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Plant development regulated by cytokinin sinks

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Morphogenetic signals control the patterning of multicellular organisms. Cytokinins are mobile signals that are perceived by subsets of plant cells. We found that the responses to cytokinin signaling during *Arabidopsis* development are constrained by the transporter PURINE PERMEASE 14 (PUP14). In our experiments, the expression of *PUP14* was inversely correlated to the cytokinin signaling readout. Loss of *PUP14* function allowed ectopic cytokinin signaling accompanied by aberrant morphogenesis in embryos, roots, and the shoot apical meristem. PUP14 protein localized to the plasma membrane and imported bioactive cytokinins, thus depleting apoplastic cytokinin pools and inhibiting perception by plasma membrane–localized cytokinin sensors to create a sink for active ligands. We propose that the spatiotemporal cytokinin sink patterns established by PUP14 determine the cytokinin signaling landscape that shapes the morphogenesis of land plants.

Multicellular organisms depend on differential cell functions controlled by signaling systems. The precise determination of signal-perceiving cells is important to ensure normal development. Cytokinins are chemical plant signals that control morphogenesis, integrate environmental cues, and mediate biotic interactions (*1*–*3*). Cytokinins are perceived by largely redundantly acting hybrid kinases that activate a phosphorelay circuitry to stimulate the transcription of target genes. The spatiotemporal precision of the signaling patterns in different plant organs (*4*, *5*) raises the question of how control is established.

Each step involved in eliciting a signaling response, including ligand biosynthesis and expression of signaling components, could be differentially regulated and contribute to defining the signaling patterns to a greater or lesser degree. To identify limiting and regulated steps, we used *Arabidopsis* heart-stage embryos as a model in which the cytokinin response marks the provascular tissue (Fig. 1A). First, to evaluate whether bioactive cytokinins are limited, we incubated embryos for 16 hours with the degradation-insensitive cytokinin benzyladenine (BA) (*6*). This caused a stereotypic expansion of the synthetic cytokinin reporter *TCSn::GFP* (*Two Component signaling Sensor new::green fluorescent protein*) (*5*) (Fig. 1A), thereby confirming that cytokinin levels are controlled (*7*). However, excess cytokinins did not induce *TCSn::GFP* expression in the prospective cotyledons, despite the transcription of the cognate cytokinin receptor *ARABIDOPSIS HISTIDINE KINASE 4* (*AHK4*) in these domains (Fig. 1A); this finding suggests that failure to turn on signaling cannot be explained by missing receptors. To test whether signaling downstream of receptors is functional, we expressed *CYTOKININ INDEPENDENT 1* (*CKI1*). *CKI1* encodes a hybrid kinase with cytokinin-independent constitutive activity (*3*). Its short-term expression caused ubiquitous *TCSn::GFP* activation (Fig. 1A). Together, these results suggest that cells of the prospective cotyledons fail to activate cytokinin signaling despite a functional signaling system, and even upon addition of abundant active ligand.

We hypothesized that productive ligand-receptor interactions within organs could depend on cytokinin transporters that guide differential cellular localization of cytokinins. To test whether members of the *Arabidopsis* PURINE PERMEASE (PUP) family of transmembrane proteins implicated in cytokinin translocation (*8*) control the spatiotemporal landscape of cytokinin signaling, we first established a transcription profile of all family members, based on our own analysis and published transcriptome data (*9*–*11*) (fig. S1). *PUP14* expression was unique in that it prevailed in all organs and stages analyzed, including embryos. To determine the *PUP14* expression pattern, we analyzed *PUP14::PUP14-GFP* transgenic plants. In heart-stage embryos, PUP14-GFP localized to cells that failed to respond to cytokinins, including cells of the prospective cotyledons (Fig. 1B). We confirmed this pattern by mRNA in situ hybridization with a *PUP14* antisense probe (Fig. 1B and fig. S2A). The exclusive nature of *PUP14* expression and the cytokinin signaling pattern are compatible with an inhibitory function of PUP14 in the cytokinin response.

To eliminate *PUP14* function during defined time windows, thereby avoiding secondary effects and potential lethality issues, we constructed an ethanol-inducible artificial microRNA (*amiR*) (*12*) targeting *PUP14* (*35S>ALC>amiRPUP14*). Upon induction of the *amiRPUP14* transgene, *PUP14* mRNA and PUP14-GFP levels were reduced within 24 hours (Fig. 1D and fig. S2, B and C). Inducing *amiRPUP14* expression for 16 hours caused widespread ectopic cytokinin signaling in the embryo (Fig. 1B), even in cells of the prospective cotyledons that are nonresponsive to treatments with exogenous cytokinins (Fig. 1A); this result supports a role of PUP14 in confining the cytokinin response. The same treatment regime did not affect the auxin response (fig. S2D), indicating that PUP14 acts specifically on cytokinin signaling. After 2 days of *amiRPUP14* induction, morphological defects in the prospective cotyledons and the nascent root meristem became apparent (Fig. 1C), consistent with the ectopic cytokinin responses in these domains. The *amiRPUP14*-induced phenotypes were complemented by an *amiRPUP14*-resistant transgene (*PUP14**) encompassing the *PUP14* locus (fig. S2, E to G), which suggests that the inducible *amiRPUP14* acts specifically. In addition, an inducible *amiR* against nonexpressed *PUP19* and *PUP20* (fig. S1) did not cause obvious phenotypes (fig. S2, E to G). Finally, a T-DNA insertion to the *PUP14* promoter causing a reduction in *PUP14* mRNA levels showed qualitatively similar but weaker phenotypes in embryos, seedlings, and adult shoots relative to *amiRPUP14*-induced

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phenotypes (Fig. 1C and fig. S3, A to F), whereas a second T-DNA insertion downstream of the *PUP14* locus did not affect *PUP14* mRNA levels (fig. S3B) and produced no apparent phenotypes. Together, these results validate the use of the *amiRPUP14* line to study *PUP14* function.

Because loss of *PUP14* produces ectopic cytokinin responses, we expected the overexpression of *PUP14* to reduce cytokinin output. Although we were unable to recover plants transgenic for *35S::PUP14*, inducible *PUP14* expression in the embryo reduced the endogenous cytokinin response

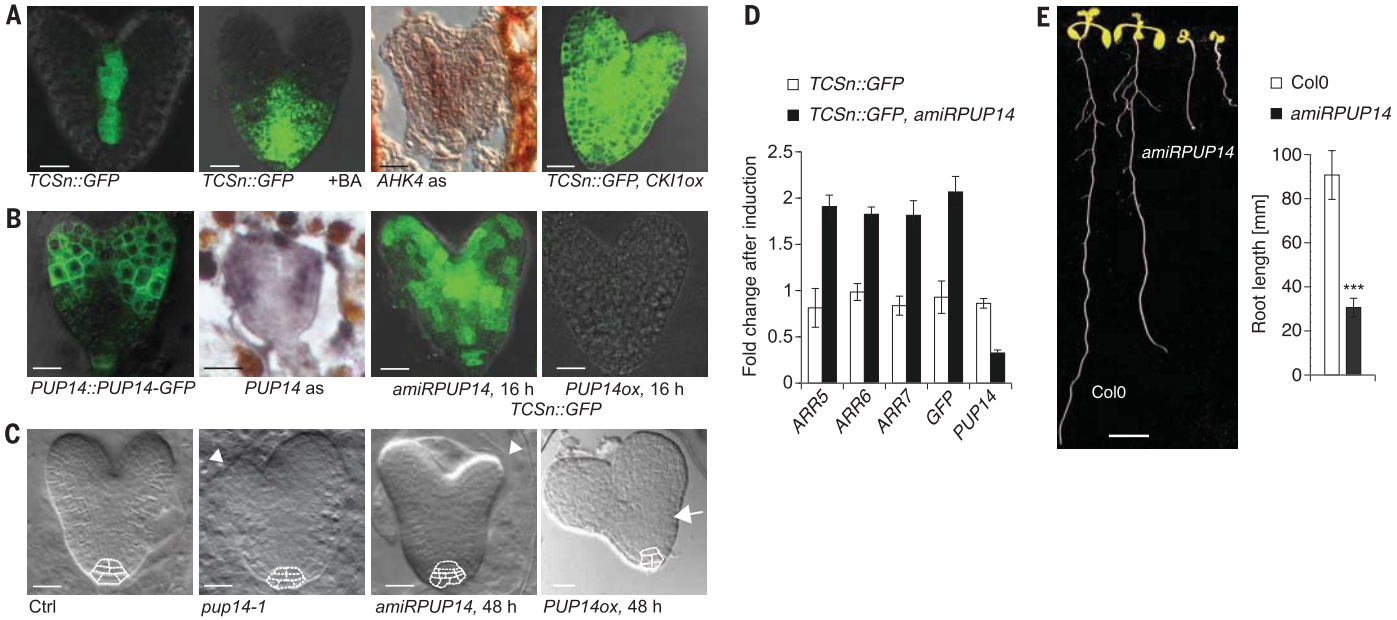
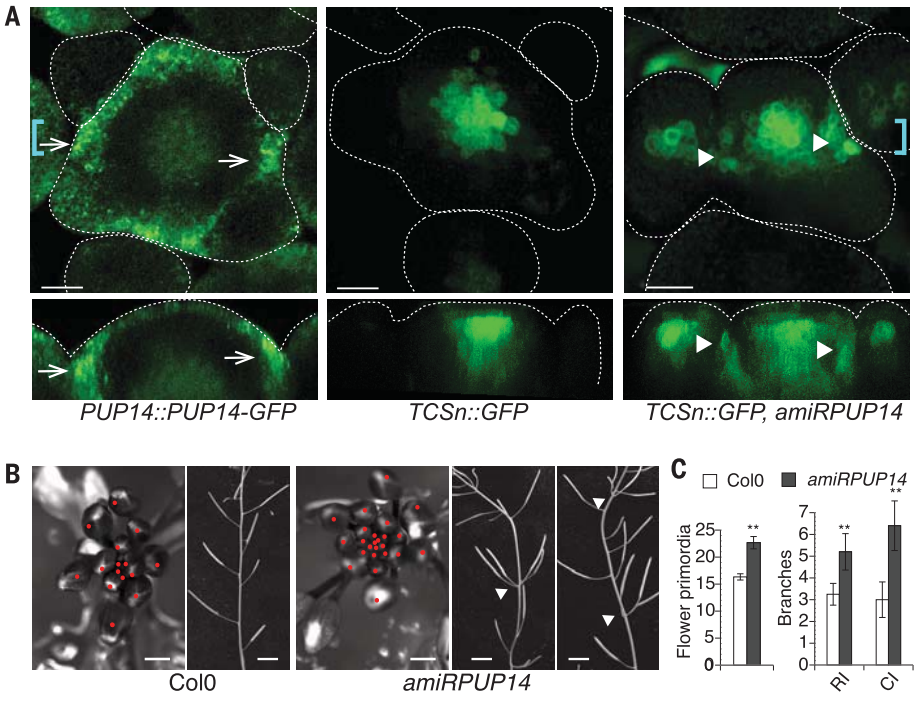


Fig. 1. PUP14 function in embryo and seedlings. (**A**) Heart-stage embryos subjected to 16 hours of mock treatment, 16 hours of treatment with 10 μM BA, 16 hours of hybridization with *AHK4* antisense (as) RNA, and 16 hours of *CKI1* expression from a *35S>ALC>CKI1* transgene (*5*) (*CKI1ox*). GFP reporter transgenes are indicated. (**B**) Left panels: *PUP14* expression detected by reporter gene and by *PUP14* as RNA probe. Right panels: *amiRPUP14* and *PUP14 35S>ALC>PUP14* transgene (*PUP14ox*) inductions. Ectopic *TCSn::GFP* in 85% of embryos, $n = 53$; loss of *TCSn::GFP* in 45% of embryos, $n = 11$. (**C**) Morphological defects 48 hours after transgene inductions and in *pup14-1*. For *amiRPUP14*, 47%, $n = 96$; for *pup14-1*, 37%, $n = 237$; for *PUP14* overexpression from *PUP14ox*, 50%, $n = 10$. Arrowheads point to affected cotyledons, arrow indicates shortened embryo root, and cell boundaries in root meristem are outlined with white dotted lines. (**D**) Relative changes of type-A *ARR5*, *ARR6*, and *ARR7* and *TCSn::GFP* [as a group, significantly different: $P < 0.001$ (unpaired *t* test); *TCSn::GFP*, $n = 4$; *TCSn::GFP, amiRPUP14*, $n = 4$] and *PUP14* mRNA levels [significantly different: $P < 0.001$ (unpaired *t* test); *TCSn::GFP*, $n = 4$; *TCSn::GFP, amiRPUP14*, $n = 7$] after 16 hours of *amiRPUP14* induction in 7-day-old seedlings of indicated genotype, assessed by quantitative real-time polymerase chain reaction; error bars represent SEM. (**E**) *amiRPUP14* and *Col0* seedlings after 7 days on ethanol-containing medium; growth retardation of seedling roots, $n = 10$. Scale bars, 20 μm [(A) and (B)], 1 cm (E). ***$P < 0.001$ (unpaired *t* test).

Fig. 2. PUP14 confines the cytokinin response in the SAM. (**A**) Floral SAM. Longitudinal optical sections (lower panels) are in cyan-colored brackets; dotted lines mark organ boundaries. Transgenes are indicated. Arrows indicate peak *PUP14-GFP* levels at organ-organ boundaries. Arrowheads indicate ectopic *TCSn::GFP* after *amiRPUP14* induction. (**B** and **C**) Comparisons of ethanol-treated *Col0* and *amiRPUP14* phenotypes. (B) Inflorescences and inflorescence stems. Red dots denote flower primordia; arrowheads indicate perturbations. (C) Numbers of flower primordia at stages 6 to 12 on the main apex (*30*), $n = 6$; numbers of primary rosette (RI) and primary cauline branching (CI), $n = 6$. Data are means ± SD. **$P < 0.01$ (unpaired *t* test). Scale bars, 20 μm (A), 1 mm [flower primordia in (B)], 1 cm [stems in (B)].



after 16 hours (Fig. 1B); after 48 hours of transgene induction, morphological defects in the embryo root were apparent (Fig. 1C). Similar to the embryo, we found *PUP14* expression in the meristematic region of the seedling's main root (fig. S4A), in the lateral root primordia (LRP) (fig. S4B), and in ovules and seeds (fig. S6, A and B), exhibiting patterns complementary to those of cytokinin signaling. As in the embryo, short-term *amiRPUP14* induction resulted in ectopic cytokinin signaling in the seedling root, particularly in the meristematic region of the root tip (fig. S4A) and in the LRP (fig. S4B). Accordingly, transcription of the immediate-early cytokinin target genes type-A *ARABIDOPSIS RESPONSE REGULATOR 5* (*ARR5*), *ARR6*, and *ARR7* (*13*) was induced in seedlings (Fig. 1D).

Continuous induction of *amiRPUP14* led to growth retardation of the seedling root and shoot, with suppression of lateral roots (*14*, *15*) (Fig. 1E); this finding suggests that the root and shoot meristem activities were both affected when cytokinin signaling patterns were perturbed by the inducible *amiRPUP14*. In contrast to the embryo (Fig. 1, B and C), *TCSn::GFP* expression remained unchanged after 24 hours of inducing ectopic *PUP14* in the seedling root (not shown). We visualized PUP14-GFP expressed from the inducible *35S>ALC>PUP14-GFP* transgene and found that, relative to *35S>ALC>GFP*, cells of the root apex and vasculature failed to express PUP14-GFP (fig. S5, A to C). This result suggests that ectopic PUP14 is not tolerated, which can explain the absent effects on *TCSn::GFP* in the seedling root apex and the lethality of the *35S::PUP14* transgene. In addition, relative to endogenous PUP14-GFP, the subcellular localization of ectopic PUP14-GFP was disturbed (fig. S5, B and C), which may impair its normal function.

In the shoot, cytokinin controls the homeostasis of the shoot apical meristem (SAM) (*16*), where increased cytokinin causes a more active meristem with more primordia (*17*). As observed in other developmental contexts (Fig. 1, A and B, fig. S4, and fig. S6, A and B), *PUP14* expression in the SAM was inversely correlated with cytokinin signaling output assayed by *TCSn::GFP* (Fig. 2A). Inducing *amiRPUP14* expression in adult plants that were allowed to complete embryogenesis and the early vegetative phase of development undisturbed caused ectopic cytokinin output in the SAM, which was accompanied by a 37% increase in number of primordia, a 94% increase in shoot branching relative to ethanol-treated wild-type plants, and disturbed phyllotaxis (Fig. 2, B and C). Similar phenotypes have been observed in plants mutant for *CYTOKININ OXIDASE 3* (*CKX3*) and *CKX5* (*17*), *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6* (*18*), and *ARR3* to *ARR9* (*19*), which also display ectopic cytokinin activities. Thus, PUP14 functions to limit the cytokinin response domains throughout development to support morphogenesis.

Next, we addressed the cellular function of PUP14. PUP14-GFP fusion proteins, supported by brefeldin A-sensitive vesicular transport, localized to the plasma membrane (fig. S6C). To test PUP14's cytokinin transport capacity, we conducted uptake experiments using labeled transzeatin (tZ), an abundant natural cytokinin (*20*). Transient expression of *PUP14* in mesophyll protoplasts or tobacco microsomes stimulated the uptake of labeled tZ (Fig. 3, A and B). Transport activity in PUP14 was higher than in PUP1 and was dependent on the presence of adenosine triphosphate (*8*) (Fig. 3B). Uptake was inhibited by unlabeled tZ, by the common natural cytokinin isopentenyl adenine (iP), by the aromatic cytokinin BA, and also by adenine, but not by tZ riboside, the major cytokinin transport form (*21*), nor by auxin (IAA) or allantoin, which is an unrelated substrate (Fig. 3, C and D). Energy-dependent cytokinin uptake into a microsomal cell-free system excludes the possibility that uptake is dependent on cytoplasmic metabolization. Conversely, relative to control seedlings, seedlings with decreased *PUP14* levels exhibited a reduced uptake rate for exogenously added tZ (Fig. 3E).

Our data show that plasma membrane–localized PUP14 imports bioactive cytokinins to the cytosol, implying that PUP14 activity depletes ligands from the apoplast, which leads to a suppression of the cytokinin response. In this scenario, extracellular cytokinins binding to the sensing domains of plasma membrane–localized receptors (*22*, *23*) are important to initiate the signaling response, whereas the cytoplasm represents a sink for bioactive ligands. To test this hypothesis, we devised experiments that compared the effects of differentially targeted cytokinin-degrading enzymes on the cytokinin signaling response. Mesophyll protoplast cells respond to as little as 100 pM exogenously added tZ by activating cytokinin signaling (*4*), which suggests that they depend on exogenous cytokinins and thus constitute a suitable model to study cytokinin perception independent of production. Transient transfection of PUP14 localizing to the plasma membrane (fig. S7A) caused a reduction of cytokinin-dependent *TCS::LUCIFERASE* (*LUC*) activity (Fig. 3F), recapitulating the phenotypes from *PUP14* overexpression in the embryo (Fig. 1B). Transient expression of wild-type CKX2 that is targeted for secretion to the apoplast (*20*) (fig. S7B) attenuated the cytokinin response triggered by tZ but not by the degradation-resistant BA. To target CKX2 to the exofacial side of the plasma membrane, we added a glycosylphosphatidylinositol (GPI) anchor (*24*), resulting in CKX2-GPI (fig. S7C), which also caused a reduction in the cytokinin response. In contrast, a variant of CKX2 that lacks the N-terminal signal

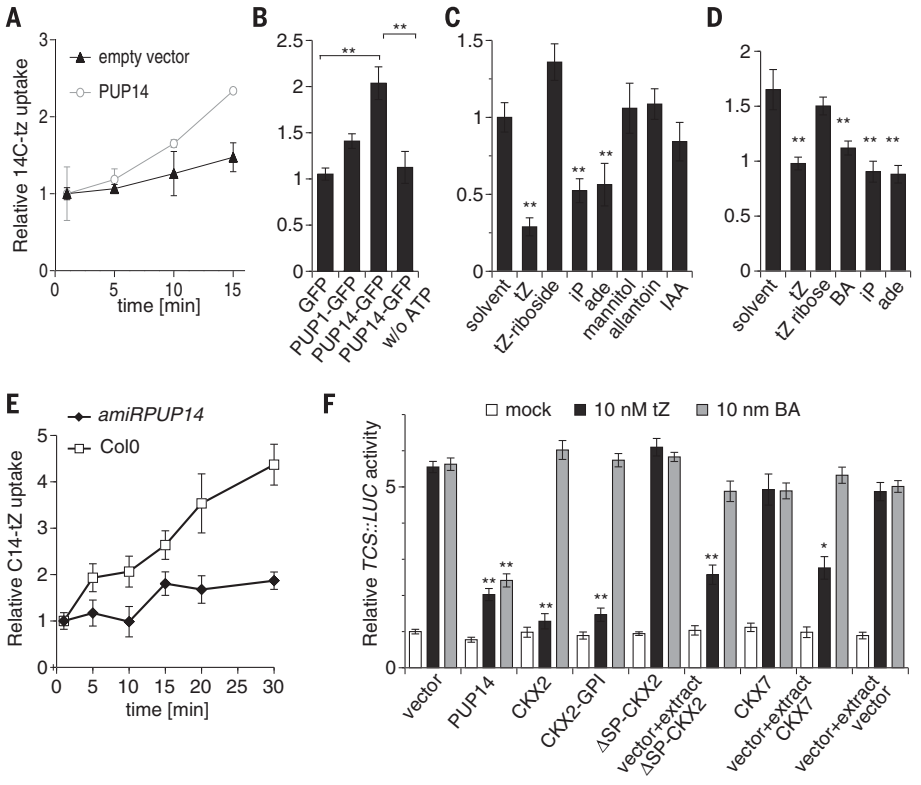


Fig. 3. PUP14 cellular function. (**A** to **E**) PUP14 transport assays, with relative ^{14}C-tZ uptake rates on *y* axis. (A) *PUP14*-transfected mesophyll protoplasts. (B) Microsomes derived from *GFP-*, *PUP1-*, or *PUP14*-transfected *Nicotiana benthamiana*. (C) Competition by indicated substances in *PUP14*-transfected protoplasts. (D) Competition in microsomes of *35S::PUP14*-transfected *N. benthamiana.* (E) *amiRPUP14* versus *Col0* seedlings. (**F**) Relative *TCSn::LUC* induction in protoplasts treated with 10 nM tZ or BA, cotransfected with effector genes, or with addition of cell extracts as indicated, normalized to empty vector control. Data are means ± SD [(A) and (E)] or means ± SEM [(B), (C), (D), and (F)]. $^{**}P < 0.01$, $^{*}P < 0.05$ (analysis of variance with Tukey HSD post hoc test).

peptide (ΔSP-CKX2) and colocalizes with a cytoplasmic marker (fig. S7D) did not affect the cytokinin response, and neither did CKX7, which also localizes to the cytoplasm (*25*) (fig. S7E). Crude cell extracts obtained from ΔSP-CKX2–transfected or CKX7-transfected cells added to the medium reduced the response triggered by tZ, indicating that these proteins are active. These data suggest that apoplastic cytokinins initiate signaling, whereas cytoplasmic cytokinins are inactive.

PUP14 imports cytokinins from the apoplast to the cytosol, away from sensing domains of plasma membrane–localized receptors, which causes a reduction in cytokinin signaling. Thus, PUP14 activity inversely correlates with the capacity of a cell to sense cytokinins (fig. S8), and PUP14 spatiotemporal activities cause region-specific depletion of cytokinins from the apoplast. In animals, the importance of such clearing activities is demonstrated by the powerful action of drugs that target dopamine influx transporters, thereby increasing dopamine's residence time in the synaptic cleft (*26*). Feeding experiments with radiolabeled bioactive cytokinin suggest that the bulk of imported cytokinins are inactivated by conversion to monophosphates by ADENINE PHOSPHORIBOSYL TRANSFERASE enzymes (*27*, *28*). Furthermore, N- or O-glycosylation, oxidative cleavage, or transport to other cells may contribute to clearance of intracellular cytokinins (*2*). PUP14 is the only family member to be linked to cytokinin signaling in all organs. In specific developmental contexts, additional PUP family members likely have overlapping functions with PUP14. The fact that *PUP* genes are specific to vascular plants (*29*) may suggest that *PUP* genes are needed to support more complex cytokinin signaling patterns associated with the bauplan of land plants. Because hormonal transporters are numerous and universal in plant and animal systems, transporters in other systems may also regulate patterning during morphogenesis.

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SUPPLEMENTARY MATERIALS