Auxin inhibits stomatal development through MONOPTEROS repression of a mobile peptide gene *STOMAGEN* in mesophyll

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Plants, as sessile organisms, must coordinate various physiological processes to adapt to ever-changing surrounding environments. Stomata, the epidermal pores facilitating gas and water exchange, play important roles in optimizing photosynthetic efficiency and adaptability. Stomatal development is under the control of an intrinsic program mediated by a secretory peptide gene family—namely, *EPIDERMAL PATTERNING FACTOR*, including positively acting *STOMAGEN/EPFL9*. The phytohormone brassinosteroids and environment factor light also control stomatal production. However, whether auxin regulates stomatal development and whether peptide signaling is coordinated with auxin signaling in the regulation of stomatal development remain largely unknown. Here we show that auxin negatively regulates stomatal development through MONOPTEROS (also known as ARF5) repression of the mobile peptide gene *STOMAGEN* in mesophyll. Through physiological, genetic, transgenic, biochemical, and molecular analyses, we demonstrate that auxin inhibits stomatal development through the nuclear receptor TIR1/AFB-mediated signaling, and that MONOPTEROS directly binds to the *STOMAGEN* promoter to suppress its expression in mesophyll and inhibit stomatal development. Our results provide a paradigm of cross-talk between phytohormone auxin and peptide signaling in the regulation of stomatal production.

Auxin is the first identified phytohormone, which exerts multifaceted influences on plant growth and development, such as embryonic root initiation (1, 2), shoot apical meristem function (3), and floral primordia initiation (4). As a "molecular glue," auxin facilitates the formation of its coreceptor complexes comprising F-box proteins (TIR1/AFBs) and AUXIN/INDOLE-3-ACETIC ACID proteins (AUX/IAAs), and subsequent AUX/IAAs ubiquitination and degradation by 26S proteasome, thus releasing auxin response factors (ARFs) from AUX/IAAs repression to regulate auxin-responsive gene expression by either activation or repression (2, 3, 5–11). Although many physiologic processes are reported to be regulated by auxin (1–4, 6, 12), the full understanding of the functions of this versatile phytohormone has not been reached.

Stomata, the pores flanked by a pair of guard cells, mainly constitute the epidermis of plant leaves together with trichomes and neighboring pavement cells that separate stomata to maintain the one-cell spacing rule (13, 14). As a gas and water passage between external environment and internal plant tissue, stomata play important roles in photosynthesis and global carbon and water circulation (15). Stomatal generation undergoes several stages, including meristemoid mother cell, meristemoid, guard mother cell, and guard cells, which is modulated by an intrinsic program (14) mainly involving putative peptide ligands [EPIDERMAL PATTERNING FACTOR (EPF) family] (16–20), membrane proteins (receptor-like protein TMM and receptor-like kinase ERECTA family) (21–23), MAPK cascades (protein kinase YDA, MKK4/5/7/9, and MPK3/6) (24–26), and transcription factors (bHLH and MYB type) (23, 27–31). EPF factors, the small secretory peptides, are proposed to act at the top of this hierarchical signaling pathway (16–20). Interestingly, the EPF family is comprised of members with completely opposite functions (32), such as negatively acting EPF1 and EPF2 (16, 17, 20) and positively acting STOMAGEN/EPFL9 (18). *EPF1* and *EPF2* are expressed in the epidermis, and their encoding peptides were recently shown to be ligands of ERECTA and TMM, negatively regulating stomatal development (16, 17, 19, 20). In contrast, *STOMAGEN* is expressed in mesophyll, and its encoding peptide then migrates to the epidermis where it is proposed to promote stomatal development by competitively inhibiting TMM-mediated signaling (18). Modulation of *EPF* expression could drastically alter stomatal development (16–18, 20), which might be due to cascade amplification from the top signals. Thus, it was proposed that EPFs are a novel class of peptide hormones (32, 33). Although intrinsic program regulating stomatal development has been well characterized, how the top signals from EPFs are regulated remains elusive.

Phytohormones and external stimuli, such as brassinosteroids (BRs), light, and carbon dioxide, are also involved in modulating stomatal production (13, 14, 34–36). Here we show that nuclear receptor-mediated auxin signaling negatively regulates stomatal development, and that ARF5/MONOPTEROS (MP) is involved in regulating this process. MP directly associates with the *STOMAGEN* promoter and represses *STOMAGEN* expression in an auxin response element (AuxRE)-dependent manner. The regulation of *STOMAGEN* by MP occurs in mesophyll, where photosynthesis mainly takes place, providing a possibility of specifically manipulating auxin signaling in mesophyll to coordinate

Significance

Stomata are widespread in aerial part of plants as passages exchanging gas and water with environment. Therefore, stomata are crucial for photosynthesis as well as global carbon and water circulation. Auxin, as the first identified phytohormone, participates in many aspects of plant growth and development, but whether auxin regulates stomatal development is unknown. This study establishes that auxin negatively regulates stomatal development through MONOPTEROS (MP) repression of mobile peptide gene *STOMAGEN* expression in mesophyll cells, which is mediated by direct binding of MP to auxin response elements in the *STOMAGEN* promoter. This study advances our knowledge about the roles of auxin and the versatile regulator MP in plant growth and development.

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The authors declare no conflict of interest.

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stomatal development with photosynthesis without disturbing the whole-body plan.

Results

Nuclear Receptor-Mediated Auxin Signaling Inhibits Stomatal Development. To explore the role of auxin in stomatal development, we treated germinating seeds with various concentrations of auxin analog 2,4-D for 8 d and found that the stomata and meristemoids indexes (SMIs; stomata plus meristemoids per total epidermal cells) in the abaxial epidermis of cotyledons are progressively reduced with the increasing concentration of 2,4-D, with 150 nM being the minimal concentration tested for the maximal inhibition (Fig. 1 *A*, *B*, and *Q*), indicating that auxin inhibits stomatal development in a dose-dependent manner. Moreover, the *35S::iaaM* transformants, which produce elevated levels of auxin in vivo (37), display significantly reduced SMIs compared with wild type (Fig. 1 *C*, *D*, and *R*). These results indicate that excess auxin from either exogenous application or genetic manipulation inhibits stomatal development. We further examined transgenic and mutant plants with deficiencies in endogenous auxin biosynthesis or signaling pathways. It is known that *taa1 tar1 tar2* triple mutant is defective in the main route for auxin biosynthesis in vivo (38–41), and that the *35S::iaaL* transformants have reduced free auxin levels due to the transformation of active auxin to inactive auxin–lysine conjugate (42). Both *taa1 tar1 tar2* mutant and *35S::iaaL* seedlings exhibit clustered stomata and significantly increased SMIs in the abaxial epidermis of cotyledons (Fig. 1 *E*–*G* and *S*), indicating that reduced auxin promotes excess stomatal production and abnormal stomatal patterning. Taken together, these results demonstrate that auxin inhibits stomatal development.

To determine how the auxin signal is transduced to influence stomatal development, we examined the auxin receptors' quadruple mutant *tir1 afb1 afb2 afb3* and observed prominent stomatal clusters and increased SMI (Fig. 1 *E*, *H*, and *S*), indicating that nuclear receptor-mediated auxin signaling plays a pivotal role in the regulation of stomatal development.

Because TIR1/AFB-mediated auxin signaling regulates gene expression eventually through ARF transcription factors (6, 43), we sought to determine which of the 23 ARFs in *Arabidopsis* are involved in the regulation of stomatal development. It is proposed that the genes implicated in regulating asymmetric divisions of stomatal precursor cells may also act to regulate earlier development (e.g., embryogenesis), such as those encoding components in MAPK signaling pathway (*YDA*, *MPK3*, and *MPK6*) (24, 25, 44). We focused on MP based on previous demonstrations. First, prominent embryo defects are observed in the well-characterized *mp* mutant, but not in any of the other *arf* mutants available so far (45). Second, an extraembryonic cell adjacent to the embryonic cell—namely, hypophysis, which is specified to become the primary root meristem founder cell, often undergoes aberrant division in both *yda* and *mp* mutants, leading to varied degrees of rootless phenotypes (1, 2, 44). Moreover, abnormal cotyledon development is obvious in *yda*, *mpk3 mpk6*, and *mp* mutants (1, 25, 44). Therefore, we examined stomatal development phenotype in *mp*/*arf5* mutants, and observed significantly increased SMI and stomatal clusters in the lethal *mp* allele *arf5-1* seedlings (Fig. 1 *E*, *I*, and *S*). A weak allele mp^{S319} homozygote is reported to have only a small portion of seedlings with root deficit (2, 46). To determine whether mp^{S319} homozygotes also exhibit stomatal development variation, we examined stomatal patterning of mp^{S319} homozygous seedlings with and without roots, respectively, and found that only rootless seedlings (~8.6% of progenies of mp^{S319} heterozygote) exhibit a prominent stomatal cluster phenotype (Fig. 1 *E* and *J* and Fig. S1).

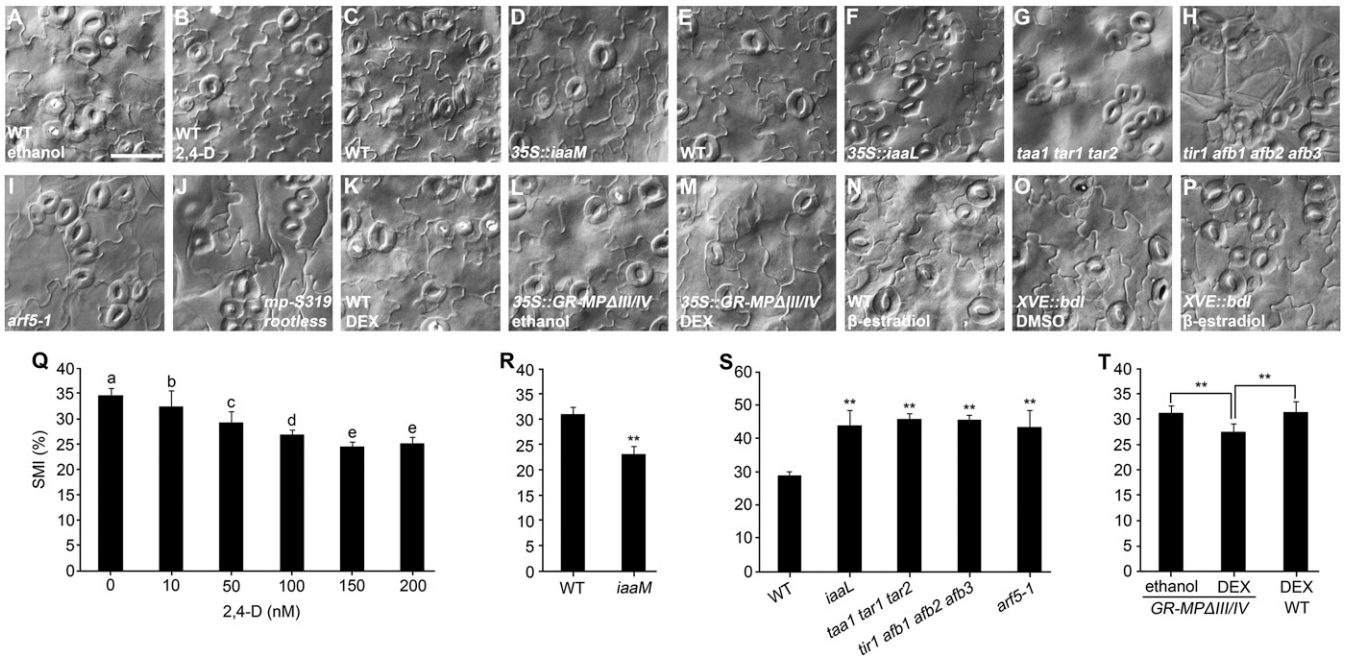

Fig. 1. Auxin negatively regulates stomatal development. (*A*–*P*) Differential interference contrast microscopy images of abaxial cotyledon epidermis of 8-dpg seedlings with indicated genotypes. Comparisons should be made in the same group, such as *A* and *B*; *C* and *D*; *E*–*J*; *K*–*M*; and *N*–*P*. The concentration of 2,4-D used in *B* is 150 nM. GR-MPΔIII/IV could be conditionally nucleus-localized on DEX (*M*) but not ethanol induction (*L*). MPΔIII/IV lacks domain III/IV, which mediates dimerization with AUX/IAA proteins. (*O* and *P*) *XVE::bdl* line expresses stabilized bdl on β-estradiol induction (*P*) rather than on solvent DMSO induction (*O*). (*Q*) SMIs obtained from various concentrations of 2,4-D treatment. Values indicated by distinct letters are significantly different ($P < 0.01$; Tukey's least significant difference, $n = 10$). (*R*–*T*) SMIs obtained from *C* and *D*; *E*–*I*; and *K*–*M*, respectively. Data are means ± SDs ($n = 10$ for *R* and *T*; $n = 9$ for *S*) with Student *t* test (**$P < 0.001$). (Scale bar: 50 μm.)

To explore whether *MP* overexpression might reduce SMI, we created *35S::GR-MPΔIII/IV* transgenic lines expressing glucocorticoid receptor (GR)-fused MP lacking domain III/IV (MPΔIII/IV). MPΔIII/IV is known not to interact with AUX/IAA proteins, thus possessing higher activity than the full-length of MP (47, 48). GR-MPΔIII/IV is imported into nucleus upon dexamethasone (DEX) treatment. We found that GR-MPΔIII/IV significantly reduces SMI on DEX induction (Fig. 1 *K–M* and *T*).

Because ARF proteins are shown to be inhibited by AUX/IAA proteins, we examined the effects of a stabilized and gain-of-function version of BDL—namely, *bdl* (49), on stomatal development. We generated transgenic plants expressing *bdl* under the control of a chemical-inducible promoter *XVE* (50). As expected, on β-estradiol induction, *pXVE::bdl* transgenic seedlings produce excess stomatal clusters (Fig. 1 *N–P*), indicating that AUX/IAA–ARF module is also involved in the regulation of stomatal development. Because BDL and MP is a well-studied IAA-ARF pair regulating embryonic root initiation, shoot apical meristem function, and floral primordia initiation (2–4), and our findings implicate MP in the regulation of stomatal development, it is probable that the BDL-MP pair is also involved in the regulation of stomatal development. Confirmation of this possibility can be made by investigating whether *BDL* is expressed in mesophyll and whether the ARFs other than MP regulate stomatal development. Taken together, these results indicate that MP-mediated nuclear auxin signaling inhibits stomatal development.

MP Functions to Regulate Stomatal Development at Early Seedling Developmental Stage. To determine how stomatal clusters are generated in the *arf5-1* mutant, we tracked and compared time-course developmental progression in *arf5-1* with that in WT using stomatal lineage marker *TMM* promoter (22). Nucleus-localized luciferase (LUC) fused with GFP driven by the *TMM* promoter (*pTMM::LUC-NLS-GFP*) was constructed to visualize stomatal lineage cells. Abnormal cell divisions appear at the third day postgermination (3 dpg) in *arf5-1* with adjacent meristemoids and immature stomata highly marked with GFP signals, which indicate potential cell-division abilities (Fig. 2 *A–C* and *F–H*). The increasingly prominent stomatal clusters are generated in *arf5-1* at 4 dpg and 6 dpg (Fig. 2 *D*, *E*, *I*, and *J*). These results indicate that, as early as 3 dpg, the role of *MP* in restraining aberrant stomatal patterning starts to be apparent.

MP Acts to Repress *STOMAGEN* Expression in Mesophyll to Inhibit Stomatal Development. We investigated the expression pattern of *MP* in leaves of transgenic seedlings expressing *MP* fused to *GFP* under the control of the *MP* native promoter (*pMP::MP-GFP*) (2). Strong nucleus-localized GFP signals were observed in mesophyll cells rather than in epidermal cells at 3 dpg (Fig. 3*A*). A previous report showed that MP–GFP is strongly expressed in mesophyll but weakly in epidermis at 1 dpg, and restricted to mesophyll at 2 dpg (48). Combined with our observation, these results indicate that epidermal cells should express very low, if any, levels of *MP* after 2 dpg. The strong expression of *MP* in mesophyll suggests that MP might function non–cell-autonomously in the regulation of stomatal development. So far, the short peptide STOMAGEN is known to be a unique mobile factor that is expressed in mesophyll cells and then migrates to the epidermis to regulate stomatal development (18). Because MP acts as a transcription factor mediating auxin signaling (51), we entertained the possibility that MP-mediated auxin signaling negatively regulates stomatal development likely through repressing *STOMAGEN* expression. Indeed, *STOMAGEN* expression peaks at 2 dpg and drastically decreases at 3 dpg in WT seedlings as shown by quantitative RT-PCR (Fig. 3*B*). In contrast, *STOMAGEN* transcripts in *taa1 tar1 tar2*, *tir1 afb1 afb2 afb3*, and *arf5-1* sustain significantly higher levels than those in WT at 1, 3, and 4 dpg, respectively, and hardly decline at 3 and 4 dpg (Fig. 3*B*), which might account for the phenotypic differences between *arf5-1* and WT appearing at 3 dpg (Fig. 2 *C* and *H*). Because the regulation of *STOMAGEN* expression at 2 and 3 dpg appears to be critical for stomatal development, hereafter only *STOMAGEN* expression at 2 and 3 dpg will be analyzed.

Our findings that both 2,4-D treatment and *iaaM* overexpression significantly reduce SMI (Fig. 1 *A–D*, *Q*, and *R*) prompt us to analyze *STOMAGEN* expression in these conditions. Consistent with the phenotypes, 2,4-D treatment results in a significant decrease in *STOMAGEN* expression at 2 and 3 dpg compared with mock treatment (Fig. 3*C*), and the expression of *STOMAGEN* in *iaaM* overexpressors is significantly reduced compared with that in WT (Fig. 3*D*), respectively, indicating that auxin inhibits stomatal development likely through repressing *STOMAGEN* expression. To explore whether *STOMAGEN* expression is sensitive to transiently applied auxin, we monitored a time-course response of *STOMAGEN* expression to auxin treatment and found that *STOMAGEN* expression is significantly reduced on 30 min auxin treatment, and then progressively elevated to the untreated level at 120 min (Fig. 3*E*). The rapid response of *STOMAGEN* expression to auxin treatment indicates that *STOMAGEN* should be one of the primary auxin-responsive genes. The progressive recovery of *STOMAGEN* expression after 30 min of auxin treatment might be a consequence of negative feedback conferred by rapidly up-regulated primary auxin-responsive genes, including genes of *AUX/IAA* and *GH3* families, which inhibit ARF activity and reduce active auxin level (52, 53), respectively.

To confirm the MP repression of *STOMAGEN* expression, we analyzed the effects of GR-MPΔIII/IV on *STOMAGEN*

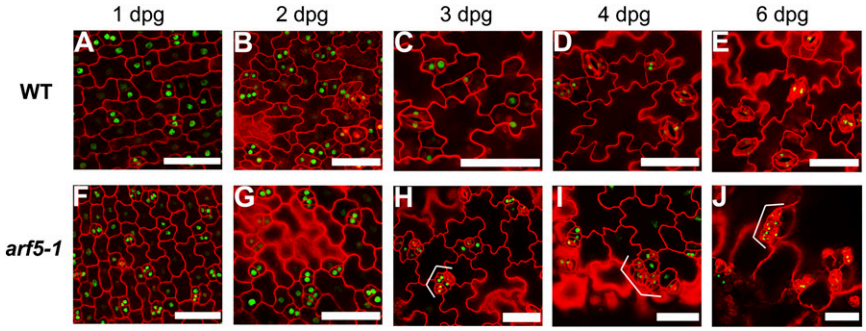

Fig. 2. MP functions to restrain aberrant stomatal patterning at an early seedling developmental stage. (*A–J*) Confocal images of *pTMM::LUC-NLS-GFP* cotyledons in WT (*A–E*) and *arf5-1* (*F–J*) background, respectively. Epidermal cell periphery is highlighted by propidium iodide (red) staining. Brackets indicate clustered stomata or abnormal stomatal lineage cells. (Scale bars: 50 μm.)

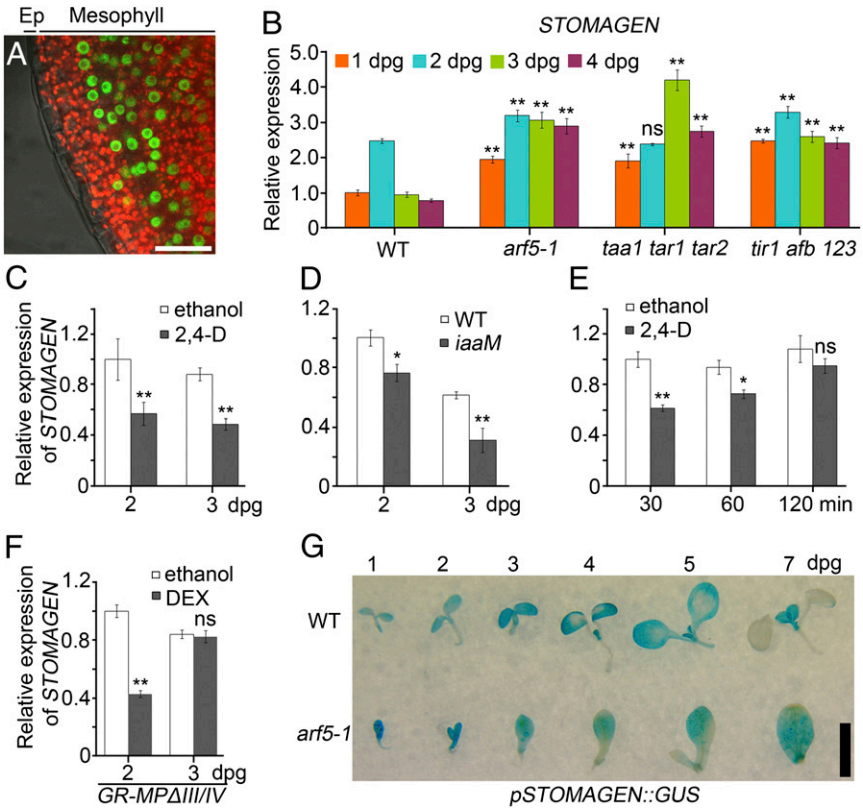

Fig. 3. MP-mediated auxin signaling represses *STOMAGEN* expression. (*A*) Confocal images of *pMP::MP-GFP* leaves at 3 dpg. The merged signals of bright field, Cy5, and GFP channels are shown. Cy5 shows chlorophyll autofluorescence (red) in mesophyll. The nuclear GFP signals (green) indicate MP-GFP. Ep, epidermis. (*B–F*) *STOMAGEN* qRT-PCR in the seedlings with indicated genotypes and/or treatments. Data are means ± SDs ($n = 3$) with Student *t* test (*$P <$ 0.01; **$P < 0.001$; ns, no significant differences). Statistical analyses are made by comparing data from mutants (*B*) and *35S::iaaM* (*D*) with those from WT at the same age, respectively. (*C*) Seeds were grown in medium with ethanol control or 150 nM 2,4-D for indicated time, and statistical analyses are made by comparing data from different treatments at the same age. (*E*) The 2-d-old seedlings were treated in liquid medium with ethanol control or 150 nM 2,4-D for indicated time, and statistical analyses are made by comparing data from different treatments at the same time point. (*F*) *35S::GR-MPΔIII/IV* seeds were grown in medium with ethanol control or 5 μM DEX for indicated time, and statistical analyses are made by comparing data from different treatments at the same age. (*G*) Time-course GUS staining of WT-like (WT) and *arf5-1* siblings segregated from *pSTOMAGEN::GUS;arf5-1*/+. (Scale bars: *A*, 25 μm; *G*, 2.5 mm.)

transcription. Compared with mock-treated *GR-MPΔIII/IV*, DEX-treated *GR-MPΔIII/IV* seedlings express significantly reduced *STOMAGEN* transcripts at 2 but not at 3 dpg (Fig. 3*F*), indicating that the down-regulation of *STOMAGEN* expression at 2 dpg might be sufficient to reduce SMI. It is important to note that DEX-induced GR-MPΔIII/IV has no such dramatic effects as 150 nM of 2,4-D on stomatal development (Fig. 1 *Q* and *T*), which might be ascribed to their differential influences on *STOMAGEN* expression at 3 dpg (Fig. 3 *C* and *F*). The reasons for our failure to observe the reduction of *STOMAGEN* expression in DEX-induced *GR-MPΔIII/IV* seedlings at 3 dpg remains to be determined. Moreover, LUC activity assay was performed in the populations including WT-like seedlings (WT) and the *arf5-1* homozygous siblings (*arf5-1*/−) segregated from the transgenic *pSTOMAGEN::LUC;arf5-1*/+ plants. In 10 independent transgenic lines, the LUC activities in *arf5-1*/− are consistently much higher than those in WT (Fig. S2). For visual inspection, histochemical staining of GUS activity with *pSTOMAGEN::GUS;arf5-1*/+ transgenic seedlings was performed. Consistent with a previous report (18), GUS activity is only detected in the mesophyll rather than in the epidermis (Fig. S3*A*), and broad at 1 dpg in WT cotyledons, gradually confined to the tips of cotyledons (4 and 5 dpg) until eventually almost invisible (7 dpg) with the development of cotyledons (Fig. 3*G*). In contrast, GUS activity in *arf5-1*/− always broadly expands throughout the cotyledons even at as late as 7 dpg (Fig. 3*G*), indicating that MP regulates the spatiotemporal expression pattern of *STOMAGEN*. Interestingly, the GUS activity appears to expand to the epidermis in *arf5-1*/− (Fig. S3*B*), indicating that trace amounts of MP and/or other ARFs might efficiently restrict *STOMAGEN* expression at an undetectable level in WT epidermis, whereas the repression of *STOMAGEN* by MP in mesophyll is likely much more elastic, making mesophyll a critical place where MP controls stomatal development by regulating *STOMAGEN* expression.

***MP* Regulates Stomatal Development Through Repressing *STOMAGEN* Expression.** To examine the genetic interaction between *MP* and *STOMAGEN*, we silenced *STOMAGEN* in *arf5-1*/+ through artificial miRNA (18). The results demonstrate that *STOMAGEN* expression is significantly reduced in both WT and *arf5-1*/− seedlings segregated from the same *STOMAGEN-RNAi;arf5-1*/+ transgenic line (Fig. 4*F*), and that the down-regulation of *STOMAGEN* leads to a prominent decrease in SMIs in WT and *arf5-1*/− cotyledons, respectively (Fig. 4 *A–E*). It is important to note that the large stomatal clusters consisting of over four stomata, otherwise obviously present in *arf5-1*/− mutant, are absent in the *STOMAGEN-RNAi;arf5-1*/− transgenic seedlings (Fig. 4 *C* and *D*). The intermediate stomatal phenotype observed for *STOMAGEN-RNAi;arf5-1*/− may indicate that there exist more genes other than *STOMAGEN* that are modulated by *MP* to regulate stomatal development. These results indicate that *MP* regulates stomatal development, at least in part, through repressing *STOMAGEN* expression.

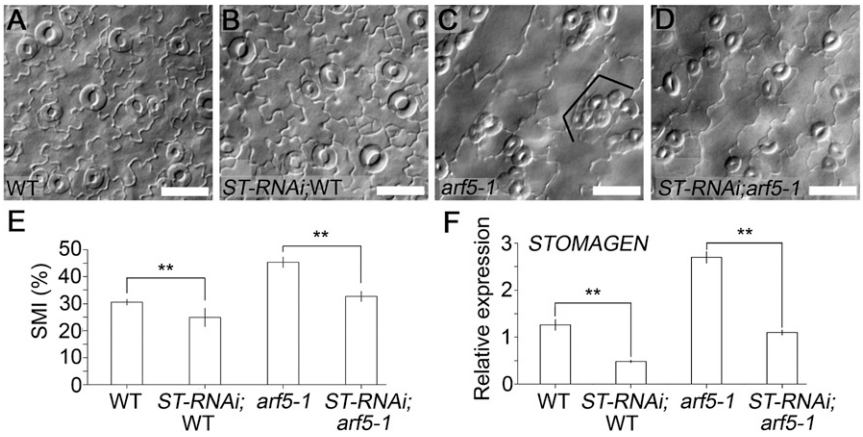

Fig. 4. *STOMAGEN* acts genetically downstream of *MP* to regulate stomatal development. (*A–D*) Differential interference contrast microscopy images of abaxial cotyledon epidermis of 8-dpg seedlings with indicated genotypes. (Scale bars: 50 μm.) *ST-RNAi;*WT (*B*) and *ST-RNAi;arf5-1* (*D*) is segregated from the same *ST-RNAi;arf5-1*/+ line. Bracket indicates clustered stomata. (*E*) SMIs calculated from *A–D*. Data are means ± SDs ($n = 9$). (*F*) *STOMAGEN* qRT-PCR in the seedlings with indicated genotypes at 4 dpg. Data are means ± SDs ($n = 3$). Significant differences were determined with Student *t* test; $^{**}P < 0.001$. *ST*, *STOMAGEN*.

MP Directly Binds to the *STOMAGEN* Promoter. Based on the demonstration that ARFs mediate auxin signaling through binding to AuxREs (54), and our finding that *STOMAGEN* expression is regulated by MP (Fig. 3 *B*, *F*, and *G* and Fig. S2), we examined whether these elements exist in the *STOMAGEN* promoter. Indeed, we identified 17 putative (TGTC) and three canonical (TGTCTC) AuxREs in the *STOMAGEN* promoter (Fig. 5*A*). To determine whether MP binds to the *STOMAGEN* promoter in vivo, we generated transgenic *Arabidopsis* plants expressing *MP* fused to a fragment encoding MYC-HA epitope under the control of the *MP* native promoter in the *arf5-1* mutant background (*pMP::MP-MYC-HA;arf5-1*), and performed ChIP-quantitative PCR (qPCR) assay. The results demonstrate that the specific MP-occupied sites are spread throughout the 1,500 base pairs upstream of the translational start codon (−1,500 bp; Fig. 5*B*). Consistent with the finding that the AuxRE-rich region is mainly located within −500 bp, multiple sites within this region were found to be bound by MP (Fig. 5*C*).

We then performed EMSA to confirm the specific interactions of MP with these sites of *STOMAGEN* promoter in vitro, and found a site (P3) within the −500-bp AuxRE-rich region that strongly interacts with recombinant maltose binding protein tagged MP (MBP-MP) (Fig. 5*D*). In the EMSA assay, smear-like signals or no clear shifted bands are obvious (Fig. 5*D*). To circumvent this problem, we performed a DNA–protein pull-down assay. The results show that the truncated fragments of MBP-MP with various molecular weights exist (Fig. 5*E*), and some of the fragments (comparing pull-down with input) together with the full-length of MBP-MP are strongly pulled down by P3 and weakly by P1, P2, and P4, whereas MBP is hardly pulled down by these probes (Fig. 5 *E* and *F*), consistent with the EMSA results (Fig. 5*D*). Furthermore, DNA–protein pull-down assay demonstrates that biotin-labeled mutated P3 fails to pull down MBP-MP (Fig. 5*G*) and that nonlabeled intact but not mutated P3 reduces the association of MBP-MP with biotin-labeled P3 (Fig. 5*H*), indicating that the interaction of MBP-MP with P3 is specific and depends on AuxREs. Moreover, His-tag MP-DNA binding domain (DBD, residues 120–274) (55), which was shown to be able to homodimerize rather than multimerize because of lacking C-terminal domain III/IV (56), was expressed in bacteria and purified. The purified MP-DBD and biotin-labeled P3 probe were used for EMSA assay. The results show that a single shifted band is obvious when MP-DBD is added, and the band intensity is reduced by the competition from intact rather than mutated cold competitor (Fig. 5*I*), indicating that the MP-DBD-P3 interaction is specific and depends on AuxREs, consistent with the results from DNA–protein pull-down assay (Fig. 5 *F–H*). The lack of higher-order shifted bands in the MP-DBD EMSA assay indicates the possible contribution of higher-order MP-DNA complexes due to multimerization to smear-like signals in full-length of MP EMSA assay (Fig. 5*D*). Taken together, these results indicate that MP directly binds to *STOMAGEN* promoter in an AuxRE-dependent manner.

MP Regulates *STOMAGEN* Expression in an AuxRE-Dependent Manner in Vivo. To identify which AuxREs within the *STOMAGEN* promoter might be mainly responsible for responding to MP *in planta*, we performed dual-LUC assay, which has been extensively used to analyze gene expression regulation (57, 58). Surprisingly, the reporter genes driven by the *STOMAGEN* promoter are activated, but not suppressed, by MP in tobacco (Fig. S4 *A–C*), which is seemingly contradictory to the negative regulation of *STOMAGEN* by MP in *Arabidopsis*. To further address this question, we generated another reporter gene driven by the *ARR15* promoter, which is shown to be repressed by MP in *Arabidopsis* (3), and found that *ARR15* promoter is also significantly activated by MP in tobacco (Fig. S4*D*). These results indicate two possibilities: one is that the activation of *STOMAGEN* and *ARR15* by MP in tobacco is nonspecific (i.e., MP could constitutively activate the reporters regardless of upstream elements in tobacco), and the other is that the dual-LUC results could reflect the specific regulation of *STOMAGEN* and *ARR15* promoters by MP in tobacco, regardless of activation or repression. To distinguish these two possibilities, we first made deletion fragments of *STOMAGEN* promoter and examined their responses to MP in tobacco using dual-LUC assay. The results show that the deletion of −500-bp AuxRE-rich region of the *STOMAGEN* promoter leads to a complete loss of response to MP (Fig. S4 *A–C*), consistent with the ChIP-qPCR, DNA–protein pull-down, and EMSA results (Fig. 5), demonstrating that MP cannot constitutively activate the reporter in tobacco. Next, we made a series of multiple mutations of AuxREs within −500-bp region of the *STOMAGEN* promoter, and demonstrated that at least three AuxREs (Fig. S4*E*, *i–iii*), which are shown to be bound by MP in ChIP-qPCR, DNA–protein pull-down, and EMSA assays (Fig. 5), are mainly responsible for the reporter to respond to MP (Fig. S4*F*), and that the *STOMAGEN* reporters bearing quadruple to sextuple AuxRE mutations (designated

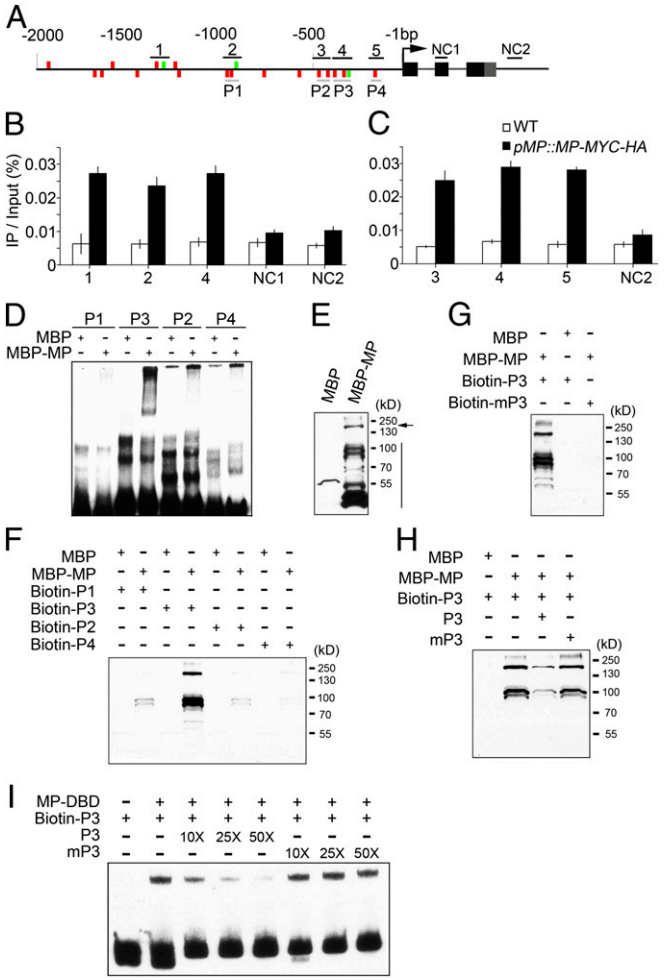

Fig. 5. MP directly associates with the AuxREs in a *STOMAGEN* promoter. (*A*) *STOMAGEN* locus comprising the 2-kb promoter region and transcribed region. AuxREs including TGTCTC (green bar) and TGTC (red bar) on the plus (*Upper*) and minus strand (*Lower*) of a 2-kb upstream *STOMAGEN* gene are indicated. Black lines denote fragments amplified in ChIP-qPCR (*B* and *C*). Negative controls 1 and 2 (NC1 and NC2) for ChIP-qPCR are located in the second exon (black box) and the region downstream of 3′ UTR (gray box), respectively. Gray lines indicate probes used in EMSA (*D* and *I*) and DNA–protein pull-down assays (*F*–*H*). (*B* and *C*) qPCR of fragments (as in *A*) from ChIP of *pMP::MP-MYC-HA* seedlings and WT with anti-HA antibody. Data are means ± SDs ($n = 3$). (*D*) EMSA assay using indicated proteins and probes. Various probes as indicated in *A* were used for EMSA assay (*D*). (*E*–*H*) Western blot analyses of the indicated input (*E*) and pulled-down proteins in DNA–protein pull-down assay (*F*–*H*) with anti-MBP monoclonal antibody. Arrow and vertical line indicate the full-length and degradation fragments of MBP–MP, respectively. Biotin-labeled probes (as in *A*) were used for DNA–protein pull-down assay with MBP and MBP–MP being as prey (*F*). Biotin-labeled wild-type (biotin-P3) or mutated (biotin-mP3) AuxRE-containing probes were used in *G*. Biotin-P3 was used to pull down MBP–MP with unlabeled P3 and mP3 as competitors, respectively (*H*). (*I*) EMSA assay using indicated proteins and probes. MP–DBD and biotin-P3 are protein and probe, respectively. Unlabeled P3 and mP3 were used as cold competitors, respectively. 10×, 25×, and 50× indicate the amount of cold competitors relative to that of labeled probe.

MIV, MV, and MVI; Fig. S4*E*) fail to respond to MP (Fig. S4*F*), indicating that MP specifically regulates *STOMAGEN* promoter in an AuxRE-dependent manner in tobacco.

To further determine whether the AuxRE-mediated association of *STOMAGEN* promoter with MP identified in EMSA, DNA–protein pull-down, and dual-LUC assays is meaningful in *Arabidopsis*, we made four constructs expressing *LUC* or *GUS* driven by wild-type (*pSTOMAGEN::LUC* or *pSTOMAGEN::GUS*) and mutant *STOMAGEN* promoters bearing quintuple or sextuple AuxREs mutations (*pSTOMAGEN-MV::LUC* or *pSTOMAGEN-MVI::GUS*), respectively, and introduced them into *Arabidopsis*. Analysis of luciferase activity shows that *pSTOMAGEN-MV::LUC* seedlings express considerably more LUC proteins than *pSTOMAGEN::LUC* seedlings (Fig. 6*A*). Time-course GUS staining demonstrates that the GUS activity is elevated, spatially expanded, and temporally extended in the cotyledons of *pSTOMAGEN-MVI::GUS* seedlings, compared with that in the cotyledons of *pSTOMAGEN::GUS* seedlings (Fig. 6*B*). In an attempt to test the binding capacity of MP to *STOMAGEN* promoter harboring sextuple AuxRE mutations in *pSTOMAGEN-MVI::GUS* seedlings, we used an anti-MP antibody that we generated to perform the ChIP assay. Western blot analysis indicates that this antibody is able to detect the induced MP in transgenic plants (*pXVE::MP-Myc* and p*XVE::MP*), but not the endogenous MP (Fig. S5 *A*–*C*), probably due to its low abundance in the wild type. We tried to use this antibody to perform a ChIP assay with wild-type seedlings, but found no significant binding of MP to *STOMAGEN* promoter (Fig. S5*D*), indicating that the antibody is not suitable for ChIP assay in

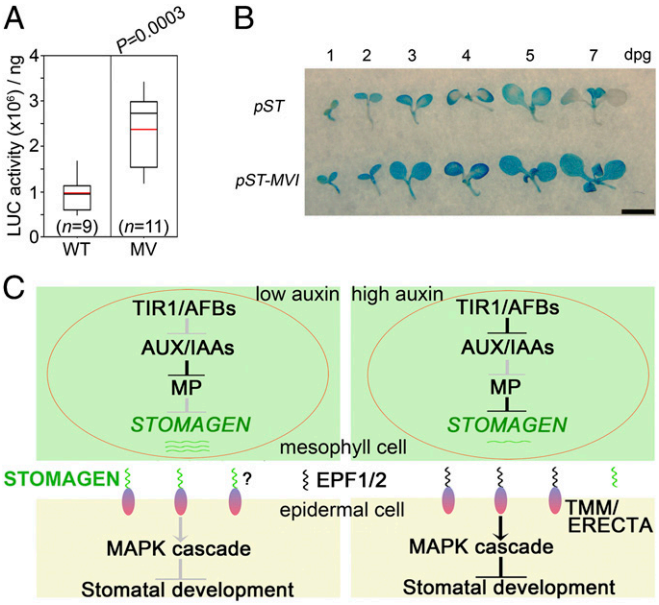

Fig. 6. AuxREs within the −500-bp region are critical for repression of *STOMAGEN* expression in vivo. (*A*) Box plot showing luciferase activities in *pSTOMAGEN::LUC* and *pSTOMAGEN-MV::LUC* transgenic lines. WT and MV indicate WT and quintuple AuxRE-mutated *STOMAGEN* promoters as described in Fig. S4 *E* and *F*, respectively. Box comprises values within median 50%. Error bars indicate highest or lowest value. Black and red lines represent median and average, respectively. *n* represents the number of individual lines. *P* value (two-tailed unpaired Student *t* test) is shown above. (*B*) Time-course examination of GUS activities in *pSTOMAGEN::GUS* and *pSTOMAGEN-MVI::GUS* transgenic lines. WT and MVI indicate WT and sextuple AuxRE-mutated *STOMAGEN* promoters as described in Fig. S4 *E* and *F*, respectively. (Scale bar: 2.5 mm.) (*C*) A model for auxin regulation of stomatal development. When auxin levels are low (*Left*), AUX/IAA proteins are stabilized and repress MP, leading to high *STOMAGEN* expression in mesophyll. The accumulated STOMAGEN peptides, which migrate to the epidermis, might compete for TMM or ERECTA with EPF1/2 and inhibit the TMM/ERECTA-MAPK signaling cascade, thus promoting stomatal development. Auxin signaling through TIR1/AFBs activates MP (*Right*) in mesophyll cells, resulting in repression of *STOMAGEN* and inhibition of stomatal development by probable activation of EPF1/2-TMM/ERECTA-MAPK signaling. Arrows and T-bars indicate attenuated (gray) or enhanced (black) activation and inhibition, respectively.

wild-type seedlings. Although we do not have the necessary materials to detect the requirement of AuxREs within the *STOMAGEN* promoter for MP binding to the *STOMAGEN* promoter in *Arabidopsis* seedlings by ChIP assay, the results from the LUC assay with *pSTOMAGEN-MV::LUC* seedlings (Fig. 6*A*) and GUS staining with *pSTOMAGEN-MVI::GUS* seedlings (Fig. 6*B*) strongly indicate that the AuxRE-mediated regulation of *STOMAGEN* by MP obtained in EMSA, DNA–protein pull-down, and dual-LUC assays is meaningful *in planta*. Therefore, we conclude that AuxREs within the −500-bp region are mainly responsible for the MP repression of *STOMAGEN* expression in *Arabidopsis*.

Discussion

The present work shows that auxin inhibits stomatal development (Fig. 1), extending our knowledge about the physiological roles of auxin. Furthermore, we revealed the molecular mechanism underlying auxin control of stomatal development by linking auxin signaling with a core stomatal development pathway. When auxin level in mesophyll is low, AUX/IAA proteins are stabilized and accumulated, thus inhibiting MP activity and resulting in high *STOMAGEN* expression in mesophyll. The resulting STOMAGEN peptide then migrates from mesophyll to epidermis to promote stomatal production probably by competitively inhibiting the EPF1/2-TMM/ERECTA-MAPK pathway (Fig. 6*C*, *Left*). When auxin is at high levels in mesophyll, AUX/IAA proteins undergo nuclear auxin receptors TIR1/AFB-mediated degradation by 26S proteasome, thus releasing MP and leading to repression of *STOMAGEN* transcription. The low production of STOMAGEN peptide probably makes the EPF1/2-TMM/ERECTA-MAPK pathway highly activated in epidermal cells, thus causing attenuated stomatal production (Fig. 6*C*, *Right*).

During the revision of our paper, a study reports that altered auxin transport, made either by mutation of auxin efflux transporters or by inhibition of auxin transport with inhibitors, affects stomatal patterning (59). However, the precise physiological role of auxin in the regulation of stomatal development, the regulatory signaling pathway, as well as the underlying molecular mechanisms have basically not been revealed. In the present study, we demonstrate that either altered auxin level or modified auxin signaling could significantly influence stomatal development. Moreover, we established a molecular model for auxin inhibition of stomatal development by showing that MP-mediated nuclear auxin signaling acts non–cell-autonomously to repress a peptide gene *STOMAGEN* expression in mesophyll. This work advances our knowledge about auxin's role in stomatal development and the underlying molecular mechanism. To note, it is shown in this recent work that exogenously applied auxin fails to lead to pronounced stomatal development phenotype, based on the production of stomatal clusters but not SMI (59). In the present study, exogenous 2,4-D application results in a significant decrease in SMI (Fig. 1 *A*, *B*, and *Q*). Because WT rarely produces clustered stomata according to the one-cell spacing rule, and auxin acts to inhibit but not promote stomatal production (This study), statistical analysis of SMI rather than stomatal cluster number should be informative for elucidating the role of exogenously applied auxin in stomatal development.

It is reported that *MP* is expressed in embryonic cells and acts non–cell-autonomously to specify an extraembryonic cell (hypophysis) to become the primary root meristem founder cell during embryogenesis by facilitating auxin transport into hypophysis, and directly transactivating *TMO7*, a gene encoding a mobile transcription factor that moves from embryonic cell to hypophysis to specify embryonic root initiation (2). In this study, we found that MP inhibits stomatal development mainly through directly repressing *STOMAGEN* expression in mesophyll, although we could not rule out the epidermal role, if any, of MP in stomatal development, providing new insights into the non–cell-autonomous action of MP in the regulation of postembryonic development. The mutants or genetically modified plants with abnormalities (enhancement or attenuation) in either auxin biosynthesis or signaling usually display phenotypic alterations in the whole plant, including altered plant height, branching, and floral organ development, or extreme phenotypes, including rootless seedlings, single cotyledon, and infertility (1, 4, 12, 40, 60–62). The regulation of *STOMAGEN* expression and stomatal development by MP-mediated auxin signaling occurs in the mesophyll where photosynthesis mainly takes place (Fig. 6*C*), providing the possibility that coordinating stomatal development with photosynthesis can be achieved by manipulating auxin signaling specifically in the mesophyll without disturbing the whole body plan.

In this study, we performed dual-LUC assay in tobacco to analyze MP regulation of *STOMAGEN* expression and found regulatory effects opposite to those obtained in *Arabidopsis*. Of 23 ARFs in *Arabidopsis*, five Q-rich ARFs, including ARF5/MP, ARF6, ARF7, ARF8, and ARF19, are thought to activate gene expression according to transient expression assay with DR5 or P3(4×) reporter (63–65). However, it is known that MP not only activates (2, 64) but also represses (3) gene expression in different developmental contexts, indicating that activation or repression might be determined by the spatiotemporal availabilities of MP-interacting partners, coactivator or corepressor (65). According to this prediction, MP might constitutively exhibit activation or repression effects on a AuxRE-containing promoter when transiently expressed in a given context (such as specific tissue or cell type) where either coactivator or corepressor is predominant. To test this possibility, we used the *ARR15* promoter, which is shown to be repressed by MP in an AuxRE-dependent manner in *Arabidopsis* (3), in dual-LUC assay. The results show that the *ARR15* promoter is also significantly activated by MP in tobacco (Fig. S4*D*), indicating that the MP-interacting coactivator might be predominant over the corepressor in tobacco leaves. To verify the specificity, we made deletion and mutation in the *STOMAGEN* promoter, respectively, and found that both deletion of the −500-bp AuxRE-rich region and mutations of AuxREs in the −500-bp region lead to complete loss of response of *STOMAGEN* promoter to MP (Fig. S4 *C* and *F*), indicating that the regulation of *STOMAGEN* promoter by MP is specific and depends on AuxREs in the −500-bp region. These results are further confirmed by results from LUC and GUS activity analyses in *Arabidopsis* (Fig. 6 *A* and *B*). Taken together, these results demonstrate that, regardless of activation or repression, the AuxREs responsible for response to MP could be reliably identified through transient dual-LUC assay in tobacco. Importantly, our combined results from analyses of *STOMAGEN* gene expression in *mp* mutant (Fig. 3*B*) and transgenic plants overexpressing *GR-MPΔIII/IV* (Fig. 3*F*), *STOMAGEN* promoter activity in *mp* mutant (Fig. 3*G* and Fig. S2), and genetic interaction between *MP* and *STOMAGEN* (Fig. 4 *A*–*E*) strongly demonstrate that *STOMAGEN* is repressed, but not activated, by MP in *Arabidopsis*. Nevertheless, the mechanisms that render the opposite regulation of *STOMAGEN* by MP in tobacco and *Arabidopsis* remain to be explored.

MP is a versatile regulator of plant growth and development. The physiological processes involving MP include embryonic root initiation (2), SAM function (3), floral primordial initiation (4), and vascular development (1). It is not surprising that MP is implicated in other developmental processes, including stomatal development. Although MP regulates vascular development, the expression of *MP* is not restricted to vascular tissue. In fact, *MP* is strongly expressed in mesophyll at the early seedling developmental stage, as shown by our and others' results (Fig. 3*A*) (48). The expression pattern of *MP* makes it possible that MP regulates mesophyll-derived signals. Stomata, the epidermal pores facilitating gas and water exchange, play important roles in

optimizing photosynthetic efficiency and adaptability. The regulation of stomatal development by MP indicates that MP should play a role in photosynthesis. The vascular tissue of plants functions to transport water, salt, and phytohormones as well as organic compounds, including photosynthetic products, thus connecting all plant tissues. The efficient coordination of photosynthesis and the transportation system is critical for optimizing plant growth and development, which could be easily achieved by placing them under control of the same regulator; this might be the biological significance for MP regulation of stomatal development and vascular development. That the regulation of *STOMAGEN* by MP occurs in mesophyll makes such regulation sense the changes of both photosynthesis and vascular cargoes efficiently and rapidly, thus timely adjusting *STOMAGEN* expression and stomatal development to coordinating photosynthesis and the transportation system.

Previous studies reported that GLV/RGF/CLE-like peptides interplay with auxin to regulate root gravitropism by regulating auxin transport (66, 67). Another report showed that ARF7-mediated induction of peptide gene *IDA* by auxin is involved in the regulation of lateral root initiation (67, 68). However, the molecular mechanism underlying auxin-induced *IDA* expression is unknown. In this study, we provide another example of auxin–peptide cross-talk using stomatal development as a model system and reveal the underlying mechanism by showing that MP directly binds to the *STOMAGEN* promoter and represses its expression, which advances our understanding of auxin–peptide cross-talk in the regulation of plant development. Stomatal development is under complex regulation of an endogenous program, developmental cues, including phytohormones (e.g., BR and auxin), and environmental cues, including light and carbon dioxide. How these signals are integrated to optimize stomatal development and plant growth awaits further investigation.

Materials and Methods

Plant materials, growth conditions, and treatments, quantitative RT-PCR, GUS staining, ChIP, EMSA, DNA–protein pull-down, and luciferase assays as well as all primers used (Table S1) are in *SI Materials and Methods*.

Phenotypic Analyses. Cotyledons of 8-d-postgermination seedlings were transiently preserved in 5% (wt/vol) NaOH solution at 100 °C for ~10 s, washed with distilled water, and placed in the clear solution (glycerol/chloral hydrate/water, 1: 8: 1, vol/wt/vol) overnight or for a few days. Two images at 200× magnification (0.2 mm^2) were captured per cotyledon using a Leica DM2500 microscope with Nomarski optics. A Leica TCS SP5II confocal laser scanning microscope was used to capture Cy5 channel signal and GFP fluorescent images.

ChIP and EMSA Assays. ChIP was performed with seedlings of p*MP::MP-MYC-HA* transgenic lines and WT. IP/input (%) was calculated by comparing the C_t values of immunoprecipitate and input from each genotype. For the EMSA assay, a kit was used with 200 ng of recombinant MBP or MBP-MP and 20 fmol biotin-labeled probes, according to manufacturer's instructions (Thermo Scientific).

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