Carbon monoxide promotes stomatal initiation by regulating the expression of two EPF genes in Arabidopsis cotyledons

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The gaseous molecule carbon monoxide (CO) can freely pass through the cell membrane and participate in signal transduction in the cell to regulate physiological activities in plants. Here, we report that CO has a positive regulatory role in stomatal development. Exogenous CO donor CORM-2 [Tricarbonyldichlororuthenium (II) dimer] treatment resulted in an increase of stomatal index (SI) on the abaxial epidermis of cotyledons in wild-type, which can be reversed by the addition of the CO biosynthesis inhibitor ZnPPIX [Protoporphyrin IX zinc (II)]. Consistent with this result, mutation of the CO biosynthesis gene HY1 resulted in a decrease of SI in hy1-100 plants, while overexpression of HY1 led to an increase of SI. Further investigation revealed that CO acts upstream of SPCH and YDA in the stomatal development pathway, since the loss of function mutants spch-1 and yda-2 were insensitive to CORM-2. The expression of EPF2 was inhibited by CORM-2 treatment in wild type and is lower in HY1-overexpression lines. In contrast, the expression of STOMAGEN was promoted by CORM-2 treatment and is higher in HY1-overexpression lines. Loss of function mutants of both epf2 and stomagen are insensitive to CORM-2 treatment. These results indicated that CO positively regulates stomatal initiation and distribution by modulating the expression of EPF2 and STOMAGEN.

KEYWORDS
Arabidopsis thaliana, stomatal development, carbon monoxide, EPF2, STOMAGEN

Abbreviations: bHLH, basic Helix-Loop-Helix; BV, Biliverdin IXa; CO, Carbon monoxide; CORM-2, Tricarbonyldichlororuthenium (II) dimer; EPFs, Epidermal Patterning Factors; ER, ERECTA; GC, Guard Cell; GMC, Guard Mother Cell; HO, Heme Oxygenase; MAPK, Mitogen-Activated Protein Kinase; MMC, Meristemoid Mother Cell; SI, Stomatal Index; SLGC, Stomatal Lineage Ground Cell; SPCH, SPEECHLESS; TMM, TOO MANY MOUTHS; YDA, YODA; ZnPPIX, Protoporphyrin IX zinc (II).
Introduction

Stomata are small pores in plants that mediate gas exchange on the epidermis of above ground organs such as the cotyledon, leaf, and stem. Stoma can respond quickly to multiple stimuli and its movement (opening and closing) affect the efficiency of both photosynthesis and respiration. In the dicotyledonous plant *Arabidopsis thaliana*, the formation of a stoma requires at least one asymmetric cell division (ACD) and one symmetric cell division (SCD), which is accompanied by a successive transition of cell fate (Pillitteri and Torii, 2012). Early in the development of young leaves, specific cells (meristemoid mother cells, MMCs) enter into the stomatal lineage and acquire the capability to divide asymmetrically, which produces two daughter cells, a small meristemoid (M) and a large SLGC (stomatal lineage ground cell). Meristemoids can undergo more ACDs (usually 1 to 3 times) to renew itself before the transition into a guard mother cell (GMC) (Nadeau and Sack, 2002). Finally, the GMC divide equally and produce two kidney-like guard cells (GCs) (Bergmann and Sack, 2007). Transcription factors, especially the basic helix-loop-helix (bHLH) family members, play core roles in stomatal development. SPEECHLESS (SPCH), MUTE, and FAMA (bHLH097) function in the successive divisions of stomatal lineage precursors, by forming heterodimers with members of the bHLH family (Pillitteri et al., 2007). The FAMA/SCRMs module regulates the ACD of MMCs and Ms and maintains their self-renewing capability (MacAlister et al., 2015). In addition, crosstalk with NO, H2O2, and other small signaling molecules also play vital roles in CO-mediated stress responses to salt, drought, and heavy metals (Wang and Liao, 2016).

Distribution of stomata on the epidermis obey the rule of "one-cell-spacing", which is ensured by a complex cell-cell signaling pathway (Nadeau and Sack, 2002). Signaling peptides from EPFs (EPIDERMAL PATTERNING FACTOR) and CLVs (CLAVATAS/ESR-RELATED) families are secreted and perceived by receptors from ER (ERECTA) family and co-receptors TMM (TOO MANY MOUTHS) (Hara et al., 2007; Hara et al., 2009; Sugano et al., 2010; Lee et al., 2015; Vate et al., 2018), which initiates a MAPK (MITOGEN-ACTIVATED PROTEIN KINASE) signaling cascade by phosphorylation. The MAPK cascade consists of the MAPKK protein YDA (YODA), MAPKK protein MKK4/5/7/9, and the MAPK proteins MPK3 and MPK6. Activated MPK3/6 can phosphorylate SPCH and decrease its stability (Bergmann et al., 2004; Wang et al., 2007; Lampard et al., 2009). Members from the EPF family play vital roles in stomatal development. EPF1 and EPF2 secreted from epidermal cells act as a negative regulator, while STOMAGEN/EPFL9 from mesophyll acts as a positive regulator of stomatal density (Hara et al., 2009; Hunt and Gray, 2009; Sugano et al., 2010). EPF2 binds with ER-TMM and activates MAPK, which restricts the initiation of stomatal development by phosphorylating SPCH and decreasing its abundance (Bergmann et al., 2004; Lee et al., 2015). STOMAGEN competitively binds to ER-TMM to inhibit the activation of the MAPK cascade triggered by EPF2 (Lee et al., 2012; Lee et al., 2015). Thus, peptides from the same family fine-tune the density and patterning of stomata on the epidermis.

Carbon monoxide (CO), as a signaling molecule, functions in the regulation of multiple plant development processes and stress responses, such as lateral root formation (Guo et al., 2008; Chen and Kao, 2012; Samma et al., 2014), seed germination (Liu et al., 2007; Liu et al., 2010; Jia et al., 2018), cadmium tolerance (Han et al., 2008; Han et al., 2014), salt acclimation (Xie et al., 2011), stomatal movement (Cao et al., 2007; Song et al., 2008; Xie et al., 2016), and boron and iron homeostasis (Wei et al., 2011; Li et al., 2013; Yang et al., 2016; Lv et al., 2017). CO interacts with hormones and other signaling molecules to regulate the above processes. For example, CO promotes the accumulation of IAA during lateral root formation and dependent on the function of NO (Nitric Oxide) in tomato (Guo et al., 2008) and rice (Chen and Kao, 2012). CO can mediate ABA-induced closure of stomata in *Vicia faba* through interaction with NO and ROS (Cao et al., 2007; Song et al., 2008; Xie et al., 2016). In addition, crosstalk with NO, H2O2, and other small signaling molecules also play vital roles in CO-mediated stress responses to salt, drought, and heavy metals (Wang and Liao, 2016).

In plant cells, CO is mainly produced by heme oxidation, which is catalyzed by four HOs (heme oxygenase enzymes) in Arabidopsis (Muramoto et al., 2002; Emborg et al., 2006; Gisk et al., 2009). The four HOs are HY1(HO1), HO2, HO3, and HO4. HO1, encoded by HY1, can decompose heme and produce CO, as well as ions (Fe2+) and biliverdin IXa (BV) (Emborg et al., 2006; Gisk et al., 2009). BV is a precursor of the phytochrome chromophore, which participates in plant photomorphogenesis (Emborg et al., 2006).

Although broadly involved in multiple developmental processes, whether CO participates in the regulation of stomatal development remains unknown. Here we demonstrate that CO acts upstream of YDA to promote stomatal development in Arabidopsis cotyledons. Promotion of CO biosynthesis both endogenously and exogenously results in the increase of SL while blocking of CO production chemically or genetically inhibits stomatal development. CO acts upstream of YDA to promote the stomatal index, as yda-2 is insensitive to CORM-2 treatment. We also found that CO can modulate the expression of EPF2 and STOMAGEN, and through their action regulate stomatal development.
Results

CO promotes stomatal development

To determine whether CO is involved in the regulation of stomatal density and distribution, Arabidopsis wild-type Columbia-0 (Col-0) seedlings were treated with CO donor CORM-2 at concentrations of 0, 10, 20, and 40 μM for 14 days (Figure 1A). We found that the stomatal index (SI) was significantly increased in treated seedlings compared with the control plants. The promotion effect of CORM-2 is concentration-dependent (Figures 1A, B). An increased SI (26.11±2.55) was found at a concentration of 10 μM compared with the control (24.09±1.90). The strongest promotion of SI was observed with 40 μM CORM-2 (33.91±1.90) (Figure 1B). Next, wild-type seedlings (Col-0) were treated with the HY1 inhibitor.
ZnPPiX to suppress CO production, which caused a significant decrease of SI in the wild type (19.87±1.37) (Figures 1A, B). Furthermore, ZnPPiX treatment also reversed the CORM-2-induced increase in SI (23.90±1.92) (Figures 1A, B). These results suggested that the CO donor treatment facilitated stomatal development.

Further experiments showed that endogenous CO has the same effects on the development of stomata. We examined the stomatal phenotypes of hy1-100 (a CO-deficient mutant) and the HY1-OE line, which overexpressed HY1 to generate higher endogenous CO (Xie et al., 2011; Yang et al., 2016). hy1-100 is a loss of function mutant (CS236) which has an A to G substitution in the first intron (Muramoto et al., 1999), and no HY1 protein can be detected in this mutant (Xie et al., 2011). As previously reported, the mutation led to a longer hypocotyl (Figure 2A) in hy1-100 homozygous plants than in wild type seedlings (Xie et al., 2011; Yang et al., 2016). We also found that the SI of hy1-100 (18.85±1.42) was lower than that of wild type (24.09±1.90) (Figures 2B, D). In contrast, the SI was higher in HY1-OE transgenic plants (30.44±2.24) than in wild type (24.09±1.90) (Figures 2B, D). Most importantly, CORM-2 treatment effectively increased the SI in hy1-100 to a level higher than the wild type, which can be effectively reversed by the addition of ZnPPiX (Figures 2C, E). ZnPPiX alone also led to a small decrease of SI in the hy1-100 background (Figures 2C, E), which might be due to the presence of the other HOs in this mutant. In addition, treatment with hemin led to an increased SI in cotyledons of wild type, which can be effectively reversed by the addition of ZnPPiX (Figures 2C, E). ZnPPiX. Compared with the control (in which the number of cells expressing pSPCH:SPCH-GFP was 124.20±30.22 per mm²), more cotyledon epidermal cells (319.80±33.92 per mm²) expressed the marker pSPCH:SPCH-GFP in response to 20 µM CORM-2 treatment, while less cells (32.20±14.04 per mm²) in response to 10 µM ZnPPiX (Figures 4A, B). A third reporter, pFAMA:FAMA-GFP, which expresses in GMC and young GCs (Ohashi-Ito and Bergmann, 2006) was also used for treatment. We found that FAMA-GFP-positive cells also increased in CORM-2-treated cotyledons (234.0±25.18 per mm²) and decrease in ZnPPiX-treated cotyledons (116.20±17.80 per mm²) than in controls (177.60±17.80 per mm²). These results show that exogenous CORM-2 can lead to the increase of the number of stomatal lineage cells on the cotyledon epidermis, while ZnPPiX has the opposite effect.

**CO acts upstream of the SPCH and YDA cascade to promote stomatal development**

The bHLH transcription factor SPCH is needed for the first entry division of stomatal lineage precursors. Without SPCH, as in the loss of function mutant spch-1, no cell can enter into the stomatal lineage leading to the formation of an epidermis with only pavement cells (MacAlister et al., 2007). The promotion of stomatal initiation by CO also needs the function of SPCH, as both CORM-2 and ZnPPiX treatment cannot rescue stomata formation in the spch-1 mutant background (Figure 5A), which also suggested that CO functions upstream of SPCH to promote the initiation of stomatal divisions.

YDA is an upstream regulator of SPCH. It encodes for a MAPKKK protein in the MAPK cascade in stomatal development. YDA can integrate environmental factors with stomatal initiation by phosphorylation of SPCH through its downstream targets MPK3 and MPK6 (Gudesblat et al., 2012; Kim et al., 2012). To detect whether the promotion of stomatal development by CO is dependent on the function of YDA-initiated MAPK signaling, a loss of function mutant of yda-2 was treated with CORM-2 and ZnPPiX. The results showed that yda-2 was insensitive to CORM-2 and ZnPPiX. As shown in Figure 5B, no significant difference of SI was found in yda-2 cotyledons in the presence of CORM-2 and ZnPPiX. These results indicated that CO regulated stomatal development in a manner dependent on the YDA-initiated pathway and functions upstream of the YDA cascade.

TMM-ERs complexes are membrane receptor kinases which bind different ligands from outside and transduce the signaling into the cell though activating the YDA cascade (Nadeau and Sack, 2002; Lee et al., 2015). To detect whether TMM and ERs were required for CO to regulate stomatal development, loss of function mutants of tmm-1, er-105, erl1, and erl2 were treated with CORM-2. CORM-2 treatment induced a slight, but significant
increase of SI in \textit{tmm1} at a concentration of 20 \textmu M, which indicated that \textit{tmm-1} was less sensitive to CORM-2 treatment compared with wild type (Figures 6A, B). The \textit{er-105}, \textit{erl1}, and \textit{erl2} mutants were also less sensitive to CORM-2 treatment because their SIs were less affected by treatment than wild type (Figures 6A, B). The changed sensitivity of the above mutants to CORM-2 indicated that regulation of stomatal development by CO may depend on the function of TMM and ERs.
CO regulates the expression of EPF2 and STOMAGEN

ER and ERL1 form a complex with TMM and specifically bind the peptides from the EPF family to modify the density and distribution of stomata (Lee et al., 2015). Since TMM and ER/ERL1 may function in CO-regulated stomatal development, we asked whether the expression of EPFs was affected by CO. We used the promoters of EPFs to express a GFP marker (pEPF1:GFP, pEPF2:GFP and pSTOMAGEN: nucGFP) to detect the effect of CORM-2 and ZnPPIX on their expression. The expression of pEPF2:GFP was inhibited by CORM-2 and promoted by ZnPPIX treatment (Figures 7A, B). In contrast, there was no difference in pEPF1:GFP expression in the presence of CORM-2 and ZnPPIX (Figures S2A, S2B). Moreover, the expression of
pSTOMAGEN:vecGFP increased after CORM-2 treatment and decreased after ZnPPIX treatment (Figures 7A, C).

Real-time PCR experiments confirmed the function of CO on the EPFs genes expression. A significant increase (by 38.33 %) of STOMAGEN and a decrease (by 53.00 %) of EPF2 were found after CORM-2 treatment in wild type (Figures 7D, E). In contrast, no difference in the expression level of EPF1 was seen with CORM-2 and ZnPPIX treatment (Figure S2B). In accordance with these results, a lower mRNA level of STOMAGEN and a higher expression of EPF2 were found in hy1-100 than in wild type (Figures 7D, E). In addition, exogenous CORM-2 treatment can restore the expression of
FIGURE 5
CO acts upstream of SPCH and YDA during stomatal development. (A) DIC images of the abaxial cotyledon epidermis of 14d old Wild–type (Col-0), spch-1, and yda-2 plants with or without CORM-2 (20µM or 40µM) treatment. Bars = 50 µm. (B) SIs of Wild–type (Col-0), spch-1, and yda-2 plants with or without CORM-2 (20µM or 40µM) treatment. Error bars show standard deviation (SD) (n = 16). Statistical significance of different results was analyzed using Student’s t-test: ns Difference is not significant (p-value > 0.05). ** Difference is highly significant (p-value < 0.01). **** Difference is highly significant (p-value < 0.0001).
**STOMAGEN** and **EPF2** in *hyl-100* to wild type levels (Figures 7D, E). These results showed that CO can promote the expression of **STOMAGEN** and inhibit the expression of **EPF2**.

**CO promotes the stomatal development in a way dependent on EPF2 and STOMAGEN**

Since CO can regulate the expression of **EPF2** and **STOMAGEN**, we then asked whether the promotion of stomatal development by CO was dependent on the function of **EPF2** and **STOMAGEN**. First, a CRISPR/Cas9 system was employed to edit the **STOMAGEN** gene and produce a loss-of-function mutant. The homozygous **stomagen-cr** mutant contained a 1-bp insertion between 798 and 799bp of the coding region (Figure 8A), which caused a decrease of the mRNA level (by 84.34 %) (Figure 8B) and SI (by 7.58 %) on the cotyledon epidermis (Figure 8C), a phenotype similar to previously reported RNAi lines (Sugano et al., 2010). We then treated this mutant with 40 μM CORM-2, and found that it was insensitive to the chemical as no significant change of SI was found after treatment (Figures 8C, D). As well, an **epf2** loss of function mutant was also treated with 40 μM CORM-2 and the SI did not significantly change (Figures 8C, D). Taken together,
FIGURE 7
CO regulates the expression of EPF2 and STOMAGEN. (A) Confocal images of the abaxial cotyledon epidermis of 4d old Wild–type (Col-0) harboring pEPF2:GFP and pSTOMAGEN:nucGFP with or without CORM-2 (20 μM) treatments and ZnPPIX (10 μM) treatment. Bars = 50 μm. (B, C) Number of GFP-positive cells on the cotyledon epidermis of 4d old Wild–type (Col-0) plants expressing pEPF2:GFP (B) and pSTOMAGEN:nucGFP (C). Error bars show standard deviation (SD) (n = 5). (D, E) Relative mRNA level of EPF2 (D) and STOMAGEN (E) in Wild–type (Col-0) and hy2–100 seedlings (4dap) with or without CORM-2 (20 μM) treatments and ZnPPIX (10 μM) treatment. Error bars show standard deviation (SD) (n = 3). Statistical significance of different results was analyzed using Student’s t-test: ns Difference is not significant (p-value > 0.05). ** Difference is highly significant (p-value < 0.01). *** Difference is highly significant (p-value < 0.001). **** Difference is highly significant (p-value < 0.0001).
FIGURE 8
Regulation of stomatal development by CO is dependent on the function of EPF2 and STOMAGEN. (A) Insertion site of CRISPR-Cas9 line of the stomagen mutant. (B) Expression level of STOMAGEN in Wild-type (Col-0) and stomagen-cr mutant produced by CRISPR-Cas9. Error bars show standard deviation (SD) (n = 3). (C) DIC images of the abaxial cotyledon epidermis of 14d old Wild-type (Col-0), epf2, and stomagen-cr plants with or without CORM-2 (40 μM) treatment. Bars = 50 μm. (D) SIs of Wild-type (Col-0), epf2, and stomagen-cr plants with or without CORM-2 (40 μM) treatments. Error bars show standard deviation (SD) (n = 16). Statistical significance of different results was analyzed using Student’s t-test. ns Difference is not significant (p-value > 0.05); **** Difference is highly significant (p-value < 0.0001).
these results verified that CO regulates stomatal development in a way dependent on the function of EPF2 and STOMAGEN.

Discussion

CO is a positive regulator of stomatal development

Mounting evidence indicates that CO can participate in a variety of developmental processes and stress responses in plants (Wang and Liao, 2016). For instance, CO positively affects seed germination (Liu et al., 2007; Liu et al., 2010; Jia et al., 2018) and lateral root formation (Guo et al., 2008; Chen and Kao, 2012; Samma et al., 2014), and protects plant against heavy metals (Han et al., 2008; Wei et al., 2011; Zheng et al., 2011; Yang et al., 2016), osmotic stress (Liu et al., 2010; Xie et al., 2011) and oxidative stress (Wu et al., 2010). Stomata are small pores through which plants exchange water and gases with the atmosphere, and both environmental factors and gaseous signaling molecules can modulate their development (Wu et al., 2010; Fu et al., 2016; Han et al., 2018; Deng et al., 2020). CO has been extensively reported to affects stomatal movement (She and Song, 2008; Song et al., 2008). However no reports focus on the effect of CO on stomatal development. In this study, we report that CO can promote stomatal initiation to increase stomatal index. Treatment with CORM-2 facilitates a dose-dependent increase of stomatal index (Figures 1A-C). CORM-2 treatment also promotes the expression of marker genes, and GMC- and GC-expressed pSPCH:SPCH-GFP and pTMM:TMM-GFP, and GMC- and GC-expressed pFAMA:FAMA-GFP (Figure 4). In contrast, treatment with the CO biosynthesis inhibitor ZnPPIX and a mutation of HY1 that disrupted CO generation dampened stomatal development in Arabidopsis cotyledons (Figures 1, 2B). In addition, the restoration of the capacity for CO generation through CORM-2 restored a wild type stomatal index in the hy1-100 mutant plants (Figures 2C, E).

Possible regulation mechanism of CO in stomatal development

In Arabidopsis, peptides from the EPFs family are perceived by membrane-located receptors to fine-tune the initiation and distribution of stomata (Lee et al., 2012; Jewaria et al., 2013; Lee et al., 2015). Environmental factors and hormonal signaling molecules regulate stomatal initiation by mediating the expression of EPFs (Engineer et al., 2014; Zhang et al., 2014; Hepworth et al., 2015; Caine et al., 2016; Wang et al., 2021). EPF2 is perceived by ER and its co-receptor TMM. Binding of EPF2 with the ER-TMM complex triggers rapid phosphorylation of the downstream of MAPK cascade (Bergmann et al., 2004; Lee et al., 2012; Lee et al., 2015). STOMAGEN can competitively bind to ER-TMM to replace EPF2, and diminish the downstream activation of MAPK elicited by EPF2 binding (Lee et al., 2015). In this study, we found that CO regulates the expression of STOMAGEN and EPF2. First, CORM-2 treatment downregulated EPF2 expression and upregulated STOMAGEN expression in the wild type (Figures 7B, C). Secondly, the expression level of EPF2 was higher in hy1-100 than in the wild type (Figure 7D). A lower expression level of STOMAGEN in hy1-100 compared to the wild type were also detected (Figure 7E). Third, the higher expression of EPF2 and lower expression of STOMAGEN in hy1-100, were rescued by treatment with CORM-2 (Figures 7D, E). Fourth, loss of function mutants of epf2 and stoma were insensitive to CORM-2 treatment (Figures 8G, D), which confirmed that CO needs the functions of EPF2 and STOMAGEN to promote stomatal development. In conclusion, our data suggest that CO plays a positive role in the regulation of stomatal initiation in Arabidopsis cotyledons via modulating the expression of EPF2 and STOMAGEN.

HY1 is involved in the regulation of two pathways for stomatal development in Arabidopsis

HY1 plays a crucial role in the endogenous production of CO, as HY1, encoding a heme oxygenase, degrades hemin producing Fe^{2+}, BV, and CO. In this report, we precluded the possibility that the effect of HY1 on stomatal development is due to the byproducts Fe^{2+} and BV (Figure S1). Moreover, a previous study reported that the hy1-100 mutant has a higher JA level than wild type (Zhai et al., 2007), and JAs negatively regulates stomatal initiation in Arabidopsis cotyledons (Han et al., 2018). Therefore, based on our results and these reports, we propose the possible regulation mechanism of HY1 catalyzed hemin oxidation in stomatal development as shown in Figure 9. On one hand, as a product of heme oxidation catalyzed by HY1 in the chloroplast, CO promotes the expression of STOMAGEN in mesophyll cells and also diffuses to epidermal cells where it inhibits the expression of EPF2. Less EPF2 from epidermal cells and more STOMAGEN synergistically release downstream inhibition of SPCH through the MAPK pathway. On the other hand, HY1 inhibits the accumulation of JAs and removes the inhibition of JAs in stomatal initiation. In addition, JAs inhibits the expression of HY1 conversely (Zhai et al., 2007), which leads to the formation of a feedback loop between the production of JAs and HY1 activity. In the wild type, the formation of the feedback loop fine-tunes the expression level of SPCH and thus the stomatal production. In the hy1 mutant, the block of this feedback resulted in low levels of CO and overaccumulation of JAs which synergistically inhibit the formation of stomata on the cotyledon epidermis. In our previous research, another gaseous
signaling molecule, H\textsubscript{2}S, also regulates stomata through JA signaling (Deng et al., 2020). Therefore, JA signaling is important to regulate stomata development through gaseous signaling molecules. Nevertheless, the mechanism underlying the crosstalk of JA and CO/H\textsubscript{2}S remains largely unknown.

Moreover, CO is also involved in the regulation of darkness-induced NO synthesis (She and Song, 2008), and the crosstalk between CO and NO has been shown in seed germination regulation (Liu et al., 2010). As a positive regulator, NO can promote the expression of SPCH, MUTE, and SCR2 and inhibit the expression of MPK6 (Fu et al., 2016). Furthermore, CO is also involved in IAA-regulated adventitious root development (Xuan et al., 2008). A previous study reported that the auxin-related transcription factor ARF5/MONOPTEROS (MP) can repress the expression of STOMAGEN by directly binding to its promoter (Zhang et al., 2014). While little is known about the cross talk between CO and auxin in stomatal development, it is reasonable to deduce that CO may regulate the expression of STOMAGEN through the ARF5-mediated auxin pathway, a hypothesis which requires further experimental verification. As well, further investigation is required to establish the relationship between CO and auxin through the EPF family, which will provide insights into the complicated regulatory mechanism governing the initiation of stomata.

Materials and methods

Materials and plant growth conditions

*Arabidopsis thaliana* plants were grown on half-strength Murashige and Skoog (MS) medium at 22°C under a 16-h light/8-h dark photoperiod. Chemicals were purchased from the Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China). COMR-2 and ZnPPIX were obtained from Sigma (St. Louis, Missouri, USA), and Taq DNA polymerases were from Takara Biotechnology (Dalian, China). The mutants used in this study are: *tmm-1* (Yang and Sack, 1995), *spch-1* (MacAlister et al., 2007), *yda-2* (CS6393, Lukowitz et al., 2004), *epf2* (Hara et al., 2007), and *35S:HY1* transgenic plants (Xie et al., 2011). Transgenic plants with stomatal lineage-specific markers (*pTMM:TMM-GFP*, *pSPCH:SPCH-GFP*, *pFAMA:FAMA-GFP*, *pEPF1:GFP*, and *pEPF2:GFP*) were obtained from Fred Sack (University of British Columbia). The mutants *hyl-100* (CS236), *er-105* (CS6588), *erl1* (SALK_08169C), and *erl2* (SALK_144166C) were from the Nottingham Arabidopsis Stock Centre (NASC).

Chemical treatment

Stock solution (at 10 mM) of COMR-2 and ZnPPIX (Sigma) were dissolved in DMSO (dimethylsulphoxide). Final use
concentration solutions were made by diluting stocks with sterilized medium. In this study, 10, 20, and 40 μM were used as final concentrations for CORM-2, and 10 μM for ZnPPIX. An equivalent concentration of DMSO was added in the plates as control. Seeds were sterilized and germinated on half-MS medium supplemented with or without chemicals. All the treatments have at least three replicates.

Microscopy

An OLYMPUS BX51 microscope was used to take DIC images from samples which were stored in Hoyer’s solution. The 14 dag (day after germination) cotyledons were used for observation. The clearing of samples was as follows: first seedlings were immersed in 70% ethanol. After being cleared overnight at room temperature, samples were transferred into the Hoyer’s solution for storage. To observe the expression of marker genes, a ZEISS LSM800 laser confocal scanning microscope was used for shoot GFP fluorescence images and PI (propidium iodide) (Sigma-Aldrich) staining. Confocal images were cleared using magenta for the PI channel.

Stomatal index measurement

An OLYMPUS BX51 microscope was used to visual the samples placed in water. Stomatal index (SI) is defined as the ratio of stomata and stomatal lineage precursors (MMC and M) in total epidermal cells per unit area. For stomatal index, number of stomata, stomatal lineage precursors (MMC and M) and all epidermal cells of 0.37 mm² were counted to acquire a SI value for different cotyledons from different plants. Cotyledons from at least eight different plants were selected from the used genotypes.

RNA extraction and qRT-PCR

Total RNA extraction and qRT-PCR were performed as described previously (Han et al., 2018; Deng et al., 2020). Trizol reagent (Invitrogen) was used to extract total RNA. For qRT-PCR, 1 μg DNase-treated RNA in 20 μl reaction volumes were used to synthesis the first-strand using M-MuLV reverse transcriptase (Monad, China) with oligo (dT)18 primer. 2×TB Green Premix Ex Taq II (Tli RNaseH) was used to perform PCR on an Applied Biosystems QuantStudio™ 6 flex system real-time PCR machine, according to the manufacturer’s instructions. Three replicates for each sample were used for qRT-PCR analysis. The Actin gene was used as an internal control. Gene-specific primers used to detect transcripts are listed in Supplemental Table S1.

Construction of plasmids

For the construction of stomagen-cr lines, target sites were designed by the use of CRISPR-GE to avoid off-targets (http://skl.scau.edu.cn; Ma et al., 2015). The editing vectors were constructed as described in a previous report (Zhang et al., 2019). The sgRNA containing a single target site driven by the AtU3b promoter was cloned into the pMH-SA vector by the restriction enzyme sites Ascl. Homozygous mutant lines were identified by sequencing.

For the construction of pSTOMAGEN:nucGFP, the 2,000 bp promoter sequence of AT4G12970 (STOMAGEN) was amplified by PCR and cloned in front of nucGFP in the Poca30 binary vector. The primers and restriction enzyme sites used to amplify sequences and generate vectors are listed in Supplemental Table S1.

Accession numbers

Arabidopsis Genome Initiative numbers for the genes used in this article are as follows: HY1 (AT2G26670); TMM (AT1G80080); ER (AT2G26330); ERL1 (AT5G62230); ERL2 (AT5G07180); YODA (AT1G63700); SPCH (AT5G53210); FAMA (AT3G24140); EPF1 (AT2G20875); EPF2 (AT1G34245); STOMAGEN (AT4G12970) and ACTIN (AT3G18780).

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

LJZ and XLC designed experiments. XJW, LYZ, SSY, YL, YYR and ZJZ performed experiments. XJW, LJZ, SSY and ZRH analyzed data. XLC, LYZ, and LJZ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1029703/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Fe-EDTA and BV have no effect on the stomatal development of wild type. (A) DIC images of the abaxial cotyledon epidermis of 14d old Wild-type (Col-0) plants with or without Fe-EDTA (40 μM). BV (20 μM) and CORM-2 (40 μM). Bars = 50 μm. (B) Sts of Wild-type (Col-0) plants with or without Fe-EDTA (40 μM), BV (20 μM) and CORM-2 (40 μM) treatments. Error bars show standard deviation (SD) (n = 8). Statistical significance of different results was analyzed using Student’s t-test: ns Difference is not significant (p-value > 0.05). **** Difference is highly significant (p-value < 0.0001).

SUPPLEMENTARY FIGURE 2
Expression of EPF1 is not affected by CO. (A) Confocal images of the abaxial cotyledon epidermis of 4 d old Wild-type (Col-0) harboring pEPF1:GFP with or without CORM-2 (20 μM) and ZnPPIX (10 μM) treatment. Bars = 50 μm. (B) Number of GFP-positive cells on the cotyledon epidermis of 4d old Wild-type (Col-0) plants expressing pEPF1:GFP with or without CORM-2 (20 μM) and ZnPPIX (10 μM) treatment. Error bars show standard deviation (SD) (n = 5). (C) Expression of EPF1 in Wild-type (Col-0) and hy1-100 with or without CORM-2 (20 μM) and ZnPPIX (10 μM) treatment. Error bars show standard deviation (SD) (n = 3). Statistical significance of different results was analyzed using Student’s t-test: ns Difference is not significant (p-value > 0.05).

SUPPLEMENTARY TABLE 1
Primers used in this study.

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