay network in Arabidopsis. *Curr. Biol.* **16**: 1116-1122.
4. Hutchison, CE, et al. (2006) The Arabidopsis histidine phosphotransfer proteins are redundant positive regulators of cytokinin signaling. *Plant Cell* **18**: 3073-3087.
5. Dortay, H, Gruhn, N, Pfeifer, A, Schwerdtner, M, Schmülling, T, and Heyl, A (2008) Toward an interaction map of the two-component signaling pathway of *Arabidopsis thaliana*. *J. Proteome Res.* **7**: 3649-3660.
6. Higuchi, M, et al. (2004) In planta functions of the Arabidopsis cytokinin receptor family. *Proc. Natl. Acad. Sci. USA* **101**: 8821-8826.
7. Nishimura, C, Ohashi, Y, Sato, S, Kato, T, Tabata, S, and Ueguchi, C (2004) Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in Arabidopsis. *Plant Cell* **16**: 1365-1377.