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The AHP6 cytokinin signaling inhibitor mediates an auxin-cytokinin crosstalk that regulates the timing of organ initiation at the shoot apical meristem

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Abbreviations: SAM, shoot apical meristem; AHP6, Arabidopsis Histidine Phosphotransfer Protein 6; CK, cytokinin; STM, SHOOT-MERISTEMLESS; ARF, Auxin Response Factor; MP, MONOPTEROS

Phyllotaxis, the spatio-temporal pattern of organogenesis at the shoot apical meristem, emerges in large part from inhibitory fields consisting in auxin-depleted areas centered on organs. We recently demonstrated the existence of an additional hormone-based inhibitory field generated by *Arabidopsis* Histidine Phosphotransfer Protein 6 (AHP6), an inhibitor of cytokinin signaling. We have shown that the spatio-temporal distribution of AHP6 in the meristem is essential for optimizing the rhythmicity of organ initiation. Here, we further analyzed AHP6 expression using fluorescent whole mount mRNA in situ hybridization and demonstrate a precise control of AHP6 level and expression domain over time. While we previously showed a regulation of AHP6 directly downstream of auxin, we show here that AHP6 transcription is unlikely influenced by cytokinin distribution in the meristem. Finally, we provide evidence that cytokinins and auxin might act synergistically during organ initiation, providing a plausible explanation for how AHP6 regulates phyllotaxis.

The spatio-temporal pattern of lateral organ initiation at the shoot apical meristem (SAM), called phyllotaxis, emerges in large part from inhibitory fields centered on organs and generated by polar auxin transport.¹ In the *Arabidopsis* SAM, polar auxin transport is mostly mediated by the PIN-FORMED1 (PIN1) efflux carriers.^{2,3,4,5} The spatio-temporal distribution of the PIN1 pumps suggests that the PIN1 pump network generates local accumulation of auxin that trigger organ initiation and at the same time depletes auxin around the organs, thus generating inhibitory fields by depletion of the morphogenetic molecule controlling organ initiation.^{2,6,7,8,9}

Despite the important biological knowledge on the control of phyllotaxis accumulated in recent years,¹ most of the studies focused on the spatial control of organogenesis and the timing of organ initiation was mostly assumed to be regular, leading to sequential production of organs. However, in a recent publication, we found using live-imaging in the model species *Arabidopsis thaliana* that the time between consecutive organ initiations or plastochrone is noisy, contrasting with a highly robust relative angle between consecutive organs.¹⁰ More precisely, organ initiation was mostly sequential but we observed frequent organ co-initiations, where up to 3 organs

were produced at their expected position but in a very close time window.

We further identified the *Arabidopsis* Histidine Phosphotransfer Protein 6 (AHP6), an inhibitor of cytokinin (CK) signaling,¹¹ as a negative regulator of organ co-initiations.¹⁰ AHP6 gene is specifically expressed in young organs as shown by a *pAHP6::GFP* transcriptional reporter and whole-mount mRNA in situ hybridization using colorimetric revelation¹⁰ (Fig. 1A). This expression changes while the organ develops (Fig. 1A) and we proposed that this temporal pattern of transcription is crucial for AHP6 function in plastochrone regulation.¹⁰ To further characterize the dynamics of AHP6 expression, we used our protocol for whole mount mRNA in situ hybridization¹⁰ with the tyramide signal amplification fluorescent detection method¹² (Rozier et al., in preparation). Combined with confocal microscopy, the use of a fluorescent detection allows obtaining precise 3D information on the spatial distribution of the AHP6 mRNA in the meristem (Fig. 1B). These results confirm that AHP6 expression is dynamically regulated with a maximum reached in P2 or sometimes P3 primordia, i.e., one to 2 plastochrones after the onset of expression in P1, and then a progressive decrease in the intensity of expression and a

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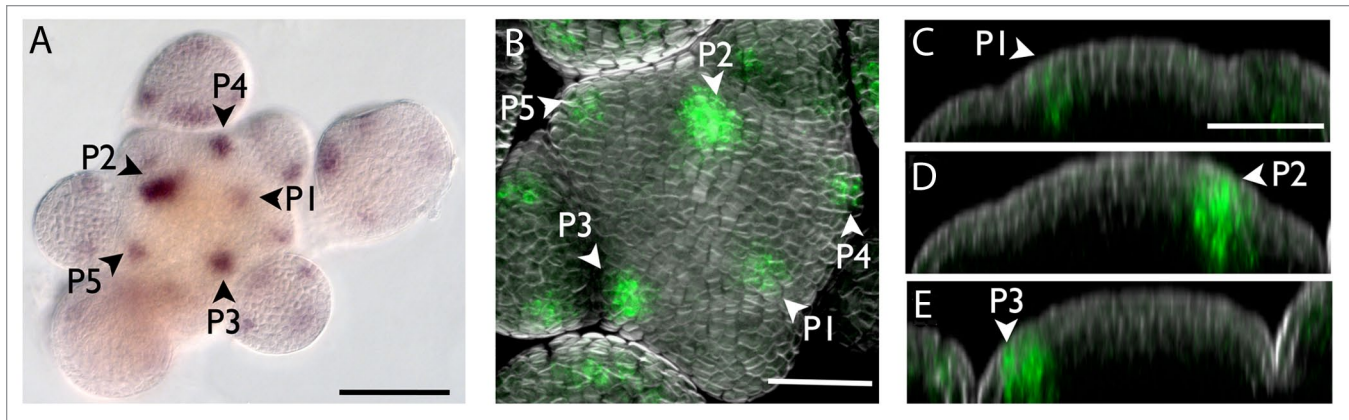


Figure 1. Visualization of the expression pattern of *AHP6* in the inflorescence meristem using whole-mount mRNA in situ hybridization. (A) Representative *AHP6* mRNA distribution in an inflorescence meristem detected using colorimetric (NBT/BCIP) revelation ($n = 8$; view from the top). (B–E) Representative *AHP6* mRNA distribution in an inflorescence meristem detected using tyramide signal amplification fluorescent revelation ($n = 7$; view from the top). (B) *AHP6* expression pattern in the inflorescence meristem (maximum intensity projection; view from the top). (C–E) Transverse optical section in lateral organs as indicated. The organ primordia (P) are numbered from youngest to oldest. Scale bar: 50 μm (A) or 25 μm (B–E; scale is identical in C–E).

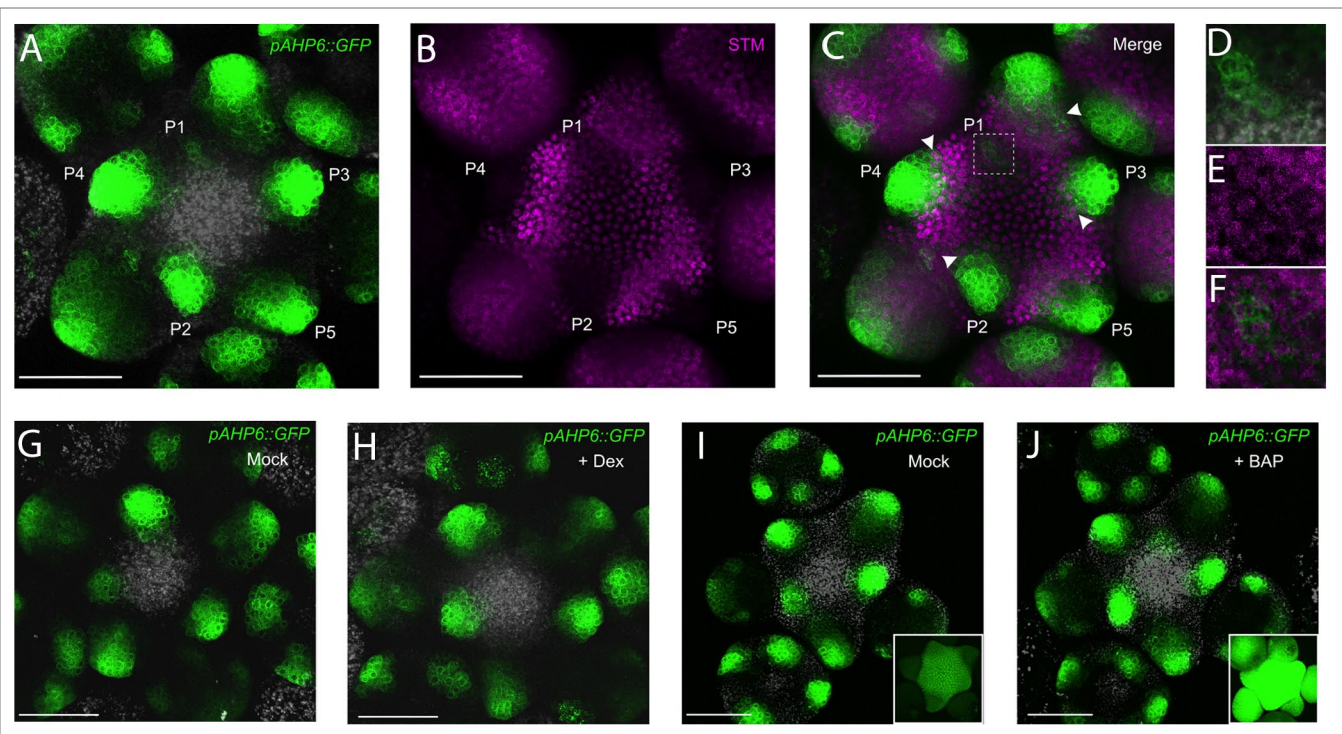


Figure 2. *AHP6* expression in the meristem is regulated independently of STM and cytokinins. (A–F) Co-expression analysis of *pAHP6::GFP* and *pSTM::STM-VENUS* (STM-VENUS). (A–C): Representative meristem ($n = 6$) expressing *pAHP6::GFP* (green) and STM-VENUS (magenta). (D–E) Close-up of P1 (square region shown in C). The channels shown corresponds to (A, D) *pAHP6::GFP* and autofluorescence (gray); (B, E) STM-VENUS; (C, F) *pAHP6::GFP* and STM-VENUS. White arrowheads in (C) indicate organs frontiers where both reporters are co-expressed. (G, H) Representative meristems ($n = 6$) co-expressing *pAHP6::GFP* and *35S::STM-GR*. Meristems were observed one day after a mock (G) or a dexamethasone (Dex) treatment (H) as described.²⁴ (I, J) Representative *pAHP6::GFP* meristems after treatment with a mock (I; $n = 3$) or a CK solution (1 μM BAP) for 48h (J; $n = 4$). Insets: control plants expressing a cytokinin-inducible *pARR5::GFP* reporter treated in parallel with the same solutions ($n = 3$ for each condition). In (G–J) *pAHP6::GFP* is in green and autofluorescence in gray. All images shown were obtained by confocal microscopy and are maximum intensity projections (view from the top). Scale bars: 50 μm .

restriction of the expression to the most central part of lateral organs (Fig. 1B). In addition, it shows that *AHP6* is expressed not only in the L1 layer but also in L2 and L3 layers from the onset of expression in P1 primordia onwards (Fig. 1C–D). Note that we

defined as P1 the first primordium where *AHP6* expression can be detected as we previously demonstrated that the expression of *pAHP6::GFP* is initiated in P1.¹⁰

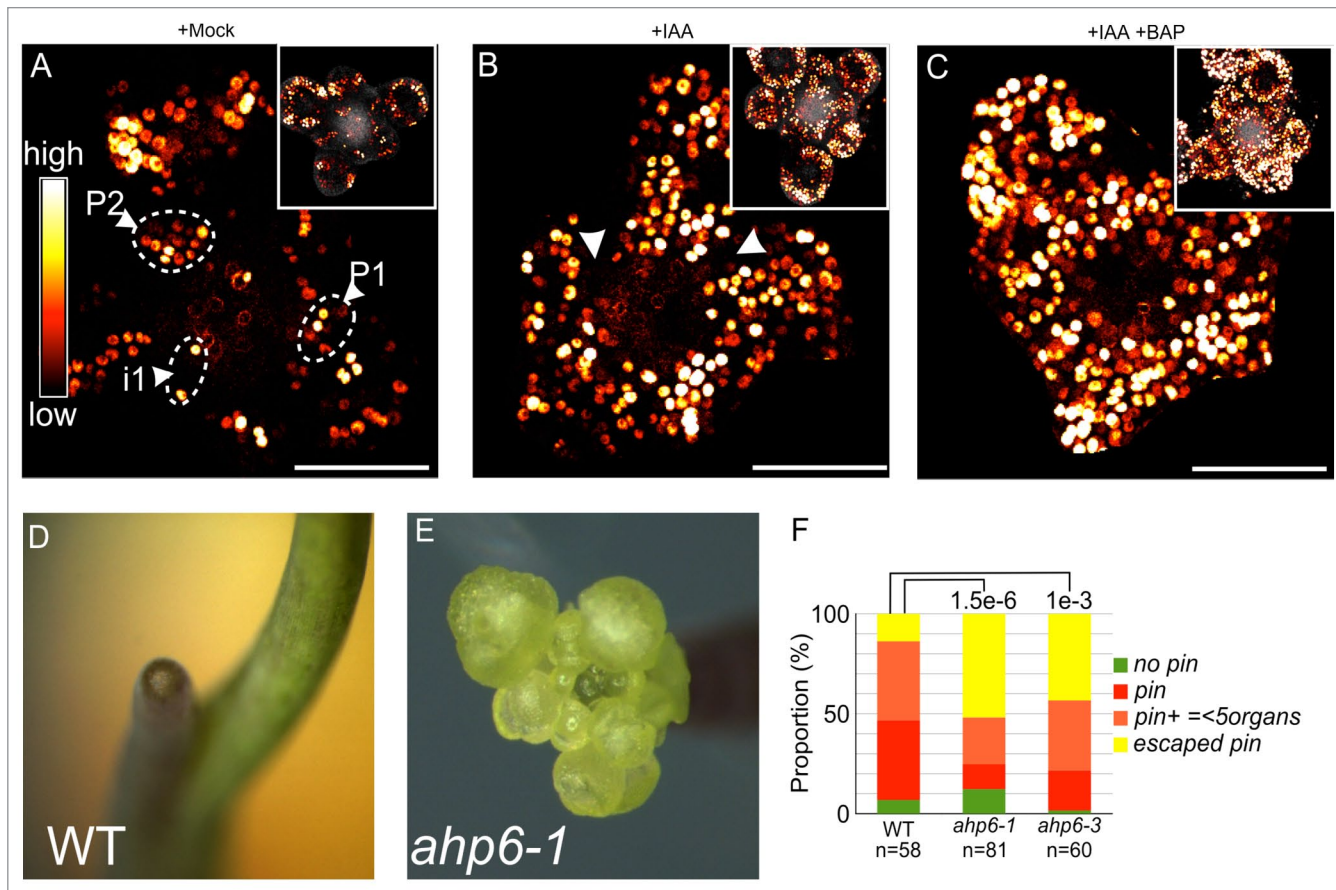


Figure 3. Cytokinin potentiates auxin responses in the SAM. (A–C) Co-treatments with exogenous auxin (IAA) and cytokinin (BAP). Representative images of *DR5::VENUS* expression in meristems with older primordia digitally removed for clarity (insets: original image, with autofluorescence in Gray) are shown after a mock (A; $n = 4$), 1 μM IAA (B; $n = 7$) or 1 μM IAA + 1 μM BAP (C; $n = 7$) treatment. Arrowheads indicate areas in the peripheral zone where there is no *DR5* activation in (B). Note the activation of *DR5::VENUS* throughout the peripheral zone in (C). Images were obtained by confocal microscopy and are maximum intensity projections (view from the top). (D–F) Organ initiation in 4 week-old NPA-grown plants. Plants were grown as previously described.²⁵ (D) Representative WT. (E) Representative *ahp6-1* escaped pin inflorescence. (F) Quantification of the phenotype of inflorescences. p -values: Fischer test. Scale bars: 50 μm .

This raises the question of which factors could control the dynamic expression of *AHP6*. In root tissues, strong evidence suggests that *AHP6* is regulated directly by auxin¹³ and we further supported this regulation by demonstrating that several elements in the *AHP6* promoter can be bound by the Auxin Response Factor 5/MONOPTEROS protein, a key transcription factor involved in auxin signaling at the SAM.¹⁰ However, we could also show that *AHP6* transcription starts with a delay of one plastochrone after the activation of the synthetic auxin-inducible *DR5::VENUS* reporter.^{10,14} We thus investigated other factors that could control *AHP6* expression. While organ initiation is triggered by auxin, it is also accompanied by a downregulation of the *SHOOTMERISTEMLESS* (*STM*) meristem identity gene.^{14,15} We thus tested a scenario where *STM* downregulation during organ initiation could regulate *AHP6* expression, either directly or indirectly through a lowering of CK levels as *STM* activates genes involved in CK biosynthesis.^{16,17} Co-expression analysis of a *STM-VENUS* translational fusion¹⁴ and of the *pAHP6::GFP* transcriptional reporter did not support this hypothesis. First, many cells co-express both reporters in organ

frontiers (Fig. 2A–F). Second, although fading away, *STM-VENUS* protein was still visible in the emerging P1 organ where *AHP6* expression starts (Fig. 2D–F), suggesting that complete *STM* downregulation is unlikely a requisite for activation of *AHP6* transcription. Then, neither an inducible mis-expression of *STM* in the organs using a *35S::STM-GR* line¹⁸ (Fig. 2G, H) nor an exogenous treatment with CK (Fig. 2I, J) could alter the expression of *pAHP6::GFP*. Taken together, these data suggest that *AHP6* is activated independently of *STM* or CK levels in the SAM and that CK are unlikely involved directly in regulating *AHP6* expression in the shoot, as shown also in the root.¹³

While expressed specifically in organ primordia in the SAM, *AHP6* proteins move outside of the transcription domain of the *AHP6* gene and establish CK signaling inhibitory fields.¹⁰ The timing of *AHP6* transcription and the movement of the *AHP6* protein result in a differential in *AHP6* protein concentration between the 2 earliest organ initia that promotes sequential organ initiation (the one with the lowest *AHP6* level/higher cytokinin signaling grows first). However, the mechanism by which a lower *AHP6* concentration facilitates organ development

is still elusive. A plausible hypothesis would be the existence of a synergistic effect of auxin and CK during the earliest steps of organ initiation, as suggested in recent studies.¹⁹ Supporting this possibility, a 12h treatment with both exogenous auxin and CK lead to an expansion of the expression domain of a *DR5::GFP* reporter,²⁰ when compared with auxin alone, and to an activation of auxin signaling in most cells at the periphery of the SAM (Fig. 3A-C). These experiments suggest that CK potentiates the response to auxin at the periphery of the SAM i.e., where organ initiation takes place. Since CK signaling activity is elevated in *ahp6* mutant SAMs,¹⁰ we wondered whether *AHP6* loss-of-function could also potentiate auxin responses when auxin is limiting. Indeed, when treated with the auxin transport inhibitor NPA, *ahp6* mutants had a significantly higher capacity to produce flowers when the inflorescence stem starts elongating (Fig. 3D-F). These data suggest that the increase in CK signaling activity resulting from the *ahp6* mutation can partially rescue the lower organ initiation capacities induced by an inhibition of auxin transport. Although we have not found yet any direct targets responding to the CK signaling increase during organ initiation, we can hypothesize that these targets might act either downstream of or in parallel with auxin during organ initiation.

By analyzing the PIN1 network in *ahp6* mutant,¹⁰ we have shown that such targets if they exist are unlikely to act on auxin transport as in roots^{13,21,22} but could possibly modify auxin concentrations²³ and/or the competence of cells to sense and respond to auxin. These different options clearly deserve more investigation in the future. Altogether, our previously published observations and the data presented here converge to indicate that higher CK signaling could potentiate auxin signaling activity during organ initiation, providing a plausible explanation for how a differential in *AHP6* concentration regulates the timing of organ initiation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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