



Cytokinin Signaling and Its Inhibitor AHP6 Regulate Cell Fate During Vascular Development

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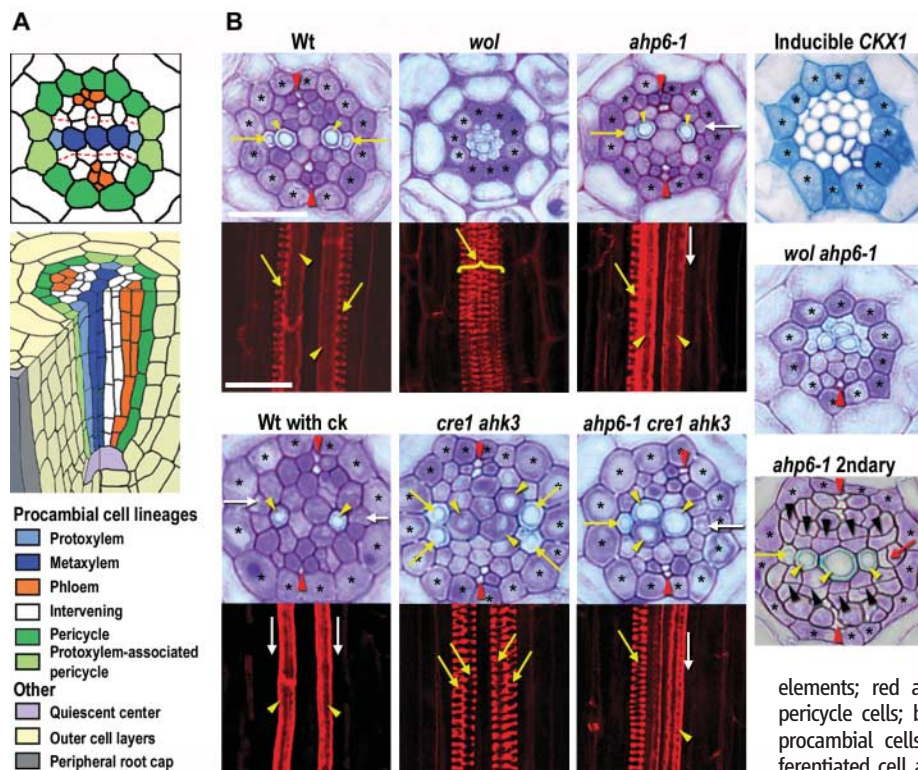


Fig. 1. Cytokinin signaling inhibits protoxylem differentiation. **(A)** Schematic of *Arabidopsis* root vasculature from a mature (transverse section, top) and meristematic procambial regions (three-dimensional representation, bottom). The pattern of periclinal cell divisions of the intervening procambial tissue characteristic to secondary development is shown with dotted red lines. **(B)** Histological analysis of root vasculature stained with toluidine blue (top) and fuchsin red (bottom, where provided) indicates that protoxylem is more abundant in genetic backgrounds with reduced cytokinin signaling, and *ahp6* is able to suppress *wol* and *cre1 ahk3*. Fuchsin staining highlights lignified regions; the helical staining pattern is characteristic of protoxylem, whereas the pitted or reticulate pattern is characteristic of metaxylem. Inducible *CKX1* indicates that *CKX1-YFP* was induced postgermination in a tissue-specific manner with the use of *CRE1prom::XVE/LexAoperator::CKX1-YFP* in wild-type roots; Wt with CK, wild-type seedling germinated with 100 nM benzyladenine (a cytokinin); 2ndary, during secondary development. Yellow arrows, protoxylem elements; white arrows, absence of protoxylem; yellow arrowheads, metaxylem elements; red arrowheads, sieve elements of protophloem; asterisks, pericycle cells; black arrowheads, periclinal cell division in intervening procambial cells; and red arrow, periclinal cell division of an undifferentiated cell at the protoxylem position. Scale bar, 20 μ m.

CRE1 promoter, in an estrogen-inducible fashion (8, 9). After induction, all cell files within the root vascular cylinder differentiated as protoxylem (Fig. 1B and fig. S1). Furthermore, when wild-type seedlings were grown on media containing cytokinin, protoxylem differentiation and green fluorescent protein (GFP) expression of the enhancer trap line J0121, which highlights the protoxylem-associated pericycle cells (10), were severely impaired or even eliminated (Figs. 1B and 2E and figs. S2 and S4B). Together, these results indicate that cytokinin signaling is required to promote and maintain cell identities other than protoxylem, and in the absence of cytokinin signaling, protoxylem is the “default” identity.

To identify molecules regulating cytokinin-mediated vascular morphogenesis, we performed a suppressor screen for the determinate root growth associated with *wol* (6). Two allelic, recessive, extragenic suppressor mutations

were identified, *ahp6-1* and *ahp6-2* (Fig. 2A). Compared with *wol*, *wol ahp6* roots display an increased number of vascular cell files with intervening procambial and phloem cell files present (Fig. 1B and fig. S3, A and C). Neither *ahp6-1* nor *ahp6-2* plants display an obvious seedling phenotype. However, histological studies revealed that protoxylem differentiation occurred sporadically along the root (Fig. 1B and figs. S3D and S4). Quantitative analysis showed that this phenotype is less prominent in *ahp6-2*, indicating that *ahp6-1* is a stronger allele (fig. S4B). Expression of a molecular marker highlighting immature xylem cells (*ZCP4prom::GUS*) (11) coincides with visible xylem differentiation in wild-type and *ahp6-1* roots (Fig. 2D). Furthermore, expression of the *GFP* transcript in the enhancer trap line J0121 (10) is slightly down-regulated in *ahp6-1* roots (Fig. 2E) (12). To further investigate cell fate at the protoxylem position in *ahp6-1* roots, we analyzed the vascular pattern during secondary development when the intervening procambial cell files undergo periclinal cell divisions in wild-type and *ahp6-1* roots (Fig. 1A). Notably, these cells underwent periclinal divisions simultaneously with the neighboring procambial cell files (Fig. 1B and fig. S5). In wild-type roots, this position is invariably differentiated as protoxylem (fig. S4B). In conclusion, the *ahp6* phenotypes in *wol* and in wild-type roots reveal a role for the *AHP6* locus in promoting the specification of protoxylem.

To characterize the *AHP6* locus at the molecular level, we determined through posi-

tional cloning that both *ahp6-1* and *ahp6-2* contain mutations in the gene *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6* (*AHP6*), At1g80100 in the *Arabidopsis* Genome Initiative database (Fig. 3A). A genomic DNA fragment containing the *AHP6* locus complemented *ahp6-1* (Fig. 2A and fig. S3C). Current evidence supports a model of signal transduction in which cytokinins first activate phosphotransfer from adenosine 5'-triphosphate (ATP) to the three CRE-family receptors, and then the phosphoryl group is transferred to the conserved histidine residue of the AHP proteins (AHP1 to AHP5) and finally to response regulators governing physiological responses (13, 14). *AHP6* lacks the conserved histidine residue (fig. S6) (Asn⁸³ in AHP6b), which is required for phosphotransfer and is present in the other AHPs (13, 15). We identified cDNA for two independent transcripts (*AHP6a*-DQ093642 and *AHP6b*-DQ093643), differing in the length of the first exon (Fig. 3, A and B). The mutation in *ahp6-1* resulted in a premature stop codon in the first exon (CAG to TAG, at Gln³⁵), and the mutation in *ahp6-2* (G to A) was located in the first intron, 5 base pairs from the 5' border of the *AHP6b* splice variant. In *ahp6-2* seedlings, only the *AHP6a* transcript is present (Fig. 3B). Given that this represents a weak allele, it is likely that both transcripts are functional. We also identified a transferred DNA (T-DNA) insertion allele (at Ser¹¹¹ of AHP6b), *ahp6-3*, which phenocopies *ahp6-1* (Fig. 3A and figs. S3D and S4B). Because the open reading frame is terminated early in *ahp6-1*, and because only

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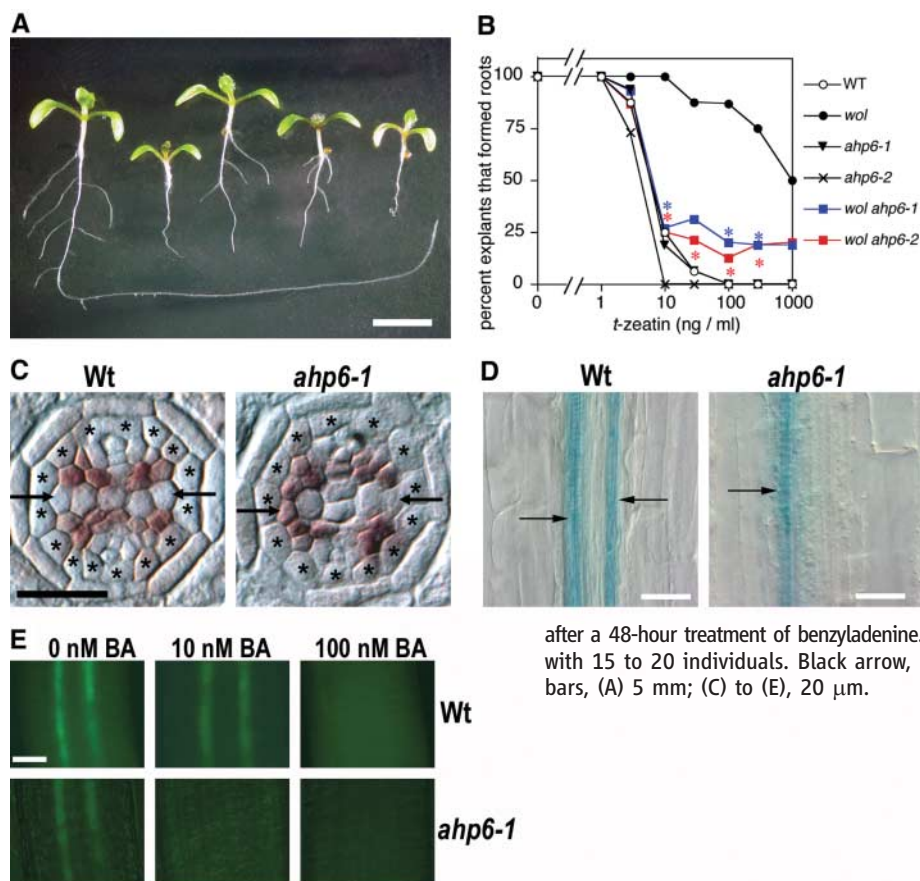


Fig. 2. Phenotypic analysis of *ahp6* mutants. **(A)** From left to right: wild type, *wol*, *wol ahp6-1*, *wol ahp6-2*, and *wol ahp6-1* complemented with *AHP6prom::AHP6*. **(B)** The adventitious root formation assay indicates that *ahp6* mutants confer increased cytokinin responsiveness in *wol* background. Cytokinins normally inhibit the formation of adventitious roots near the cut end of a hypocotyl. The percent of explants producing adventitious roots when grown on media with increasing concentrations of *t*-zeatin (a cytokinin) is presented. Significant differences existed between *wol* and *wol ahp6-1* (blue asterisk) or between *wol* and *wol ahp6-2* (red asterisk), according to the Fisher's exact probability test ($P < 0.05$). **(C)** In situ hybridization with an antisense probe reveals *ARR15* expression at the protoxylem position in *ahp6-1*. A sense probe showed no detectable signal (12). **(D)** Loss of protoxylem differentiation is accompanied with loss of *ZCP4prom::GUS* expression in *ahp6-1*. **(E)** Expression of J0121, a GFP marker line with expression specifically in protoxylem-associated pericycle cells (Fig. 1A and fig. S2) is down-regulated after a 48-hour treatment of benzyladenine. Panels shown are representative of analyses performed with 15 to 20 individuals. Black arrow, protoxylem position; asterisks, pericycle cells. Scale bars, (A) 5 mm; (C) to (E), 20 μ m.

reduced level of *AHP6* message was detected upstream and no message was detected downstream of the T-DNA insertion in *ahp6-3*, these are likely to represent null alleles (Fig. 3B).

To investigate the biochemical nature of AHP6 function, we based an in vitro assay around the already well-characterized phosphotransfer activity of the yeast SLN1 histidine kinase (16). The histidine kinase domain (SLN1HisK) and the receiver domain (SLN1Rec) were prepared as separate peptides, enabling examination of interdomain phosphotransfer (16). In this assay, the phosphoryl group is transferred from 32 P-labeled ATP to a conserved His residue of SLN1HisK, then to a conserved Asp residue within the SLN1Rec, and subsequently to a conserved His residue within an HPT protein. Using this in vitro phosphotransfer system, we demonstrated that AHP1 to AHP3, AHP5, and a mutant version of AHP6b (Asn⁸³ replaced with the conserved His required for phosphotransfer activity) were able to accept a phosphoryl group from SLN1 (Fig. 3C and fig. S7). In contrast, native AHP6 was unable to accept a phosphoryl group. These results suggest that AHP6 does not function as a phosphotransfer protein. Although AHP6 does not appear to have phosphotransfer activity, it was able to inhibit phosphotransfer from the SLN1HisK to SLN1Rec. We also examined the effect of AHP6 on phosphotransfer from AHP1 to an *Arabidopsis* response regulator, ARR1 (Fig.

3C). When phosphorylated AHP1 and ARR1 were co-incubated, phosphotransfer occurred from phosphorylated AHP1 to ARR1, and this was inhibited by AHP6 (Fig. 3C). Taken together, the evidence suggests that AHP6 acts as an inhibitor of cytokinin signaling by interacting with the phosphorelay machinery, potentially at multiple steps. Database searches using the AHP6 sequence reveal homologs in other plant species (fig. S6), indicating that the negative regulation of the His-to-Asp phosphorelay system by pseudo-HPTs may be widespread in the plant kingdom.

Using in situ RNA hybridization, and the *AHP6prom::GUS* and *AHP6prom::GFP* reporter constructs, we observed expression of *AHP6* in developing protoxylem and associated pericycle cell files, revealing a spatially specific expression pattern consistent with the mutant phenotype (Figs. 3, D and E, and 4A; fig. S8A). GFP signal is ubiquitous in the cotyledons of heart-shaped embryos and is gradually restricted to two poles by the late heart stage/early torpedo stage of embryogenesis (Fig. 3F and fig. S8C). By the mature stage, GFP expression is refined to the cotyledon apices and two poles in the embryonic root which later form protoxylem. *AHP6* is also expressed in the shoot apex and young leaves (fig. S8A). The existence of the AHP6 protein was confirmed by complementing *ahp6-1* with *AHP6prom::AHP6-GFP* and observing GFP signal (fig. S8B).

To examine the role of AHP6 in cytokinin signaling, cytokinin responsiveness conferred by *ahp6* was measured with the use of the adventitious root formation assay (17). The impaired cytokinin response in *wol* was partially restored in *wol ahp6* (Fig. 2B, consistent with the negative role of AHP6 on cytokinin signaling. We also demonstrated that *ahp6-1* could not suppress the cytokinin-insensitive *cre1 ahk2 ahk3* phenotype, indicating that the suppressor phenotype in the *wol* background is due to residual cytokinin signaling (17) (Fig. 2B and fig. S3C). To study the status of cytokinin signaling in *ahp6-1* at the position normally occupied by protoxylem files, we performed in situ RNA hybridization, with the cytokinin primary response gene *ARR15* (18) as a probe (Fig. 2C). Whereas *ARR15* expression in wild-type roots was restricted to the intervening procambial cells adjacent to the xylem axis ($n = 4$), the expression domain in four out of five *ahp6-1* roots expanded to include the protoxylem position. These results indicate that AHP6 functions to facilitate protoxylem specification by down-regulating cytokinin signaling in a spatially specific manner. To confirm this, we examined whether the cytokinin-degrading enzyme CKX2 (8) could substitute AHP6 when ectopically expressed in the protoxylem. *AHP6prom::CKX2* suppressed the *ahp6-1* phenotype (fig. S3D). Furthermore, when *ahp6-1* plants were germinated on media containing

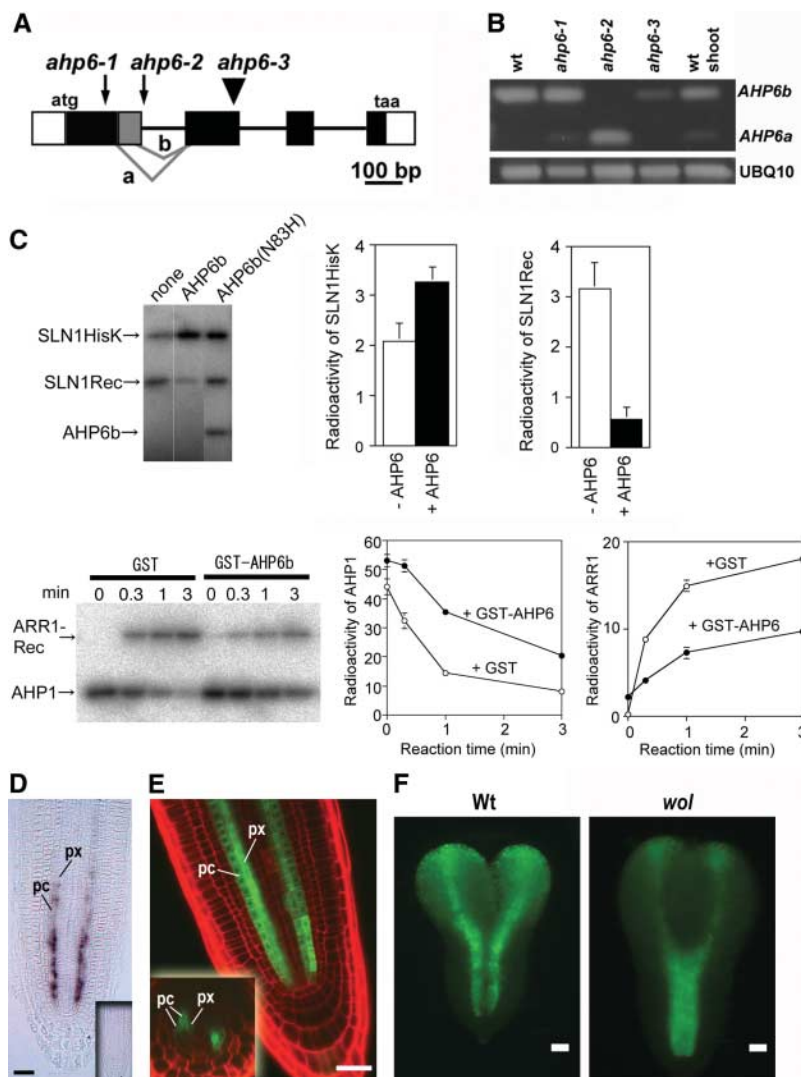


Fig. 3. Molecular characterization of the *AHP6* locus. **(A)** Structure of the *AHP6* gene. White boxes, untranslated regions; gray lines, the alternative splicing between *AHP6a* (a) and *AHP6b* (b) variants. Locations of the *ahp6* mutations are shown by arrows (single nucleotide polymorphisms) or arrowheads (T-DNA insertion, stock number SALK_058085). **(B)** Analysis of both *AHP6* transcripts by reverse transcription polymerase chain reaction (RT-PCR). *UBQ10* is an internal control. The smaller transcript only becomes apparent in wild-type and *ahp6-1* roots after a greater number of cycles (12). **(C)** *AHP6* is a pseudo-HPt because of the lack of the conserved His. Upper panels demonstrate that *AHP6* inhibits phosphorelay from the SLN1 histidine kinase domain (SLN1HisK) to the SLN1 receiver domain (SLN1Rec). Lower panels indicate that *AHP6* inhibits phosphorelay from phosphorylated *AHP1* to *ARR1*. Radioactivity is measured in relative units and each value was quantified from four independent reactions (standard deviation indicated). **(D and E)** *AHP6* is expressed in protoxylem and adjacent pericycle cells as shown by in situ hybridization and with the *AHP6prom::GFP* reporter construct. The sense *AHP6* probe did not reveal any signal (inset). px, protoxylem; pc, pericycle. **(F)** *AHP6prom::GFP* expression during embryogenesis in early torpedo stage of wild-type and *wol* embryos. Scale bars, 20 μ m.

cytokinin, the loss-of-protoxylem phenotype was enhanced (figs. S2A and S4B), supporting the role of *AHP6* as a negative regulator of cytokinin signaling and implicating additional unidentified factors.

cre1 ahk3 shows intermediate cytokinin responsiveness (17). Histological studies revealed that 91% of *cre1 ahk3* roots ($n = 35$; wild type 0%, $n = 50$) had one or more extra protoxylem files, even though the number of procambial cell files remained normal (Fig. 1B) (*cre1 ahk3*: 32.6 ± 2.0 cells, $n = 17$; wild-type: 31.8 ± 2.2 cells, $n = 20$; errors are SD). Similar phenotypes are observed in weak *CRE1prom::CKX2* lines (fig. S3B). To investigate whether the ectopic protoxylem files in these genotypes are due to an increase in the spatial domain of *AHP6* action, we first studied the expression of *AHP6* in *cre1 ahk3*. Consistent with this, the *AHP6* expression domain is slightly broader than in wild-type roots, because it is typically found in two (as opposed to one) protoxylem cell files and in three (as opposed to two) adjacent pericycle cell files (88%; $n = 17$) (Fig. 4A). This expanded domain of *AHP6*

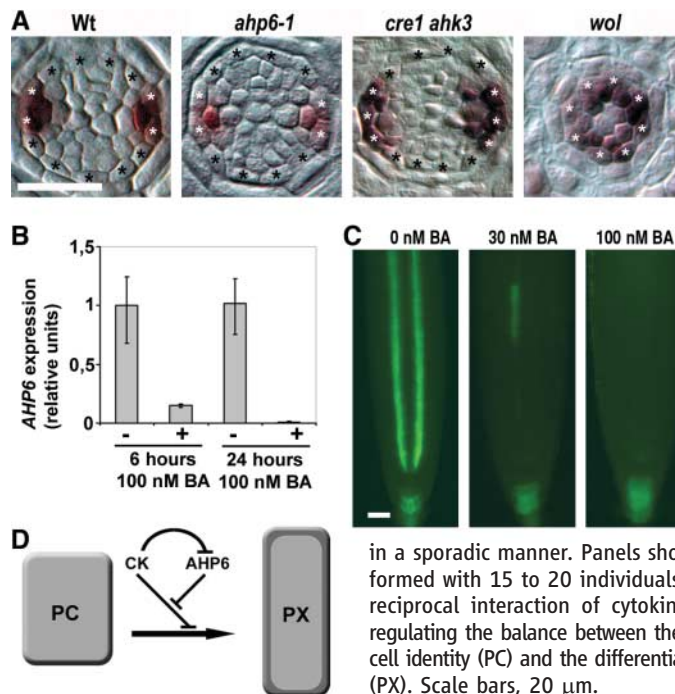


Fig. 4. Cytokinin negatively regulates *AHP6* expression. **(A)** The expression domain of *AHP6* broadens in genetic backgrounds with decreased cytokinin signaling. White asterisks, pericycle cells expressing *AHP6*; black asterisks, other pericycle cells. **(B)** *AHP6* expression is down-regulated following cytokinin treatments as shown by real-time quantitative PCR. Error bars show means $\pm$ SD. **(C)** With intermediate cytokinin concentrations *AHP6* is expressed

in a sporadic manner. Panels shown represent analyses performed with 15 to 20 individuals. **(D)** A model showing the reciprocal interaction of cytokinin signaling and *AHP6* in regulating the balance between the maintenance of procambial cell identity (PC) and the differentiation of protoxylem elements (PX). Scale bars, 20 μ m.

expression is responsible for the *cre1 ahk3* phenotype, because loss of AHP6 function in *ahp6-1 cre1 ahk3* was able to suppress the ectopic protoxylem (91%; $n = 45$) (Fig. 1B). In both *wol* and *cre1 ahk2 ahk3*, the AHP6 expression pattern expands throughout the vascular bundle (Fig. 4A) (12). The expanded expression pattern in *wol* is already evident by the early torpedo stage of embryogenesis when it occupies one broad domain within the embryonic root as opposed to two narrow strands in wild-type (Fig. 3F and fig. S8C). This indicates that cytokinin signaling specifies the spatial domain of AHP6 expression upstream of protoxylem differentiation, which occurs after embryogenesis. Next, we examined the effect of exogenous cytokinins on AHP6 expression. We observed down-regulation of the AHP6 transcript after a 6-hour treatment with cytokinins (Fig. 4B). Likewise, the level of fluorescence in the *AHP6prom::GFP* line was reduced by cytokinins, and the reduction occurred at lower levels of cytokinin in *ahp6-1* than in wild-type roots. (Fig. 4C and fig. S9). In the absence of applied cytokinin, the levels of AHP6 transcript in *ahp6-1* were slightly lower than in the wild type (12).

We report a regulatory circuit between cytokinin signaling and its newly identified inhibitor, AHP6, which specifies the meristematic versus differentiated nature of procambial cell files (Fig. 4D). In this sense, our results are consistent with requirement of cytokinins for transdifferentiation of xylem observed in *Zinnia* mesophyll cell culture (5, 19). AHP6 can be considered the founding member of a new “pseudo” subclass of HPT proteins within the wider group present in prokaryotes and eukaryotes.

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Supporting Online Material

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References

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Fishing, Trophic Cascades, and the Process of Grazing on Coral Reefs

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Since the mass mortality of the urchin *Diadema antillarum* in 1983, parrotfishes have become the dominant grazer on Caribbean reefs. The grazing capacity of these fishes could be impaired if marine reserves achieve their long-term goal of restoring large consumers, several of which prey on parrotfishes. Here we compare the negative impacts of enhanced predation with the positive impacts of reduced fishing mortality on parrotfishes inside reserves. Because large-bodied parrotfishes escape the risk of predation from a large piscivore (the Nassau grouper), the predation effect reduced grazing by only 4 to 8%. This impact was overwhelmed by the increase in density of large parrotfishes, resulting in a net doubling of grazing. Increased grazing caused a fourfold reduction in the cover of macroalgae, which, because they are the principal competitors of corals, highlights the potential importance of reserves for coral reef resilience.

Caribbean reefs were acutely disturbed in 1983 when the herbivorous urchin *Diadema antillarum* experienced mass disease-induced mortality (1). In the absence of *Diadema*, parrotfishes (Scaridae) have become the dominant grazer on most Caribbean reefs (2). Grazing performs several critical functions in this ecosystem, including the conversion of primary production to fish-based trophic pathways (3), the provision of suitable settlement substrata for new corals (4), and the mediation of competition between corals and macroalgae (5). Parrotfishes are exploited in many parts of the region, and several studies

have reported increases in their density when fishing intensity was reduced inside reserves (6, 7). However, although reserves may benefit parrotfish populations in the short term, these impacts may be reversed on longer time scales. Most reserves aim to restore the biomass of large piscivores (8) such as the Nassau grouper (*Epinephelus striatus*), but this might eventually cause cascading top-down limitation on the biomass of their prey (9, 10), which includes parrotfishes (11, 12). Therefore, with the continued scarcity of *Diadema* (13), the realization of a successful reserve may impair levels of grazing and its associated ecosystem functions.

Few reserves are either large, old, or effective enough to have had a significant impact on large predators (6, 14). An exception is the Exuma Cays Land and Sea Park (ECLSP), which lies near the center of the Bahamas archipelago. The ECLSP is large (456 km²) and was established in 1959. A ban on fishing has been enforced there since 1986, and the current biomass of *E. striatus* is seven times greater in the ECLSP than that observed in three other regions of the archipelago (15). The ECLSP is, therefore, one of the few places in the Caribbean where the long-term impacts of reserves can be investigated (16). We sampled four island systems at hierarchical scales and contrasted the results in and around the reserve with those found at equivalent spatial scales in systems that lacked reserves. Specifically, fish communities of *Montastraea* reefs were sampled at scales of sites (hundreds of

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