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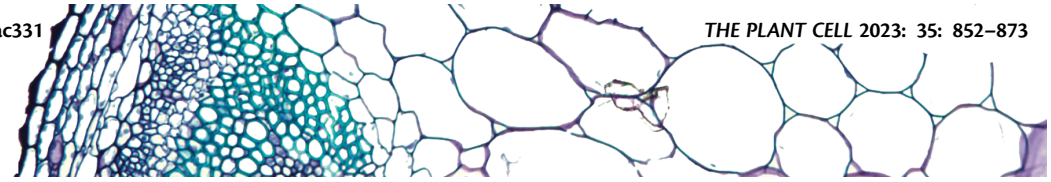

Research Article

CO interacts with JAZ repressors and bHLH subgroup IIId factors to negatively regulate jasmonate signaling in Arabidopsis seedlings

Xiao Han [1,2,†] Mengyi Kui [1,2,3,†] Tingting Xu [1,2,3] Jingwen Ye [1,4] Jiancan Du [1,2] Milian Yang [1,2] Yanjuan Jiang [1,2] and Yanru Hu [1,2,*]

1 CAS Key Laboratory of Tropical Plant Resources and Sustainable Use, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming, Yunnan 650223, China
2 Center of Economic Botany, Core Botanical Gardens, Chinese Academy of Sciences, Kunming, Yunnan 650223, China
3 University of Chinese Academy of Sciences, Beijing 100049, China
4 School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230027, China

*Author for correspondence: huyanru@xtbg.ac.cn (Y.H.)
†These authors contributed equally to this work.

Y.H., X.H., and M.K. designed this study and wrote the article; X.H., M.K., T.X., J.Y., J.D., M.Y., Y.J., and Y.H. performed experiments or interpreted data; all authors approved the final article.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (https://academic.oup.com/plcell/) is: Yanru Hu (huyanru@xtbg.ac.cn).

Abstract

CONSTANS (CO) is a master flowering-time regulator that integrates photoperiodic and circadian signals in *Arabidopsis thaliana*. *CO* is expressed in multiple tissues, including young leaves and seedling roots, but little is known about the roles and underlying mechanisms of CO in mediating physiological responses other than flowering. Here, we show that *CO* expression is responsive to jasmonate. CO negatively modulated jasmonate-imposed root-growth inhibition and anthocyanin accumulation. Seedlings from *co* mutants were more sensitive to jasmonate, whereas overexpression of *CO* resulted in plants with reduced sensitivity to jasmonate. Moreover, CO mediated the diurnal gating of several jasmonate-responsive genes under long-day conditions. We demonstrate that CO interacts with JASMONATE ZIM-DOMAIN (JAZ) repressors of jasmonate signaling. Genetic analyses indicated that CO functions in a CORONATINE INSENSITIVE1 (COI1)-dependent manner to modulate jasmonate responses. Furthermore, CO physically associated with the basic helix-loop-helix (bHLH) subgroup IIId transcription factors bHLH3 and bHLH17. CO acted cooperatively with bHLH17 in suppressing jasmonate signaling, but JAZ proteins interfered with their transcriptional functions and physical interaction. Collectively, our results reveal the crucial regulatory effects of CO on mediating jasmonate responses and explain the mechanism by which CO works together with JAZ and bHLH subgroup IIId factors to fine-tune jasmonate signaling.

Introduction

The phytohormone jasmonate is a signaling molecule that is essential for regulating a variety of physiological processes (Chini et al., 2016; Howe et al., 2018; Guo et al., 2018a; Zhou et al., 2019; Wasternack, 2020; Cao et al., 2022). Mutations blocking jasmonate biosynthesis or signaling adversely affect several developmental programs and stress responses in Arabidopsis (*Arabidopsis thaliana*), such as root-growth inhibition, stamen development, trichome formation, and anthocyanin accumulation (Chini et al., 2016; Howe et al., 2018). Jasmonate is perceived by the F-box protein CORONATINE INSENSITIVE1 (COI1), which binds to

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ASK1 and ASK2 (Arabidopsis SKP1-like), CULLIN1, and RING-BOX1 (RBX1) to form the SCF^{COI1} complex (Xie et al., 1998; Xu et al., 2002; Liu et al., 2004; Ren et al., 2005; Yan et al., 2009). After jasmonate is perceived, the SCF^{COI1} complex recruits JASMONATE ZIM-DOMAIN (JAZ) proteins, which are critical repressors of jasmonate signaling, for their subsequent degradation via the 26S proteasome pathway (Chini et al., 2007; Thines et al., 2007; Sheard et al., 2010). The removal of JAZ proteins alleviates their repressive effect on downstream components to modulate various jasmonate responses (Fonseca et al., 2009; Fernández-Calvo et al., 2011; Kazan and Manners, 2013; Guo et al., 2018b).

JAZ repressors negatively regulate jasmonate signaling by interacting with and inhibiting multiple transcription factors. For example, the basic helix-loop-helix (bHLH) subgroup IIIe transcription factors MYC2, MYC3, and MYC4, which are the most extensively characterized JAZ-binding factors, control a subset of jasmonate-related processes, such as the inhibition of root elongation and stress responses (Boter et al., 2004; Lorenzo et al., 2004; Dombrecht et al., 2007; Fernández-Calvo et al., 2011; Schweizer et al., 2013; Qi et al., 2015; Liu et al., 2019; Wang et al., 2019; You et al., 2019). Several bHLH subgroup IIId transcription factors (bHLH3, bHLH13, bHLH14, and bHLH17) also physically interact with JAZ proteins and suppress jasmonate responses (Nakata et al., 2013; Sasaki-Sekimoto et al., 2013; Song et al., 2013a; Fonseca et al., 2014). Recently, we revealed that JAZ proteins directly interact with and inhibit the activities of the transcription factors ROOT HAIR DEFECTIVE 6 (RHD6) and RHD6-LIKE1 (RSL1) to regulate jasmonate-stimulated root hair development (Han et al., 2020). Furthermore, the interacting partners of JAZ repressors include several critical transcriptional regulators, such as ETHYLENE INSENSITIVE3 (EIN3), FILAMENTOUS FLOWER (FIL), TARGET OF EAT1 (TOE1), and ABSCISIC ACID INSENSITIVE5 (ABI5) (Fernández-Calvo et al., 2011; Qi et al., 2011; Song et al., 2011; Zhu et al., 2011; Hu et al., 2013; Jiang et al., 2014; Boter et al., 2015; Zhai et al., 2015; Ju et al., 2019; Pan et al., 2020). Although diverse transcription factors involved in jasmonate responses have been identified, the transcriptional regulation underlying jasmonate signaling-related processes has not been comprehensively characterized. Further clarifying the crucial JAZ-interacting regulators and elucidating the biological significance of their interactions may provide new insights into the molecular basis of the tight modulation and fine-tuning of jasmonate signaling networks.

B-box (BBX) proteins are a class of zinc-finger transcription factors that contain a conserved BBX domain in their N-terminal regions with or without a CCT (CONSTANS, CO-like, and TIMING OF CAB2 EXPRESSION1 [TOC1]) domain at the C terminus (Gangappa and Botto, 2014). Both the N-terminal and C-terminal regions of BBX proteins are involved in protein–protein interactions, DNA binding, or their transcriptional function (Gangappa and Botto, 2014). The BBX family of transcription factors controls a wide range of physiological processes, including the photoperiodic regulation of flowering, shade avoidance, seedling photomorphogenesis, and stress responses (Sarmiento, 2013; Gangappa and Botto, 2014). CONSTANS (CO, also named BBX1), which is the most well-known member of the BBX family, is a master regulator of flowering in the photoperiodic and circadian pathways (Putterill et al., 1995; Simon et al., 1996; Suárez-López et al., 2001; Takada and Goto, 2003; An et al., 2004; Song et al., 2013b, 2015; Shim et al., 2017). Under long-day conditions, CO directly stimulates the transcription of *FLOWERING LOCUS T* (*FT*), which encodes a floral induction-related long-distance signal, to determine flowering time in Arabidopsis (Samach et al., 2000; Corbesier et al., 2007; Tiwari et al., 2010; Song et al., 2013b; Shim et al., 2017). Further research revealed that CO functions in the vascular tissue (phloem) to activate *FT* expression and trigger photoperiodic flowering (An et al., 2004; Shim et al., 2017). The expression of *CO* under the control of a phloem-specific promoter was sufficient for inducing early flowering and rescuing the late-flowering phenotype of *co* mutants (An et al., 2004). Interestingly, spatial expression pattern analyses showed that *CO* is expressed in multiple tissues, including the shoot apex, young leaves, and seedling roots (Takada and Goto, 2003; An et al., 2004). These observations suggest that CO may contribute to the regulation of other physiological or developmental processes in addition to flowering.

The objective of this study was to identify additional CO regulatory functions and to characterize the molecular mechanisms by which CO mediates jasmonate signaling. We initially confirmed the spatial pattern of *CO* expression and detected its transcripts in young leaves and seedling roots. We also observed that the *CO* transcription level decreases in seedlings treated with methyl jasmonate (MeJA). Phenotypic analyses indicated that CO negatively regulates jasmonate-induced root-growth inhibition and anthocyanin accumulation. Compared to the wild-type control, loss-of-function *co* mutants exhibited greater MeJA-induced root-growth inhibition and anthocyanin accumulation. Conversely, *CO* overexpression decreased the sensitivity of transgenic seedlings to MeJA. Moreover, CO was involved in mediating the diurnal gating of several jasmonate-responsive genes under long-day conditions. Additionally, CO was able to interact with several JAZ repressors of jasmonate signaling. Genetic analyses demonstrated that the MeJA-hyposensitive phenotype of *myc2-1* is attenuated by the *CO* mutation; however, there were no obvious differences between *coi1-2* (which overaccumulates JAZ proteins) and *co-1 coi1-2* mutants, suggesting that CO and MYC2 have the opposite effects and CO functions in a COI1-dependent manner to modulate jasmonate responses. Furthermore, CO physically interacted with the bHLH subgroup IIId transcription factors bHLH3 and bHLH17. Further analyses showed that CO works cooperatively with bHLH17 in repressing jasmonate signaling, and that JAZ proteins interfere with their transcriptional functions and physical association. Collectively, our results indicate

that CO is a critical negative regulator of jasmonate-induced root-growth inhibition and anthocyanin accumulation. The data presented herein may be useful for clarifying how CO functions together with JAZ repressors and bHLH subgroup IIId transcription factors to precisely control jasmonate signaling.

Results

CO expression is responsive to jasmonate

CO is a major positive regulator of flowering in the photoperiodic pathway (Putterill et al., 1995; Simon et al., 1996; Samach et al., 2000; Takada and Goto, 2003; An et al., 2004; Shim et al., 2017). More specifically, *CO* expression in the vascular tissue (phloem) promotes flowering (An et al., 2004). Because *CO* is expressed in multiple tissues, such as young leaves and seedling roots (Takada and Goto, 2003; An et al., 2004), we speculated that CO might be involved in other physiological or developmental processes besides flowering. We first determined the spatial expression pattern of *CO*. To this end, we cloned a *CO* promoter fragment (*proCO*; 3,576 bp) upstream of the *β-glucuronidase* reporter gene (*GUS*) to generate a reporter construct (*ProCO:GUS*), which we introduced into wild-type Columbia-0 (Col-0) plants. Consistent with the results of previous studies (Takada and Goto, 2003; An et al., 2004), GUS staining revealed *CO* promoter activity in the root and shoot apexes, young leaves, and seedling roots (Figure 1A).

Interestingly, a microarray-based analysis of Arabidopsis had suggested that *CO* transcription was repressed by jasmonate (Winter et al., 2007). To test the regulatory effect of jasmonate on *CO* transcription, we analyzed *CO* promoter activity on the basis of GUS staining and reverse transcription quantitative PCR (RT-qPCR) experiments. The GUS signal and *GUS* transcript levels decreased following exogenous MeJA treatment (Figure 1, A and B). We also performed RT-qPCR analyses to examine *CO* transcript levels in wild-type seedlings with or without MeJA treatments and observed that *CO* transcript levels decrease in response to MeJA treatment (Figure 1C). We then asked whether *CO* transcript levels are affected by critical components related to endogenous jasmonate signaling. The F-box protein COI1 is the jasmonate receptor that positively modulates jasmonate responses, whereas JAZ repressors are crucial negative regulators of jasmonate signaling (Xie et al., 1998; Chini et al., 2007; Thines et al., 2007; Yan et al., 2009). We thus examined *CO* transcript levels in *coi1* mutants and transgenic plants overexpressing *JAZ1* with a deleted Jas-encoding domain (*JAZ1-ΔJas*; Han et al., 2018). Relative *CO* transcript levels were higher in the *coi1* mutants and *JAZ1-ΔJas* seedlings than in the wild type (Figure 1D). By contrast, *CO* expression levels were lower in the quintuple mutant *jazQ* (which lacks five JAZ repressors; Campos et al., 2016) than in the wild-type control (Figure 1D). Taken together, these results suggest that jasmonate represses *CO* transcription.

Previous studies have revealed that the stability of CO is strictly regulated by various signaling pathways at the post-translational level (Valverde et al., 2004; Jang et al., 2008; Liu et al., 2008; Lazaro et al., 2012; Song et al., 2012, 2014a; Zhang et al., 2015; Hayama et al., 2017). Hence, we investigated whether jasmonate also affected CO abundance. Specifically, we generated transgenic plants (*35S:3Myc-CO*) overexpressing a sequence encoding a fusion protein between full-length CO and the 3×Myc tag under the control of the cauliflower mosaic virus (CaMV) 35S promoter (*Pro35S*; Supplemental Figure S1A). Similar to the results of earlier studies (Valverde et al., 2004; Liu et al., 2008), we detected a diurnal pattern in CO protein abundance (Figure 1E). Furthermore, we observed that CO protein accumulation clearly increases in response to MeJA (Figure 1E). Moreover, the abundance of CO further rose in seedlings treated with both MeJA and the proteasome inhibitor MG132 compared to that in seedlings treated with only MeJA or MG132 (Figure 1E), suggesting that MeJA stimulates the production of CO. However, CO protein accumulation was similar in seedlings treated with the protein synthesis inhibitor cycloheximide (CHX) with or without MeJA (Figure 1E), suggesting that MeJA may have little effect on CO degradation.

Loss of CO function stimulates jasmonate-induced root-growth inhibition and anthocyanin accumulation

Because *CO* expression is responsive to jasmonate, we hypothesized that CO may help mediate responses to jasmonate. To test this possibility, we initially evaluated jasmonate-induced root-growth inhibition in the loss-of-function mutants *co-1* and *co-9* (Col-0 background) grown on Murashige and Skoog (MS) medium with or without 30-μM MeJA under long-day conditions (16-h light/8-h dark). The roots of *co-1* and *co-9* seedlings grown on MeJA-containing medium were significantly shorter than those of wild-type seedlings (Figure 2, A and B); however, in the absence of MeJA, the root lengths of the *co* mutants and the wild-type control were similar. In response to MeJA treatment, anthocyanin contents were higher in the *co-1* and *co-9* mutants than in the wild type (Figure 2C). To confirm the MeJA-induced phenotypes of the *co-1* and *co-9* mutants, we examined the transcript levels of several jasmonate-responsive genes in MeJA-treated *co-1* and *co-9* seedlings: *JAZ2*, *LIPOXYGENASE2* (*LOX2*), and *LEUCOANTHOCYANIDIN DIOXYGENASE* (*LDOX*). After MeJA treatment, relative *JAZ2*, *LOX2*, and *LDOX* transcript levels were higher in the *co-1* and *co-9* seedlings than in the wild-type control (Figure 2D), indicating that CO negatively modulates the transcript levels of several jasmonate-responsive genes, possibly by modulating their transcription.

To further analyze the effects of the loss of CO function on jasmonate signaling, we investigated the performances of the *co-4* and *co-6* mutants (Landsberg *erecta* [*Ler*] background) grown on MS medium containing 30-μM MeJA under long-

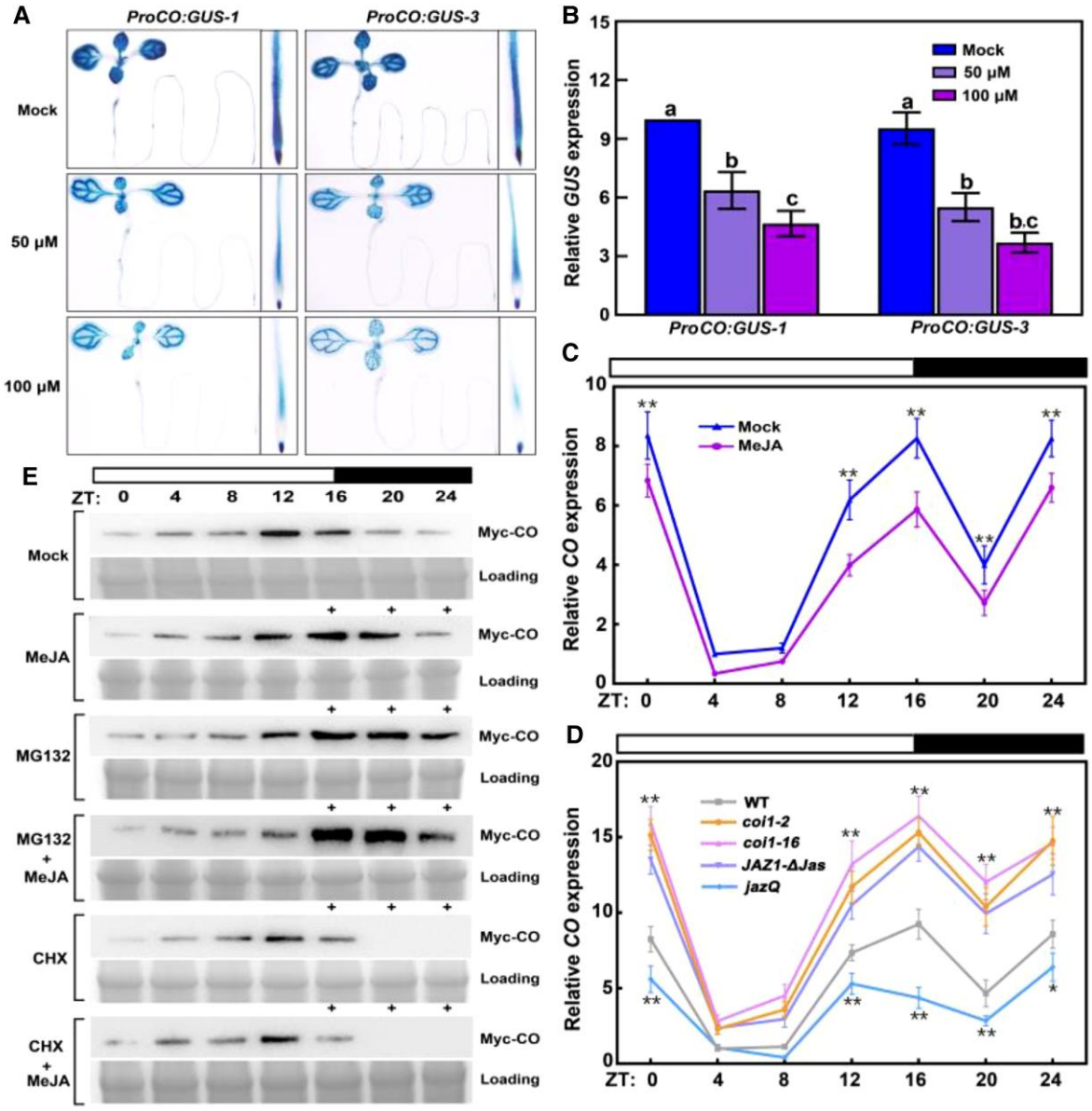

Figure 1 Expression patterns of *CO* in response to jasmonate. A, GUS staining of *ProCO:GUS* transgenic seedlings. Eight-day-old *ProCO:GUS-1* and *ProCO:GUS-3* seedlings grown in long days were treated with or without (mock) 50- or 100-μM MeJA for 4 h, and then the samples were harvested at ZT 16 for staining. In the mock treatment, an equal volume of 10% (v/v) ethanol was added. Experiments were performed three times with similar results. B, RT-qPCR analysis of relative *GUS* transcript levels in *ProCO:GUS-1* and *ProCO:GUS-3* seedlings. Total RNA was extracted from seedlings grown and treated as in (A). Relative *GUS* expression level of *GUS* in mock-treated *ProCO:GUS-1* seedlings was set to 10. Values are means ± SD from eight independent biological replicates ($n = 8$). Different lowercase letters indicate significant differences ($P < 0.05$) based on a two-way ANOVA (genotype × treatment interaction). C, RT-qPCR analysis of jasmonate-regulated *CO* expression in wild-type (WT, Col-0) seedlings. Total RNA was extracted from Col-0 seedlings grown on MS medium with or without (mock) 30-μM MeJA for 8 days under long-day conditions. The samples were harvested from ZT0 to ZT24. Time is expressed as h from dawn (ZT0). *CO* expression levels in mock-treated seedlings at ZT4 were set to 1. Values are means ± SD from eight independent biological replicates ($n = 8$). $^{**}P < 0.01$ as determined by a one-way ANOVA compared to Col-0. D, RT-qPCR analysis of *CO* expression in Col-0, *coi1-2*, *coi1-16*, *jazQ*, and *JAZ1-ΔJas* seedlings. Eight-day-old Col-0 and mutant seedlings grown in long days on MS medium were harvested from ZT0 to ZT24. *CO* expression levels in Col-0 seedlings at ZT4 were set to 1. Values are means ± SD from eight independent biological replicates ($n = 8$). $^{*}P < 0.05$; $^{**}P < 0.01$ as analyzed by a one-way ANOVA compared to Col-0. E, Involvement of MeJA in CO abundance. Whole proteins were extracted from 8-day-old *35S:3Myc-CO-1* transgenic seedlings grown in long days (harvested from ZT0 to ZT24) with or without (mock) MeJA and/or the proteasome inhibitor (MG132) or protein synthesis inhibitor CHX treatment. For MeJA and/or MG132 or CHX treatments, seedlings were treated with 100-μM MeJA and/or 100-μM MG132 or 100-μM CHX for 4 h (+), and then the samples were harvested at ZT16, ZT20, and ZT24. CO was detected by immunoblotting with an anti-Myc antibody (1:10,000) and a secondary antibody (goat anti-mouse, 1:10,000). Experiments were performed three times with similar results.

day conditions. Analyses of jasmonate-regulated root growth and anthocyanin accumulation revealed the enhanced jasmonate responses of *co-4* and *co-6*. Indeed, compared to seedlings from the wild-type L*er*, the *co-4* and *co-6* mutants had shorter roots and accumulated more anthocyanin in the presence of MeJA (Figure 2, E–G). In response to MeJA treatment, relative *JAZ2*, *LOX2*, and *LDOX* transcript levels were consistently higher in the *co-4* and *co-6* mutants than in L*er* seedlings (Supplemental Figure S2). These findings suggest that CO may negatively modulate jasmonate-induced root-growth inhibition and anthocyanin accumulation under long-day conditions.

Because jasmonate signaling is affected by photoperiod (Cagnola et al., 2018), we further analyzed the responses of *co-1* and *co-9* seedlings grown under short-day conditions (8-h light/16-h dark) to MeJA treatment. Seedlings from *co-1*, *co-9*, and the wild type were similarly affected by MeJA under short-day conditions (Supplemental Figure S3). Moreover, the MeJA-induced expression of *LOX2* and *LDOX* in *co-1* seedlings was similar to that in the wild-type control (Supplemental Figure S3). Accordingly, CO may not substantially affect jasmonate signaling under short-day conditions.

Overexpression of *CO* attenuates jasmonate-mediated root-growth inhibition and anthocyanin accumulation

Having demonstrated that *co* mutants exhibit enhanced jasmonate signaling under long-day conditions, we analyzed whether *CO* overexpression had inhibitory effects on jasmonate responses. We selected homozygous transgenic lines overexpressing *CO* (*35S:3Myc-CO-1* and *35S:3Myc-CO-3*) for phenotypic analyses (Supplemental Figure S1A). Consistent with previous studies (Putterill et al., 1995; Simon et al., 1996; Suárez-López et al., 2001; Wang et al., 2016), we observed that the T_5 progeny of *35S:3Myc-CO-1* and *35S:3Myc-CO-3* plants flowered earlier than wild-type plants. We examined jasmonate-regulated root elongation and anthocyanin accumulation in the *35S:3Myc-CO-1* and *35S:3Myc-CO-3* seedlings: their roots were much longer than those of the wild type after a 30-μM MeJA treatment under long-day conditions (Figure 3, A and B). Moreover, following MeJA treatment, anthocyanin contents were lower in the *35S:3Myc-CO-1* and *35S:3Myc-CO-3* seedlings compared to the wild type (Figure 3C). To confirm these observations, we analyzed the transcript levels of several jasmonate-responsive genes, including *JAZ2*, *LOX2*, and *LDOX*, in MeJA-treated *35S:3Myc-CO-1* and *35S:3Myc-CO-3* samples. The relative transcript levels of these jasmonate-inducible genes were lower in the *35S:3Myc-CO-1* and *35S:3Myc-CO-3* seedlings than in the wild-type control (Figure 3D). Thus, *CO* overexpression decreases the sensitivity of the transgenic seedlings to MeJA. These findings provide further evidence that CO negatively regulates jasmonate-induced root-growth inhibition and anthocyanin accumulation under long-day conditions.

CO mediates the diurnal gating of several jasmonate-responsive genes under long-day conditions

Multiple studies have confirmed that jasmonate signaling is modulated by the circadian clock (Goodspeed et al., 2012; Shin et al., 2012; Nitschke et al., 2016; Zhang et al., 2018; Thines et al., 2019). For example, Shin et al. (2012) investigated the MeJA-induced expression of several jasmonate-responsive genes throughout the day and found that the gene expression levels oscillated from dawn to dusk. On the basis of the circadian pattern of *CO* expression and the accumulation of the encoded protein (Suárez-López et al., 2001; Valverde et al., 2004; Liu et al., 2008), we speculated that CO might be involved in diurnal or circadian clock-mediated jasmonate signaling. To test this possibility, we examined the transcript levels of several well-known jasmonate-responsive genes (e.g. *JAZ1*, *JAZ2*, and *LOX2*) in MeJA-treated wild-type and *co-1* seedlings throughout the day. We grew seedlings under long-day conditions and calculated the fold-change in *JAZ1*, *JAZ2*, and *LOX2* transcript levels after a 1-h MeJA treatment in 4-h intervals over one diurnal cycle. In accordance with the findings of a previous study (Shin et al., 2012), we detected the rhythmic gating of MeJA responses in all three transcripts, with a peak induction at Zeitgeber time (ZT) 0 (dawn) (Figure 4, A–C). Moreover, the MeJA-induced expression of *JAZ1*, *JAZ2*, and *LOX2* was significantly higher in *co-1* seedlings than in the wild type at ZT12, ZT16, and ZT20 (Figure 4, A–C). These observations suggest that CO contributes to the gating of clock-mediated jasmonate signaling mainly at ZT12, ZT16, and ZT20, which corresponds to the period during which *CO* is expressed and the encoded protein accumulates (Suárez-López et al., 2001; Valverde et al., 2004; Liu et al., 2008).

CO interacts with several JAZ repressors of jasmonate signaling

Having ascertained that CO mediates jasmonate signaling, we conducted further analyses to explore the underlying molecular mechanisms. Previous research demonstrated that JAZ proteins are repressors of jasmonate signaling and negatively regulate various responses to jasmonate (Chini et al., 2007; Thines et al., 2007). Notably, JAZ repressors interact with the COI1 receptor and transcription factors to link jasmonate perception with transcriptional changes (Chini et al., 2007; Thines et al., 2007; Kazan and Manners, 2013; Guo et al., 2018b). Because CO is responsive to exogenous jasmonate and negatively modulates jasmonate responses, we hypothesized that it might interact directly with JAZ proteins. To test this possibility, we performed yeast two-hybrid (Y2H) assays to examine the physical interactions between CO and JAZ repressors. For these analyses, we cloned the full-length *JAZ* coding sequences downstream of the sequence encoding the yeast (*Saccharomyces cerevisiae*) GAL4 activation domain in the prey vector (AD-JAZ). Additionally, we cloned the

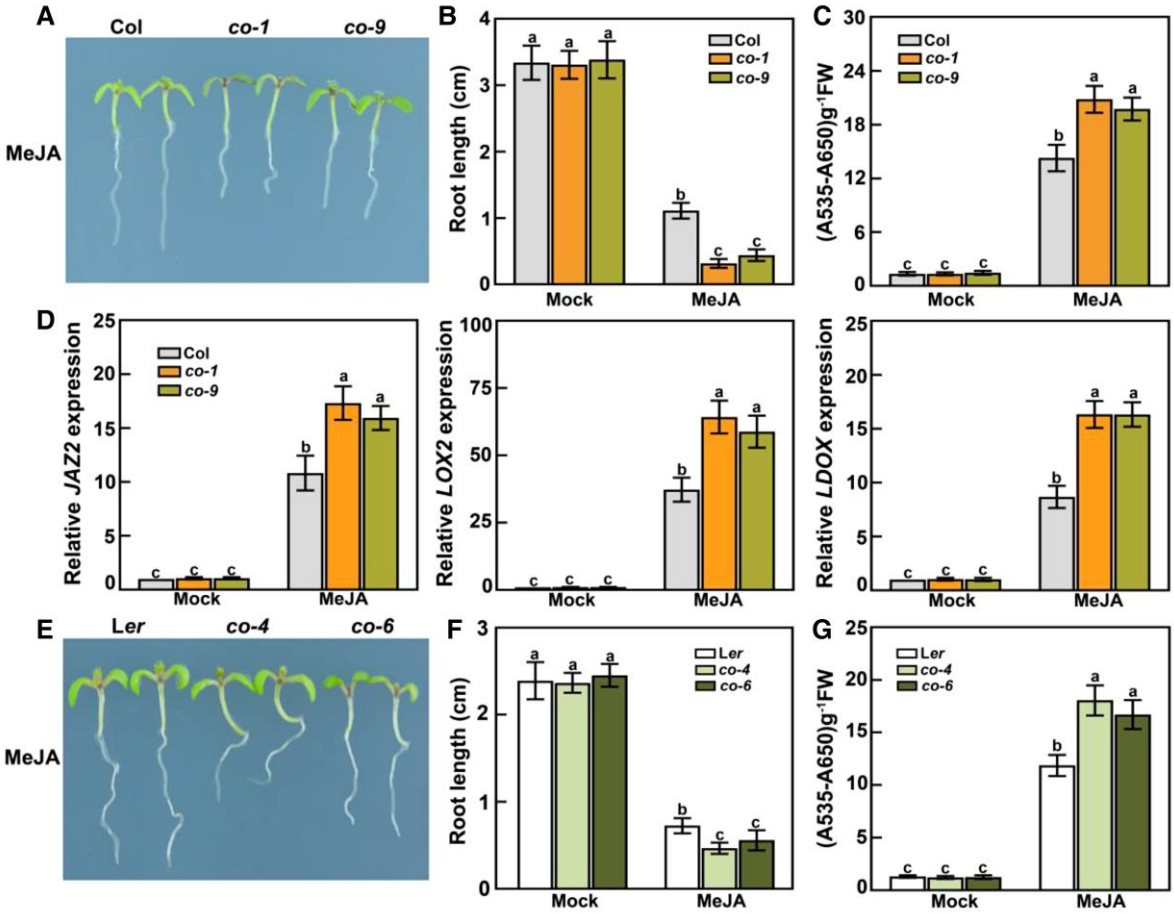

Figure 2 *Co* mutants exhibit enhanced jasmonate responses under long-day conditions. A, Root phenotypes of 8-day-old wild-type (Col-0), *co-1*, and *co-9* seedlings grown on MS medium with 30-μM MeJA under long-day conditions. B, Root length of 8-day-old seedlings grown on MS medium with or without (mock) 30-μM MeJA under long-day conditions. In the mock treatment, an equal volume of 10% (v/v) ethanol was added. The experiments were performed more than 5 times with similar results and 20 representative seedlings was measured for each repeat. Data are means ± SD ($n = 20$ representative seedlings). C, Anthocyanin contents in 10-day-old Col-0, *co-1*, and *co-9* seedlings grown on MS medium with or without (mock) 30-μM MeJA under long-day conditions. FW, fresh weight. The experiments were performed 8 times with similar results with more than 200 seedlings for each repeat. Data are means ± SD from eight independent experiments ($n = 8$). D, RT-qPCR analysis of *JAZ2*, *LOX2*, and *LDOX* expression levels in Col-0, *co-1*, and *co-9* seedlings. For *JAZ2* and *LOX2*, total RNA was extracted from 8-day-old seedlings grown in long days and treated with or without (mock) 100-μM MeJA for 4 h. For *LDOX*, total RNA was extracted from 10-day-old seedlings grown on MS medium with or without (mock) 30-μM MeJA in long days. Relative expression levels in mock-treated Col-0 seedlings were set to 1. Values are means ± SD from eight independent biological replicates ($n = 8$). E, Root phenotypes of 8-day-old L*er* wild-type, *co-4*, and *co-6* seedlings grown on MS medium with 30-μM MeJA under long-day conditions. F, Root length of 8-day-old seedlings grown on MS medium with or without (mock) 30-μM MeJA under long-day conditions. The experiments were performed more than 5 times with similar results and 20 representative seedlings were measured for each repeat. Data are means ± SD ($n = 20$ representative plants). G, Anthocyanin contents in 10-day-old seedlings of L*er*, *co-4*, and *co-6* grown on MS medium with or without (mock) 30-μM MeJA under long-day conditions. FW, fresh weight. The experiments were performed 8 times with similar results with more than 200 seedlings for each repeat. Data are means ± SD from eight independent experiments ($n = 8$). Different lowercase letters indicate significant differences ($P < 0.05$), as determined by a two-way ANOVA (genotype × treatment interaction).

sequence encoding the C-terminal region of CO containing the CCT domain (amino acids 176–373; Gangappa and Botto, 2014) downstream of the sequence encoding the GAL4 DNA-binding domain (BD) in the bait vector (BD-CO$^{176-373}$). In yeast cells, we determined that CO interacts with JAZ1, JAZ3, JAZ4, JAZ8, JAZ9, JAZ10, and JAZ11 (Figure 5A; Supplemental Figure S4). To determine which JAZ1 protein region interacts with CO, we conducted additional directed Y2H analyses, which indicated that the ZIM domain of JAZ1 (AD-JAZ1$^{122-154}$) is sufficient for the interaction (Figure 5B).

To confirm that CO interacts with JAZ repressors, we conducted bimolecular fluorescence complementation (BiFC) assays in *Nicotiana benthamiana*. We prepared a fusion construct encoding the C-terminal fragment of the yellow fluorescent protein (cYFP) fused to the full-length CO sequence

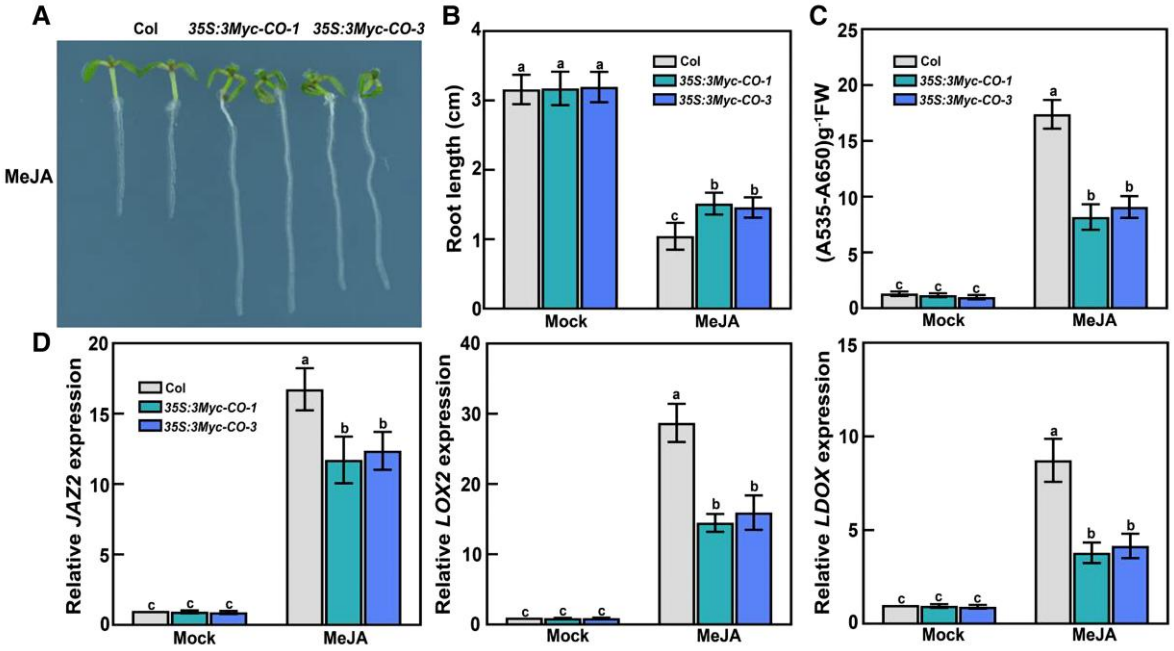

Figure 3 Overexpression of *CO* attenuates jasmonate responses under long-day conditions. A, Root phenotypes of 8-day-old wild-type (Col-0) and *35S:3Myc-CO* transgenic seedlings grown on MS medium with 30-μM MeJA under long-day conditions. B, Root length of 8-day-old seedlings grown on MS medium with or without (mock) 30-μM MeJA under long-day conditions. In the mock treatment, an equal volume of 10% (v/v) ethanol was added. The experiments were performed more than 5 times with similar results and 20 representative seedlings was measured for each repeat. Data are means ± SD ($n = 20$ representative plants). C, Anthocyanin contents in 10-day-old Col-0 and *35S:3Myc-CO* seedlings grown on MS medium with or without (mock) 30-μM MeJA under long-day conditions. The experiments were performed 8 times with similar results with more than 200 seedlings for each repeat. Data are means ± SD from eight independent experiments ($n = 8$). FW, fresh weight. D, RT-qPCR analysis of *JAZ2*, *LOX2*, and *LDOX* expression levels in Col-0 and *35S:3Myc-CO* seedlings. For *JAZ2* and *LOX2*, total RNA was extracted from 8-day-old seedlings grown in long days and treated with or without (mock) 100-μM MeJA for 4 h. For *LDOX*, total RNA was extracted from 10-day-old seedlings grown on MS medium with or without (mock) 30-μM MeJA under long-day conditions. Relative expression levels in mock-treated Col-0 seedlings were set to 1. Values are means ± SD from eight independent biological replicates ($n = 8$). Different lowercase letters indicate significant differences ($P < 0.05$) as determined by a two-way ANOVA (genotype × treatment interaction).

(CO-cYFP) under the control of the 35S promoter. Additionally, we generated fusion constructs encoding the N-terminal fragment of YFP (nYFP) fused to the full-length JAZ1 or JAZ9 sequence (JAZ1-nYFP and JAZ9-nYFP). When *CO-cYFP* was transiently co-expressed with *JAZ1-nYFP* or *JAZ9-nYFP* in *N. benthamiana* leaves, we detected strong YFP fluorescence in the nucleus, as evidenced by overlap with 4′,6-diamidino-2-phenylindole (DAPI) staining (Figure 5C; Supplemental Figure S4). Moreover, we also observed the YFP signal in *N. benthamiana* leaves when *CO-cYFP* was co-expressed with the construct encoding the N-terminal amino acids (1–200) of JAZ1 fused to nYFP (*JAZ1*$^{1-200}$*-nYFP*; Figure 5C; Supplemental Figure S4). We detected no fluorescence in the negative controls in which *CO-cYFP* was co-expressed with *JAZ1*$^{155-201}$*-nYFP* (amino acids 155–201 of JAZ1 fused to nYFP) or *JAZ5-nYFP* (JAZ5 fused to nYFP) or when *CO*$^{1-105}$*-cYFP* (amino acids 1–105 of CO fused to cYFP) was co-expressed with *JAZ1-nYFP* (Figure 5C; Supplemental Figure S4). We also confirmed the interaction between CO and JAZ1 by conducting co-immunoprecipitation (Co-IP) assays involving transgenic Arabidopsis seedlings simultaneously overexpressing *CO* and *JAZ1* (*35S:2Flag-CO JAZ1-ΔJas*; Figure 5D). These seedlings were derived from a cross between plants carrying *35S:2Flag-CO-2* (containing a 2×Flag-CO construct driven by the 35S promoter; Supplemental Figure S1B) and the previously described *JAZ1-ΔJas* plants (Han et al., 2018). Together, these results confirm that CO interacts with several JAZ repressors of jasmonate signaling, indicating that CO functions as a JAZ-binding factor that mediates jasmonate signaling.

CO functions in a COI1-dependent manner to mediate jasmonate responses

JAZ proteins suppress jasmonate signaling through their associations with multiple transcription factors. They accumulate to high levels in *coi1* mutants, wherein they repress responses to jasmonate (Chini et al., 2007; Thines et al., 2007; Katsir et al., 2008; Browse, 2009; Fonseca et al., 2009). Because CO negatively mediates jasmonate responses and interacts with crucial components (JAZ repressors) of

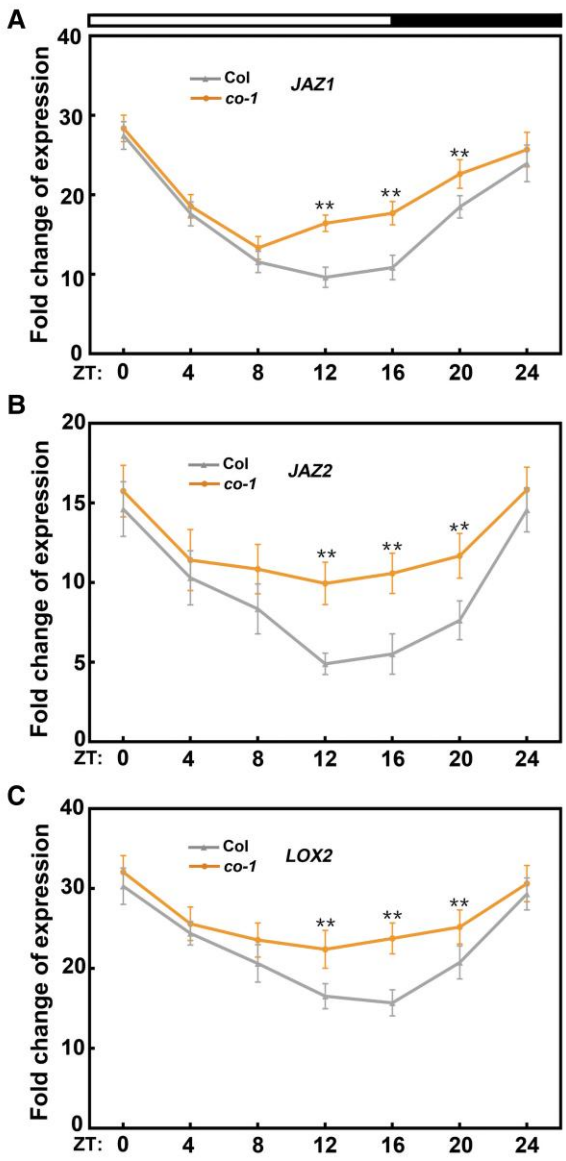

Figure 4 CO mediates the diurnal or circadian gating of several jasmonate-responsive genes under long-day conditions. Fold-induction of *JAZ1* (A), *JAZ2* (B), and *LOX2* (C) expression in response to MeJA treatment. Total RNA was extracted from 8-day-old Col-0 and *co-1* seedlings grown in long days and treated with or without (mock) 100-μM MeJA for 1 h at 4-h intervals over one diurnal cycle. In the mock treatment, an equal volume of 10% (v/v) ethanol was added. Gene expression levels at each time point were measured by RT-qPCR. The expression levels of each gene in mock-treated seedlings at each time point were set to 1. Fold-induction relative to the basal expression levels were then determined. Values are means ± SD from eight independent biological replicates ($n = 8$). $^{**}P < 0.01$ compared to Col-0, as determined by a one-way ANOVA.

jasmonate signaling, we asked whether CO function is dependent on endogenous jasmonate perception and signaling. To test this possibility, we crossed *co-1* with *coi1-2* (a mutant with a leaky mutant allele of *COI1* encoding a receptor with the missense mutation L245F; Xu et al., 2002) to obtain the *co-1 coi1-2* double mutant. We then examined the MeJA-induced phenotype of the double mutant. Compared to the wild-type control, the *co-1* mutant exhibited increased MeJA sensitivity, whereas the *coi1-2* mutant was hyposensitive to MeJA (Figure 6, A–C; Xu et al., 2002). Similar to *coi1-2*, *co-1 coi1-2* seedlings were hyposensitive to MeJA (Figure 6, A–C). These results suggest that the enhanced sensitivity of *co-1* to jasmonate is dependent on functional COI1 and the perception of jasmonate. The MYC2 transcription factor is a central regulator of jasmonate signaling (Boter et al., 2004; Lorenzo et al., 2004). To investigate whether the relationship between *CO* and *MYC2* may modulate jasmonate signaling, we crossed *co-1* to *myc2-1* to generate the *co-1 myc2-1* double mutant. Genetic analyses showed that the MeJA-hyposensitive phenotype of *myc2-1* is attenuated by the *CO* mutation (Figure 6, D–F). Additionally, relative transcript levels of jasmonate-responsive genes were higher in *co-1 myc2-1* seedlings than in *myc2-1* (Figure 6G). Accordingly, CO and MYC2 appear to have opposite effects on jasmonate responses.

CO physically associates with the bHLH subgroup IIId transcription factors bHLH3 and bHLH17

Because of the contrasting effects of CO and MYC2 on jasmonate signaling, we tested whether CO interacts with MYC2 and its homologs (MYC3 and MYC4). To this end, we generated constructs encoding full-length MYC2, MYC3, and MYC4 fused to the GAL4 activation domain in the prey vector (AD-MYC) for Y2H analyses. However, we detected no interactions between CO and MYC2, MYC3, or MYC4 in yeast cells (Figure 7A; Supplemental Figure S5). Interestingly, in addition to CO, several bHLH subgroup IIId transcription factors (bHLH3, bHLH13, bHLH14, and bHLH17) reportedly bind to JAZ repressors and negatively modulate jasmonate responses (Nakata et al., 2013; Sasaki-Sekimoto et al., 2013; Song et al., 2013a; Fonseca et al., 2014), prompting us to investigate their association with CO. We first examined their possible interactions in an Y2H assay by cloning the full-length coding sequence of each bHLH transcription factor gene downstream of the sequence of the GAL4 activation domain in the prey vector (AD-bHLH). In yeast cells, CO strongly interacted with bHLH3 and bHLH17, but not with bHLH13 or bHLH14 (Figure 7A; Supplemental Figure S5). Next, we determined which bHLH17 region is essential for interaction with CO. Specifically, we divided bHLH17 into its N-terminal fragment (amino acids 1–360) and its C-terminal fragment (amino acids 350–566) that includes the bHLH domain (Figure 7B). We determined that the C-terminal region of bHLH17 is required for interaction with CO (Figure 7B).

We confirmed the interaction of CO with bHLH3 or bHLH17 by BiFC and Co-IP assays *in planta*. When *CO-cYFP* was co-expressed with *bHLH3-nYFP* or *bHLH17-nYFP* in *N. benthamiana* leaves, we observed YFP fluorescence in the nucleus (Figure 7C; Supplemental Figure S5). We also detected

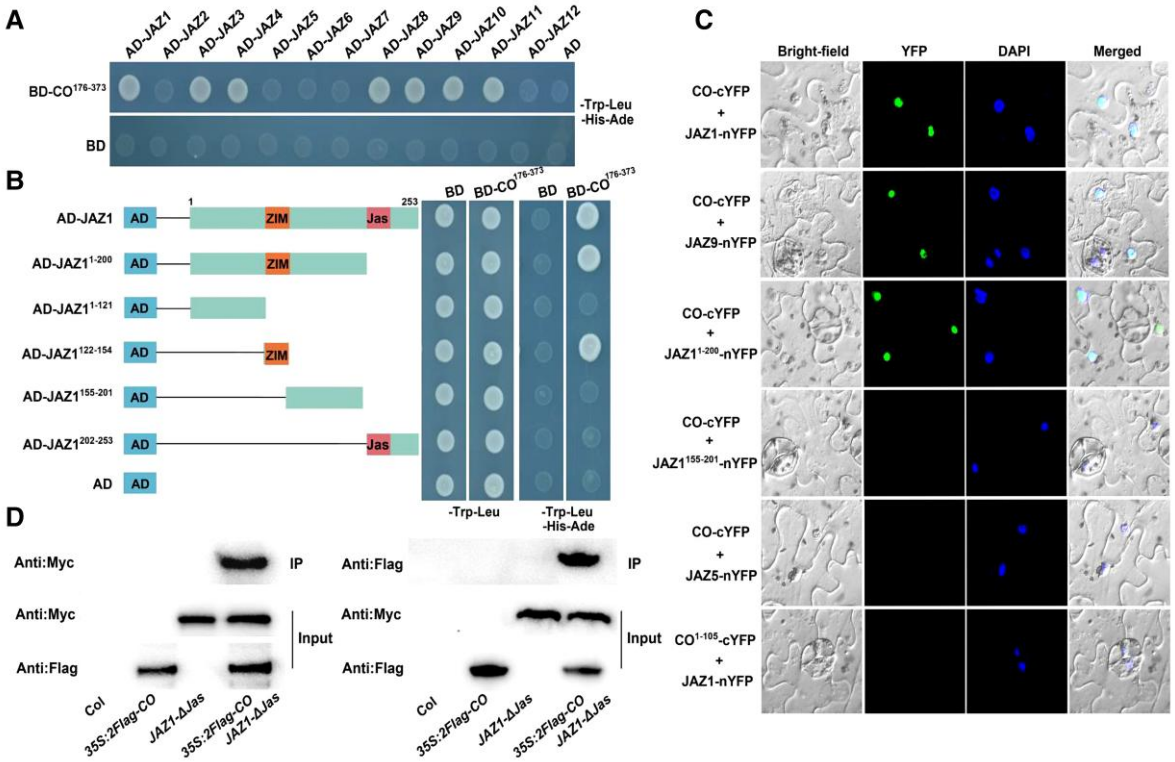

Figure 5 Physical interactions of CO with JAZ repressors. A, Yeast two-hybrid (Y2H) assays. Interactions of CO with JAZ are indicated by the ability of yeast cells to grow on SD medium lacking Leu, Trp, His, and Ade. pGBKT7 (BD) and pGADT7 (AD) vectors were used as negative controls. B, Mapping the CO-interacting domain of JAZ1 using a Y2H assay. Left, diagram of full-length and truncated JAZ1 constructs with specific regions. Right, interaction as indicated by the ability of cells to grow on SD medium lacking Leu, Trp, His, and Ade. BD and AD vectors were used as negative controls. C, BiFC assays. Fluorescence was detected in the nuclei of *N. benthamiana* cells co-expressing *CO-cYFP* and *JAZ1-nYFP*, *JAZ9-nYFP*, or $JAZ1^{1-200}$*-nYFP*. No signal was observed in the negative controls *CO-cYFP* and $JAZ1^{155-201}$*-nYFP* or *JAZ5-nYFP*, or CO^{1-105}*-cYFP* and *JAZ1-nYFP*. Nuclei are indicated by DAPI staining. D, Co-IP assays. Total proteins were extracted from *35S:2Flag-CO JAZ1-ΔJas* transgenic Arabidopsis seedlings. Flag-tagged CO was immunoprecipitated using an anti-Flag antibody (1:250) and the co-immunoprecipitated protein was detected with an anti-Myc antibody (1:10,000, monoclonal antibody) and a secondary antibody (goat anti-mouse, 1:10,000). Similarly, Myc-tagged JAZ1 was immunoprecipitated using an anti-Myc antibody (1:250) and the co-immunoprecipitated protein was detected with an anti-Flag antibody (1:10,000). Protein input for 2Flag-CO or 3Myc-fused JAZ1 in immunoprecipitated complexes was also detected. Experiments were performed three times with similar results.

YFP signal detected in *N. benthamiana* leaves when *CO-cYFP* was co-expressed with $bHLH17^{350-566}$*-nYFP* (bHLH17 C-terminal amino acids 350–566 fused to nYFP; Figure 7C; Supplemental Figure S5). We detected no YFP signal in the negative control in which *CO-cYFP* was co-expressed with $bHLH17^{1-360}$*-nYFP* (bHLH17 N-terminal amino acids 1–360 fused to cYFP) or when CO^{1-105}*-cYFP* was co-expressed with *bHLH17-nYFP* (Figure 7C; Supplemental Figure S5). Moreover, the results of the Co-IP assays provided further in vivo evidence for the direct interaction between CO and bHLH17 (Figure 7D). These findings indicate that CO physically interacts with the transcription factors bHLH3 and bHLH17 in the nucleus of plant cells.

CO works together with bHLH17 in suppressing jasmonate signaling, whereas JAZ1 affects their transcriptional functions and physical interaction

Having established that CO physically interacts with bHLH3 and bHLH17, we wondered whether they act cooperatively to mediate jasmonate signaling. Accordingly, we crossed *co-1* to the *bhlh3 bhlh17* double mutant to generate the *co-1 bhlh3 bhlh17* triple mutant, which we then treated with MeJA for a phenotypic examination. Consistent with the results of previous studies (Sasaki-Sekimoto et al., 2013; Song et al., 2013a; Fonseca et al., 2014), *bhlh3 bhlh17* seedlings accumulated more anthocyanin in the presence of MeJA than the wild type (Figure 8A). Notably, jasmonate signaling was more enhanced in the *co-1 bhlh3 bhlh17* triple mutant than in *co-1* and *bhlh3 bhlh17*. Anthocyanin levels were much higher in *co-1 bhlh3 bhlh17* seedlings than in *co-1* and *bhlh3 bhlh17* seedlings (Figure 8A). To verify this observation, we quantitatively analyzed the expression of the jasmonate-responsive gene *LDOX* in MeJA-treated *co-1 bhlh3 bhlh17* seedlings. Relative *LDOX* transcript levels were significantly higher in *co-1 bhlh3 bhlh17* than in *co-1* or *bhlh3 bhlh17* seedlings (Figure 8B). These findings suggest that CO functions together with bHLH3 and bHLH17 to negatively regulate jasmonate signaling.

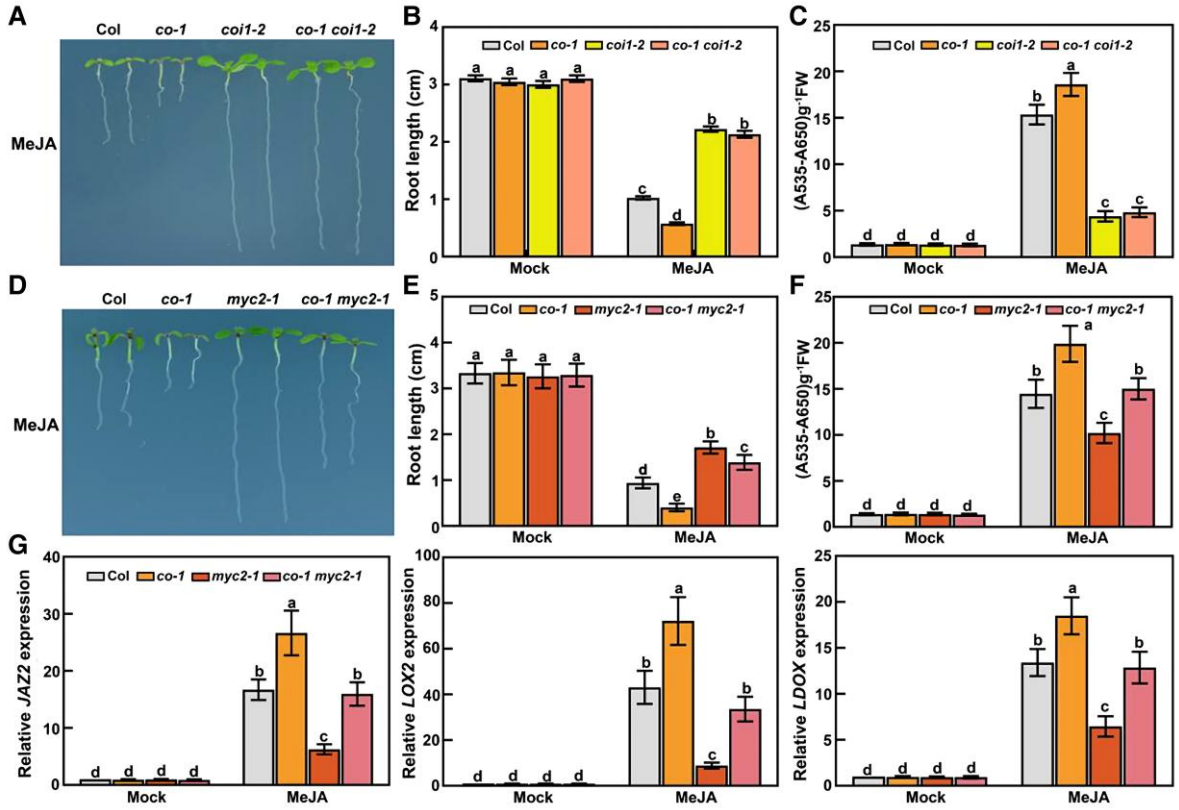

Figure 6 Jasmonate responses of *co-1 coi1-2* and *co-1 myc2-1* double mutant seedlings. A, Root phenotypes of 8-day-old wild type (Col-0), *co-1*, *coi1-2*, and *co-1 coi1-2* seedlings grown on MS medium with 30-μM MeJA under long-day conditions. B, Root length of 8-day-old seedlings grown on MS medium with or without (mock) 30-μM MeJA under long-day conditions. In the mock treatment, an equal volume of 10% (v/v) ethanol was added. The experiments were performed more than 5 times with similar results and 20 representative seedlings were measured for each repeat. Data are means ± SD ($n = 20$ representative plants). C, Anthocyanin contents in 10-day-old Col-0, *co-1*, *coi1-2*, and *co-1 coi1-2* seedlings grown on MS medium with or without (mock) 30-μM MeJA under long-day conditions. The experiments were performed 8 times with similar results with more than 200 seedlings for each repeat. Data are means ± SD from eight independent experiment ($n = 8$). FW, fresh weight. D, Root phenotypes of 8-day-old Col-0, *co-1*, *myc2-1*, and *co-1 myc2-1* seedlings grown on MS medium with 30-μM MeJA under long-day conditions. E, Root length of 8-day-old seedlings grown on MS medium with or without (mock) 30-μM MeJA. The experiments were performed more than 5 times with similar results and 20 representative seedlings were measured for each repeat. Data are means ± SD ($n = 20$ representative plants). F, Anthocyanin contents in 10-day-old Col-0, *co-1*, *myc2-1*, and *co-1 myc2-1* seedlings grown in long days on MS medium with or without (mock) 30-μM MeJA. The experiments were performed 8 times with similar results with more than 200 seedlings for each repeat. Values are means ± SD from eight independent experiments ($n = 8$). G, RT-qPCR analysis of *JAZ2*, *LOX2*, and *LDOX* expression levels in Col-0, *co-1*, *myc2-1*, and *co-1 myc2-1* seedlings. For *JAZ2* and *LOX2*, total RNA was extracted from 8-day-old seedlings grown in long days and treated with or without (mock) 100-μM MeJA for 4 h. For *LDOX*, total RNA was extracted from 10-day-old seedlings grown in long days on MS medium with or without (mock) 30-μM MeJA. Relative expression levels in mock-treated Col-0 seedlings were set to 1. Values are means ± SD from eight independent biological replicates ($n = 8$). Different lowercase letters indicate significant differences ($P < 0.05$) as determined by a two-way ANOVA (genotype × treatment interaction).

We also investigated the mechanisms underlying the additive effects of CO and bHLH17 on jasmonate signaling. More specifically, we conducted dual-luciferase (LUC) reporter assays in Arabidopsis mesophyll protoplasts (Yoo et al., 2007) to assess whether CO enhances the effect of bHLH17 on transcription. The effectors consisted of *CO*, *bHLH17*, or *GFP* (green fluorescent protein) under the control of the 35S promoter (Supplemental Figure S6). We also constructed a reporter construct comprising the *LUC* gene driven by the *JAZ2* promoter (Fonseca et al., 2014). Consistent with the findings of a previous study (Fonseca et al., 2014), expressing *bHLH17* decreased relative LUC activity from the *ProJAZ2:LUC* reporter in wild-type protoplasts (relative to the effects of GFP alone) (Figure 8, C and D; Supplemental Figure S7). We obtained similar results when *CO* was expressed in this assay with the same reporter (Figure 8, C and D; Supplemental Figure S7). Moreover, relative LUC activity decreased more in the presence of CO and bHLH17 than in the presence of GFP and bHLH17 (Figure 8, C and D; Supplemental Figure S7), suggesting that CO and bHLH17 function cooperatively to repress *JAZ2* transcription. To assess whether the loss of CO function would affect the transcriptional function of bHLH17, we analyzed the ability of bHLH17 to suppress *JAZ2* transcription in mesophyll

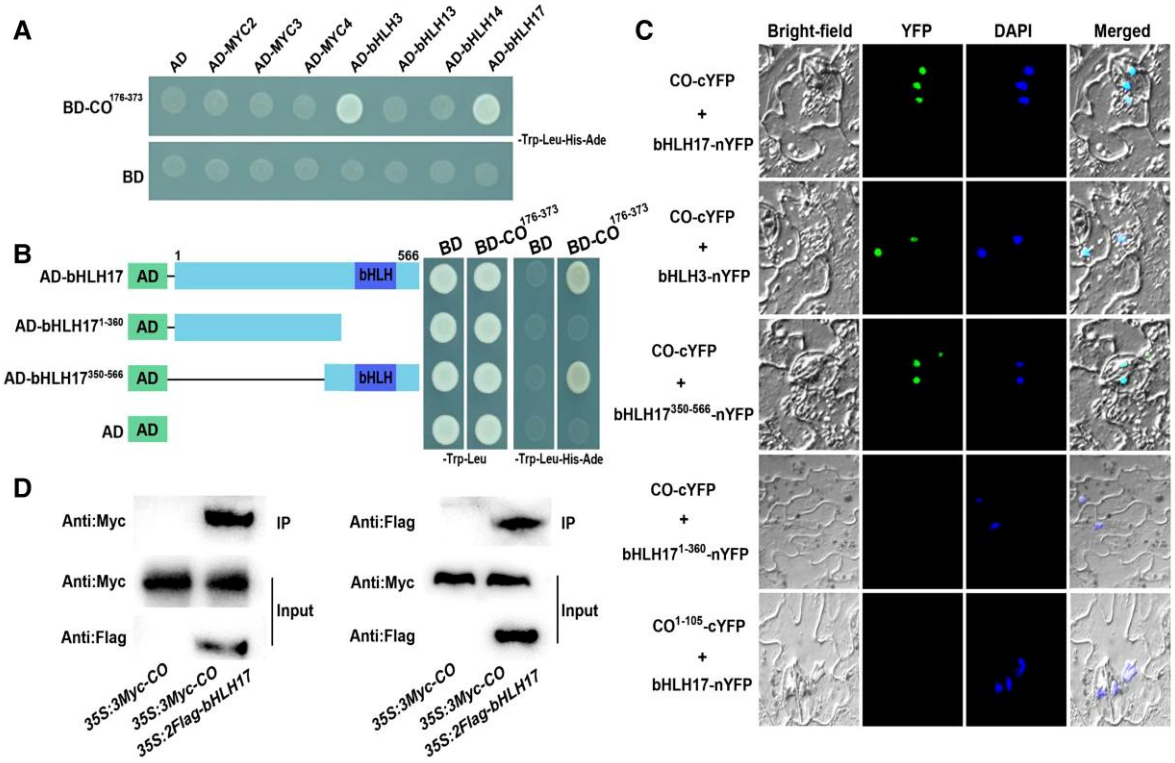

Figure 7 Physical interactions of CO with bHLH3 and bHLH17. A, Y2H assays. Interactions of CO with bHLH3 and bHLH17 are indicated by the ability of yeast cells to grow on SD medium lacking Leu, Trp, His, and Ade. pGBKT7 (BD) and pGADT7 (AD) were used as negative controls. B, Mapping the CO-interacting domain of bHLH17 using a Y2H assay. Left, diagram of full-length and truncated bHLH17 constructs with specific regions. Right, interaction as indicated by the ability of cells to grow on SD medium lacking Leu, Trp, His, and Ade. BD and AD vectors were used as negative controls. C, BiFC assays. Fluorescence was detected in the nuclei of *N. benthamiana* cells co-expressing *CO-cYFP* and *bHLH3-nYFP*, *bHLH17-nYFP*, or *bHLH17*$^{350-566}$*-nYFP*. No signal was observed in the negative controls co-expressing *CO-cYFP* and *bHLH17*$^{1-360}$*-nYFP*, or *CO*$^{1-105}$*-cYFP* and *bHLH17-nYFP*. Nuclei are indicated by DAPI staining. D, Co-IP assays. Total proteins were extracted from *35S:3Myc-CO 35S:2Flag-bHLH17* transgenic seedlings. Flag-tagged bHLH17 was immunoprecipitated using an anti-Flag antibody (1:250) and the co-immunoprecipitated protein was detected with an anti-Myc antibody (1:10,000). Similarly, Myc-tagged CO was immunoprecipitated using an anti-Myc antibody (1:250) and the co-immunoprecipitated protein was detected with an anti-Flag antibody (1:10,000). Protein input for 2Flag-bHLH17 or 3Myc-CO in immunoprecipitated complexes was also detected. Experiments were performed three times with similar results.

protoplasts prepared from the wild-type control and the *co-1* mutant. We measured lower relative LUC activity from the *ProJAZ2:LUC* reporter in *bHLH17*-expressing wild-type protoplasts than in *co-1* protoplasts (Figure 8E; Supplemental Figure S7). Similarly, relative LUC activity from the *ProJAZ2:LUC* reporter was lower in *CO*-expressing wild-type protoplasts than in *bhlh3 bhlh17* protoplasts (Figure 8F; Supplemental Figure S7). These findings support the notion that CO and bHLH17 reciprocally enhance their repressive effects on transcription. To further elucidate the regulatory effect of CO on bHLH17, we explored whether CO stimulates the enrichment of bHLH17 to the promoter regions of its downstream genes in vivo by chromatin immunoprecipitation (ChIP) assay. We observed that the enrichment of bHLH17 to the *JAZ1* and *JAZ2* promoter regions (*pJAZ1-a*, *pJAZ1-b*, *pJAZ2-a*, and *pJAZ2-b*; Supplemental Table S1) is greater in *35S:3Myc-CO 35S:2Flag-bHLH17* seedlings than in *35S:2Flag-bHLH17* seedlings (Figure 9, A and B). Thus, CO appears to enhance the enrichment of bHLH17 to the promoter regions of downstream jasmonate-responsive genes in plants.

Because JAZ repressors interact with CO and bHLH subgroup IIId transcription factors, we investigated the modulatory effects of JAZ proteins on these transcription factors in dual-luciferase reporter assays conducted using Arabidopsis mesophyll protoplasts (Yoo et al., 2007). We expressed *JAZ1* (or *JAZ9*) with *CO* and *bHLH17* in protoplasts derived from the wild-type control. Relative LUC activity from the *ProJAZ2:LUC* reporter was higher in protoplasts expressing *JAZ1* (or *JAZ9*) with *CO* and *bHLH17* than in protoplasts expressing only *CO* and *bHLH17* (Figure 8, C and D). These results suggest that JAZ1 and JAZ9 proteins antagonize the regulatory effects of CO and bHLH17 to modulate the transcription of downstream target genes. To further clarify the regulatory effects of JAZ proteins on CO and bHLH17, we performed BiFC assays to analyze the interaction between CO and bHLH17 in the presence of JAZ1. When *JAZ1* was

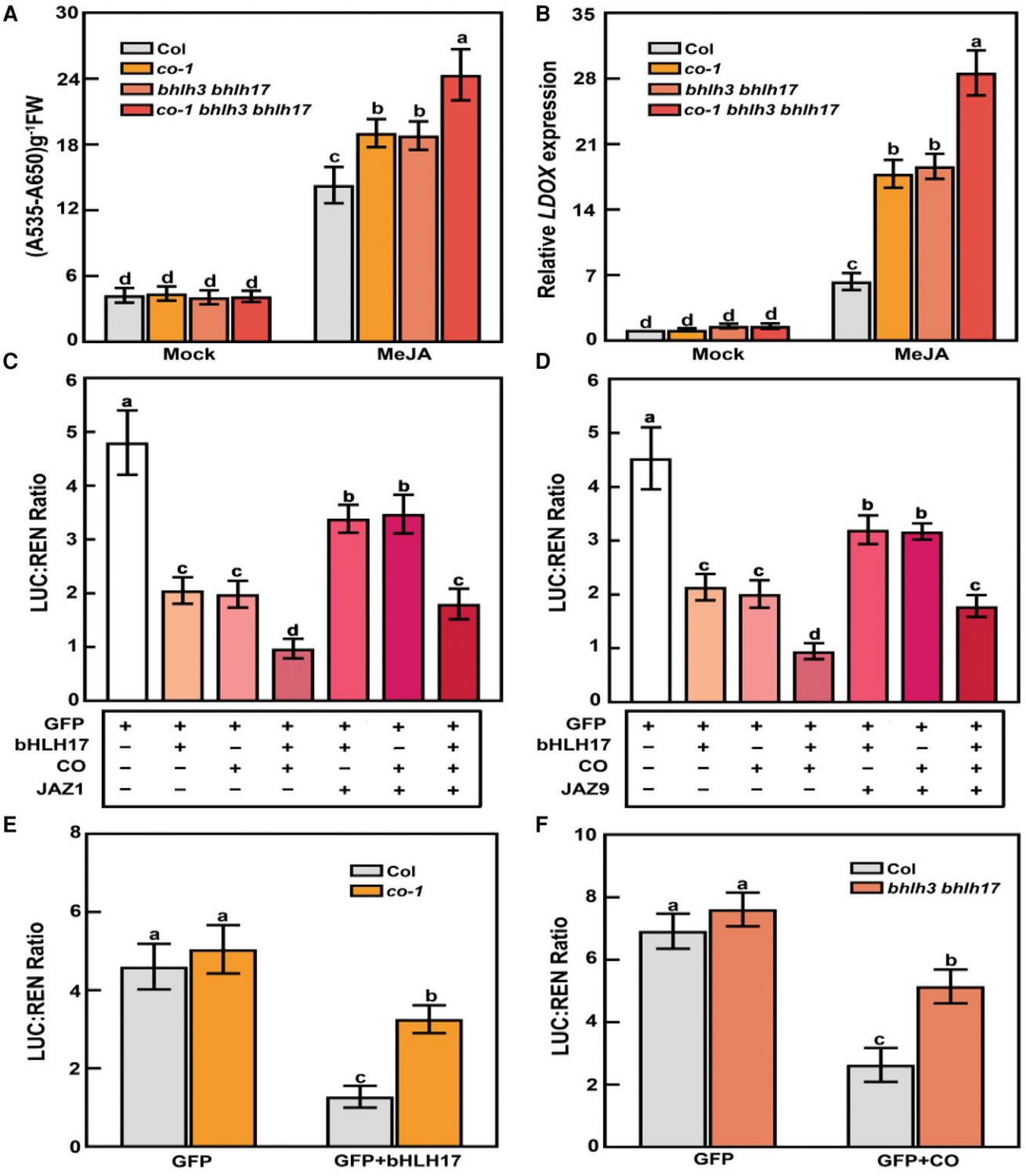

Figure 8 CO works cooperatively with bHLH17 in suppressing jasmonate signaling. A, Anthocyanin contents in 10-day-old long-day-grown seedlings of wild type (Col), *co-1*, *bhlh3 bhlh17*, and *co-1 bhlh3 bhlh17* on MS medium with or without (mock) 30-μM MeJA. In the mock treatment, an equal volume of 10% (v/v) ethanol was added. The experiments were performed 8 times with similar results with more than 200 seedlings for each repeat. Data are means ± SD from eight independent experiments ($n = 8$). FW, fresh weight. Different lowercase letters indicate significant differences ($P < 0.05$) as determined by a two-way ANOVA (genotype × treatment interaction). B, RT-qPCR analysis of *LDOX* expression levels in Col, *co-1*, *bhlh3 bhlh17*, and *co-1 bhlh3 bhlh17*. Total RNA was extracted from 10-day-old seedlings grown in long days on MS medium with or without (mock) 30-μM MeJA. The expression level of *LDOX* in mock-treated Col-0 seedlings was set to 1. Data are means ± SD from eight independent

(continued)

co-expressed with *CO-cYFP* and *bHLH17-nYFP* in *N. benthamiana* leaves, the YFP signal decreased (Figure 9, C and D). In the negative control, the expression of *GUS* with *CO-cYFP* and *bHLH17-nYFP* did not affect fluorescence intensity (Figure 9, C and D). These results suggest that JAZ1 interferes with the physical association between CO and bHLH17.

Discussion

CO is a critical negative regulator of jasmonate-imposed root-growth inhibition and anthocyanin accumulation

The BBX member transcription factor CO is a major regulator of flowering in the photoperiodic pathway. Previous studies have highlighted its regulatory effects on *FT* expression and flowering induction under long-day conditions (Putterill et al., 1995; Simon et al., 1996; Samach et al., 2000; Suárez-López et al., 2001; Takada and Goto, 2003; An et al., 2004; Corbesier et al., 2007; Tiwari et al., 2010; Shim et al., 2017). *CO* is widely expressed in multiple tissues, and its expression in the vascular tissue (phloem) is sufficient to stimulate *FT* transcription and trigger photoperiodic flowering (Takada and Goto, 2003; An et al., 2004; Shim et al., 2017). Although there has been considerable progress in recent years, other potential functions of CO and their underlying molecular mechanisms remain unknown. Investigating the direct involvement of CO in other biological processes and thoroughly elucidating the associated regulatory mechanisms will advance our understanding of CO-mediated signaling networks. In this study, we confirmed that *CO* was expressed in the shoot apex, young leaves, and roots of seedlings (Figure 1A), which is in accordance with previous studies (Takada and Goto, 2003; An et al., 2004). Intriguingly, we also revealed that *CO* transcript and CO protein levels were responsive to the phytohormone jasmonate (e.g. MeJA; Figure 1, A–E). Phenotypic analyses showed that loss-of-function *co* mutants were more sensitive to jasmonate than wild-type seedlings (Figure 2, A–G). Additionally, compared to control seedlings, *co* mutant seedlings had shorter roots and accumulated more anthocyanin following jasmonate treatment (Figure 2, A–G). By contrast, *CO* overexpression rendered the transgenic seedlings less sensitive to jasmonate than the wild type (Figure 3, A–D). These findings suggest that CO negatively regulates jasmonate-induced root-growth inhibition and anthocyanin accumulation in Arabidopsis seedlings.

Jasmonate is an essential signaling compound that controls multiple developmental processes and stress responses in plants, such as root elongation, trichome formation, stamen development, and anthocyanin accumulation (Chini et al., 2016; Hu et al., 2017; Huang et al., 2017; Zhang et al., 2017; Howe et al., 2018; Guo et al., 2018a). Jasmonate signal transduction involves profound changes in the cellular gene expression profile associated with the complex interplay between negative and positive components (e.g. JAZ proteins and downstream transcriptional regulators). Additionally, JAZ proteins inhibit jasmonate signaling by physically associating with and attenuating multiple transcription factors. For example, recent studies have revealed that JAZ proteins interact with the transcription factors ABI5 and ABI3 to modulate abscisic acid signaling and seed germination (Ju et al., 2019; Pan et al., 2020). Other targets of JAZ proteins include MYC transcription factors, essential components of the WD-repeat/bHLH/MYB transcriptional complexes, EIN3, FIL, TOE1, MYB21, RHD6, WRKY57, and INDUCER OF CBF EXPRESSION1 (ICE1), all of which regulate diverse aspects of jasmonate responses (Chini et al., 2007; Fernández-Calvo et al., 2011; Qi et al., 2011; Song et al., 2011; Zhu et al., 2011; Hu et al., 2013; Jiang et al., 2014; Boter et al., 2015; Zhai et al., 2015; Han et al., 2020). Two repressors of jasmonate signaling in apple (*Malus domestica*), MdJAZ1 and MdJAZ2, were recently reported to interact with MdBBX37 to repress the transcriptional activation of C-repeat binding factor genes by MdBBX37 (An et al., 2021). The results of the present study suggested that CO also physically associated with several JAZ repressors and that the C-terminal region with the CCT domain of CO was sufficient for these interactions (Figure 5, A–D). As the C-terminal fragment of MdBBX37 lacks a CCT domain and shares low similarity with that of CO, it is possible that the MdJAZ–MdBBX37 and JAZ–CO interactions do not occur through a conserved domain. Interestingly, previous studies have revealed that JAZ proteins exhibit physical associations with bHLH transcription factors (e.g. MYC2 and ICE1) also through different domains (Fernández-Calvo et al., 2011; Hu et al., 2013). These findings underscore the involvement of different domains from transcription factors in their interactions with JAZ proteins, and future elucidations may shed light on the distinct and complex transcriptional mechanisms of jasmonate signaling. Our further investigation demonstrated that JAZ

Figure 8 (Continued)
biological replicates using different batches of seedlings ($n = 8$). Different lowercase letters indicate significant differences ($P < 0.05$) as determined by a two-way ANOVA (genotype × treatment interaction). C and D, Transient dual-luciferase reporter assays showing that CO and bHLH17 function cooperatively to repress *JAZ2* transcription, whereas JAZ1 and JAZ9 proteins affect their transcriptional functions. Data are means ± SD from eight biological replicates using different batches of Col-0 protoplasts ($n = 8$ times). Different lowercase letters indicate significant differences ($P < 0.05$) as determined by a two-way ANOVA. E, Transient transcriptional activity assays showing that repression of the *JAZ2* promoter by bHLH17 decreases in the *co-1* mutant. Data are means ± SD from eight biological replicates using different batches of Col-0 and *co-1* protoplasts ($n = 8$). B Different lowercase letters indicate significant differences ($P < 0.05$) as determined by a two-way ANOVA (for an interaction of genotype × effector/protein expressed in the protoplasts). F, Transient transcriptional activity assays showing that repression of the *JAZ2* promoter by CO decreases in the *bhlh3 bhlh17* double mutant. Data are means ± SD from eight biological replicates using different batches of Col-0 and *bhlh3 bhlh17* protoplasts ($n = 8$). Different lowercase letters indicate significant differences ($P < 0.05$) as determined by a two-way ANOVA (for an interaction of genotype × effector/protein expressed in the protoplasts).

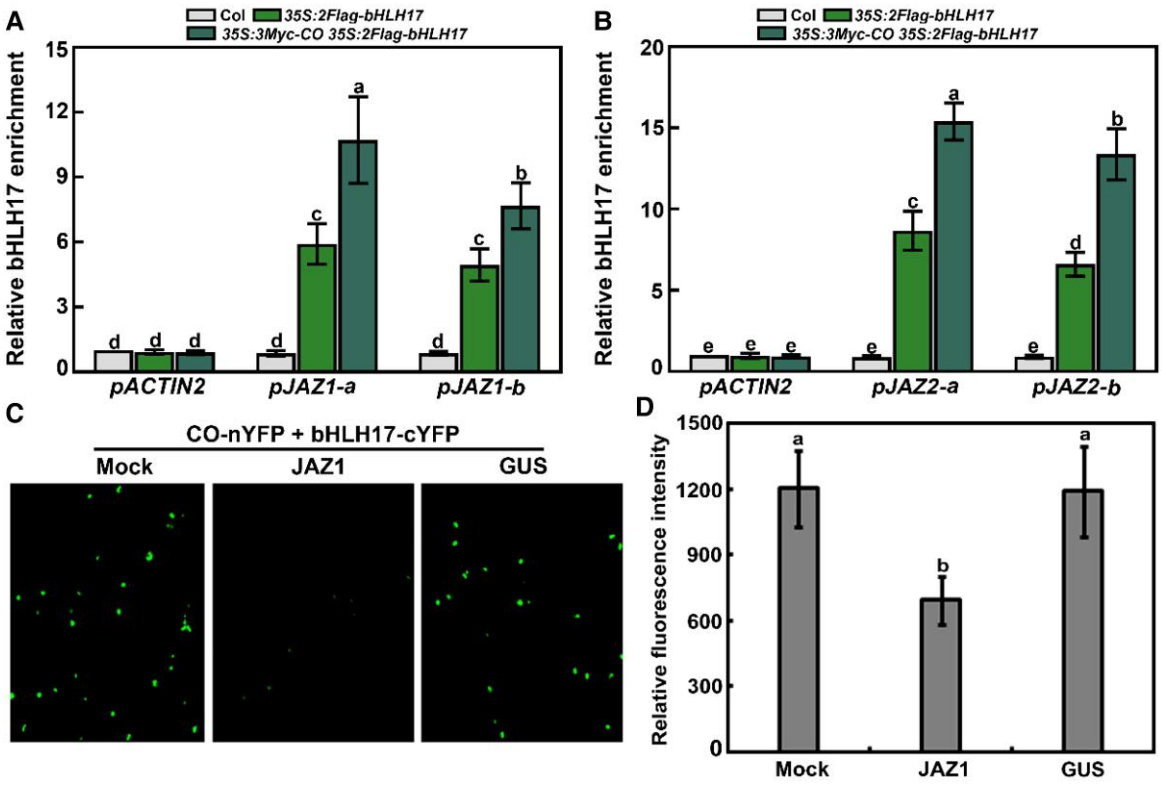

Figure 9 CO enhances the enrichment of bHLH17 at the promoter of target genes, and JAZ1 affects the CO-bHLH17 interaction. A and B, CO enhances the enrichment of bHLH17 at the promoter regions of *JAZ1* (*pJAZ1-a* and *pJAZ1-b*) and *JAZ2* (*pJAZ2-a* and *pJAZ2-b*). Eight-d-old wild-type (Col-0), *35S:2Flag-bHLH17*, and *35S:3Myc-CO 35S:2Flag-bHLH17* seedlings grown in long days and treated with 100-μM MeJA for 1 h and harvested at ZT16 were used in ChIP assays with an anti-Flag antibody (or IgG antibody as a negative control). The enrichment (nonspecific binding) level of bHLH17 at the untranslated region of *ACTIN2* (*pACTIN2*) in Col-0 seedlings was set to 1. Data are means ± SD from eight independent biological replicates using different batches of seedlings ($n = 8$). Different lowercase letters indicate significant differences ($P < 0.05$) as determined by a two-way ANOVA (for an interaction of genotype × DNA/promoter region). C, BiFC analyses showing that JAZ1 attenuates the interaction between CO and bHLH17. Fluorescence was detected 48 h after co-expression of *CO-cYFP* + *bHLH17-nYFP* (mock), *JAZ1 CO-cYFP* + *bHLH17-nYFP* (+*JAZ1*), or *GUS* + *CO-cYFP* or *bHLH17-nYFP* (+*GUS*). D, Quantitative analysis of YFP fluorescence intensity in (C). Fifty independent fluorescent spots were assessed for fluorescence intensity. Data are means ± SD ($n = 50$). One representative fluorescent spot resulting from the co-expression of *CO-cYFP* + *bHLH17-nYFP* (mock) was used as the control and its relative intensity value was set to 1,200. Experiments were performed more than three times with similar results. Different lowercase letters indicate significant differences ($P < 0.05$) as determined by a two-way ANOVA.

proteins interfered with the transcriptional function of CO and the interaction of CO with bHLH17 in mediating jasmonate signaling (Figures 8 and 9). Based on our genetic analyses, we propose that CO functions in a COI1-dependent manner and CO and MYC2 have opposite effects on jasmonate responses (Figure 6, A–G). These findings indicate that CO is a JAZ-binding factor that participates in jasmonate signaling via direct protein–protein interactions.

Previous investigations have shown that the bHLH family subgroup IIId transcription factors bHLH3, bHLH13, bHLH14, and bHLH17 interact with JAZ repressors and negatively modulate jasmonate responses (Nakata et al., 2013; Sasaki-Sekimoto et al., 2013; Song et al., 2013a; Fonseca et al., 2014). The quadruple mutant *bhlh3 bhlh13 bhlh14 bhlh17* exhibits severe jasmonate-repressed root growth and jasmonate-induced anthocyanin accumulation (Song et al., 2013a). Interestingly, we observed that CO also interacted with bHLH3 and bHLH17 and acted cooperatively with these two transcription factors to suppress jasmonate signaling (Figures 7 and 8). Through their physical interaction, CO enhanced the transcriptional function of bHLH17 and its enrichment at the promoter regions of downstream jasmonate-responsive genes (Figures 8 and 9). Moreover, our results suggested that JAZ proteins affected the transcriptional activity of CO, while also inhibiting the CO–bHLH17 interaction associated with jasmonate signaling (Figures 8 and 9). Consistent with these findings, we detected an interaction between the C-terminal region of CO with the CCT domain crucial for its transcriptional function (Gangappa and Botto, 2014) and bHLH3/bHLH17 and JAZ proteins (Figures 5 and 7). Thus, we uncovered a critical signaling module in which, CO acts together with bHLH subgroup IIId transcription factors (bHLH3 and bHLH17) to negatively regulate jasmonate signaling, but their

transcriptional functions and physical interactions are repressed by JAZ proteins. Unlike CO and bHLH subgroup IIId transcription factors, the transcription factors MYC2, MYC3, and MYC4 stimulate jasmonate-mediated root-growth inhibition and anthocyanin accumulation (Boter et al., 2004; Lorenzo et al., 2004; Dombrecht et al., 2007; Fernández-Calvo et al., 2011). Phenotypic and biochemical analyses showed that CO/bHLH subgroup IIId transcription factors and MYC2 have opposite effects on jasmonate signaling (Figure 6, D–G; Nakata et al., 2013; Sasaki-Sekimoto et al., 2013; Song et al., 2013a; Fonseca et al., 2014), which may be explained by the competitive inhibition of MYC by CO/bHLH subgroup IIId transcription factors. Because a direct interaction between MYC and CO/bHLH subgroup IIId transcription factors has not been detected (Figure 7A; Song et al., 2013a), further research is required to explore the molecular mechanisms underlying the opposite effects of MYC and CO/bHLH subgroup IIId transcription factors.

CO modulates the diurnal gating of jasmonate signaling

The circadian clock is an endogenous biological oscillator that provides an adaptive advantage to land plants (Dunlap, 1999; Pruneda-Paz and Kay, 2010; Hsu and Harmer, 2014; Webb et al., 2019; Yang et al., 2021). Several recent studies have revealed that jasmonate accumulation and signaling are controlled by the circadian clock (Goodspeed et al., 2012; Shin et al., 2012; Nitschke et al., 2016; Zhang et al., 2018; Thines et al., 2019). More specifically, Goodspeed et al. (2012) detected a circadian pattern in the accumulation of jasmonate, with peak levels in the middle of the day. Moreover, Shin et al. (2012) analyzed MeJA-induced expression of several jasmonate-responsive genes at different times over diurnal cycles and found that these genes were rhythmically expressed, with peak expression levels at dawn. Moreover, the circadian clock component TIME FOR COFFEE (TIC) physically interacts with MYC2 and prevents MYC2 from accumulating to negatively modulate jasmonate signaling. Similarly, we detected a rhythmic diurnal pattern in MeJA-induced expression of *JAZ1*, *JAZ2*, and *LOX2*, with the highest expression levels at dawn (Figure 4, A–C). Furthermore, relative *JAZ1*, *JAZ2*, and *LOX2* transcript levels were significantly higher in *co-1* seedlings than in wild-type seedlings at ZT12, ZT16, and ZT20 (Figure 4, A–C). These results indicate that CO is involved in the gating of circadian clock-mediated jasmonate signaling when CO contents increase (Suárez-López et al., 2001; Valverde et al., 2004; Liu et al., 2008). Because bHLH3 and bHLH17 interact with CO (Figure 7), we also analyzed their diurnal expression patterns in MeJA-treated wild-type seedlings. The *bHLH3* and *bHLH17* transcript levels did not show a diurnal pattern in response to MeJA (Supplemental Figure S8). Nevertheless, the precise biochemical mechanisms underlying the circadian clock-mediated gating of jasmonate signaling remain to be characterized. Elucidating the potential relationships between circadian clock components and key regulators of jasmonate signaling will enhance our understanding of circadian clock-mediated gating of jasmonate signaling networks.

The circadian clock and jasmonate signaling pathways are both involved in the regulation of plant growth and stress responses by synchronizing internal physiological processes with surrounding environmental changes. Considering that jasmonate accumulation and signaling are rhythmically regulated (Figure 4; Goodspeed et al., 2012; Shin et al., 2012; Nitschke et al., 2016; Zhang et al., 2018; Thines et al., 2019), we speculate that the circadian gating of jasmonate responses may contribute to maintaining an appropriate temporal balance so that plant growth and stress responsiveness are optimized for the prevailing conditions. Because jasmonate activates stress responses at the cost of energy for plant growth and development, such a gating mechanism underlying jasmonate signaling may allow plants to promote growth in the evening and restrict growth in the morning. Consistently, when jasmonate signaling is relatively less active at night, daily plant growth and development are promoted, which are also modulated by rhythmic gibberellin (GA) and auxin signaling (Figure 4; Covington and Harmer, 2007; Nozue et al., 2007; Rawat et al., 2009; Arana et al., 2011; Goodspeed et al., 2012; Shin et al., 2012; Thines et al., 2019). In terms of defense responses, jasmonate is vital for the establishment of resistance against necrotrophic pathogen infection and wounding by feeding insects. Previous studies have highlighted that jasmonate-mediated plant defense against necrotrophic fungal pathogens is modulated by the circadian clock and differs with a time of day (Hevia et al., 2015; Ingle et al., 2015; Zhang et al., 2019a, 2019b). Moreover, the circadian clock also gates jasmonate-regulated resistance to insect herbivory (Goodspeed et al., 2012; Sharma and Bhatt, 2015). As jasmonate signaling is stimulated in the morning, it is possible that this regulation of jasmonate helps plants establish the appropriate defense to anticipate particular pathogen infections and insect attacks during the morning phase. Despite recent advances, in-depth analyses of the direct implication of crucial regulators that integrate the circadian clock and jasmonate signaling pathways may shed light on the molecular basis of the balance between plant growth and stress tolerance.

A network of transcriptional regulators mediates jasmonate signaling

The COI1/JAZ-mediated jasmonate signaling pathway delays flowering in Arabidopsis (Robson et al., 2010; Yang et al., 2012; Zhai et al., 2015). Under long-day conditions, *coi1-2* and *JAZ1*-overexpressing plants flower earlier than wild-type plants (Zhai et al., 2015). Detailed analyses of the responsible mechanism revealed that a subset of JAZ proteins interacts with and inhibits the transcription factors TOE1 and TOE2, which directly repress *FT* transcription and flowering (Zhai et al., 2015). Similarly, the bHLH subgroup IIIe transcription

factors (MYC2, MYC3, and MYC4) are required for jasmonate-mediated inhibition of flowering (Wang et al., 2017). The *myc2 myc3 myc4* triple mutant accumulates more *FT* transcripts and flowers earlier than the wild type (Wang et al., 2017). The results of these studies provided insights into how COI1/JAZ-mediated jasmonate signaling negatively modulates flowering time. In this study, we identified CO as a JAZ-binding protein that helps mediate jasmonate responses (Figure 5, A–D). Zhang et al. (2015) showed that TOE proteins interact with CO and transmit a photoperiodic signal with inhibitory effects on CO. The *bhlh3 bhlh13 bhlh14 bhlh17* quadruple mutant exhibits a late-flowering phenotype, indicating that these bHLH subgroup IIId transcription factors also stimulate flowering (Song et al., 2013a). The effects of bHLH subgroup IIId transcription factors on flowering time may involve antagonism of the repressive effects of bHLH subgroup IIIe transcription factors. These findings collectively suggest that jasmonate-regulated flowering may require a network of transcriptional regulators that combine to delay flowering.

Considering that CO and bHLH subgroup IIId transcription factors function oppositely (or antagonistically) to MYC and TOE proteins in mediating jasmonate-regulated processes, we speculate that the effects of different JAZ-binding factors on jasmonate signaling are balanced to maintain appropriate jasmonate signaling levels. This fine-tuning of jasmonate responses may optimize the balance between establishing stress tolerance and plant growth and development. Similar dual modulations have been described in other situations. For example, jasmonate-activated EIN3 inhibits MYC2 function to regulate jasmonate-induced apical hook curvature and defense responses to the necrotrophic fungal pathogen *Botrytis cinerea* (Berrocal-Lobo et al., 2002; Lorenzo et al., 2004; Zhu et al., 2011; Zhang et al., 2014; Song et al., 2014b). In this study, we found that exogenous application of jasmonate (i.e. MeJA) down-regulated *CO* transcript levels in wild-type seedlings (Figure 1, A–C). Additionally, *CO* transcript levels increased in the *coi1* mutant and *JAZ1-ΔJas* seedlings (Figure 1D), indicative of the suppressive effects of jasmonate on *CO* transcription. By contrast, CO increased in abundance in the presence of jasmonate (Figure 1E). Considering these findings along with the fact that JAZ repressors interact with and inhibit the transcriptional function of CO (Figures 5 and 8), it is possible that jasmonate has dual (negative and positive) regulatory effects on CO that strictly control jasmonate signaling. Further analyses of the regulatory mechanisms underlying the dual effects of jasmonate on *CO* transcription and the accumulation of the encoded protein will help understand how CO precisely controls jasmonate signaling.

The transcription of *CO* and the stability of the encoded protein are strictly controlled by multiple regulators and signaling pathways. More specifically, GIGANTEA (GI) and FLAVIN-BINDING, KELCH REPEAT, F-BOX1 (FKF1) play a major role in maintaining appropriate *CO* expression levels (Imaizumi et al., 2005; Sawa et al., 2007; Song et al., 2012, 2014a). Previous studies have demonstrated that GI physically associates with FKF1 to form a complex that recruits CYCLING DOF FACTOR (CDF) proteins, which are repressors of *CO* transcription, for degradation (Imaizumi et al., 2005; Sawa et al., 2007; Fornara et al., 2009). In the *cdf1 cdf2 cdf3 cdf5* quadruple mutant, *CO* transcript levels are high in the morning, regardless of photoperiod or daylength changes (Fornara et al., 2009). Moreover, several critical light-signaling components modulate CO abundance. For example, the RING finger E3 ubiquitin ligase CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1) and its interacting partner SUPPRESSOR OF PHYA-105 (SPA) facilitate CO protein degradation at night (Laubinger et al., 2006; Jang et al., 2008). Another RING finger E3 ubiquitin ligase, HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES1 (HOS1), works synergistically with COP1 to precisely regulate the timing of CO accumulation (Lazaro et al., 2012). Additionally, photoreceptors (e.g. phytochromes and cryptochromes), ZEITLUPE (ZTL), PSEUDO-RESPONSE REGULATORs (PRRs), GI, and TOE modulate CO stability (Valverde et al., 2004; Zuo et al., 2011; Song et al., 2012, 2014a; Hayama et al., 2017). Because jasmonate also affects *CO* transcription (Figure 1, A–D), further research is needed to determine whether jasmonate operates through the known upstream CO regulators listed above. Furthermore, whether the crucial regulators of CO also participate in jasmonate signaling should be investigated. Interestingly, the results presented in Figure 1E showed that CO accumulation increased in response to MeJA, and its accumulation further rose in seedlings treated with both MeJA and MG132 compared to seedlings treated with only MeJA or MG132. These observations suggest that the production of CO is activated by MeJA. It is possible that jasmonate affects *CO* mRNA stability and influences the subsequent translation and/or it affects how efficiently *CO* is translated. The underlying mechanisms should be clarified in future investigations.

Based on our results and those of other studies, we propose the following simplified model to explain the molecular mechanism underlying CO-mediated jasmonate signaling in Arabidopsis. When the jasmonate concentration is low, JAZ proteins interact with CO and bHLH3/bHLH17, interfere with CO–bHLH3/bHLH17 interactions, and repress transcription factor functions (Figures 5, 8, and 9). When jasmonate concentrations increase, COI1 perceives jasmonate and targets JAZ proteins for degradation via the SCF^{COI1}–26S proteasome pathway (Chini et al., 2007; Thines et al., 2007; Yan et al., 2009; Sheard et al., 2010). The degradation of JAZ repressors subsequently releases CO and bHLH3/bHLH17 to form a transcriptional complex that negatively regulates jasmonate-induced root-growth inhibition and anthocyanin accumulation (Figures 2–7; Nakata et al., 2013; Sasaki-Sekimoto et al., 2013; Song et al., 2013a; Fonseca et al., 2014). Additionally, CO accumulates in response to jasmonate (Figure 1E), which also contributes to the repressive effects of CO on jasmonate signaling.

Materials and methods

Materials and plant growth conditions

Taq DNA polymerases were obtained from Takara Biotechnology (Dalian, China); MeJA was purchased from Sigma-Aldrich. Other common chemicals were obtained from Sangon Biotech (Shanghai, China). The wild-type and mutant Arabidopsis plants used in this study were in the Col-0 or Landsberg *erecta* (*Ler*) genetic background. The *co-1* (CS3325), *co-4* (CS177), *co-6* (CS179), *co-9* (CS870084), *bhlh3* (CS871836), and *bhlh17* (CS874647) mutants were obtained from the Arabidopsis Biological Resource Center at Ohio State University (http://abrc.osu.edu). The *coi1-2*, *coi1-16*, *jazQ* (*jaz1 jaz3 jaz4 jaz9 jaz10*), and *myc2-1* mutants have been described (Ellis and Turner, 2002; Xu et al., 2002; Campos et al., 2016; Wang et al., 2017). To generate transgenic plants *35S:3Myc-CO*, *35S:2Flag-CO*, or *35S:2Flag-bHLH17*, the full-length coding sequence of *CO* or *bHLH17* was cloned into the binary vector pOCA30 in the sense orientation under the control of the CaMV 35S promoter (Han et al., 2020). The transgenic plants overexpressing *JAZ1* with a deleted Jas domain (*JAZ1-ΔJas*) were described previously (Han et al., 2018). The *co-1 coi1-2*, *co-1 myc2-1*, *bhlh3 bhlh17*, and *co-1 bhlh3 bhlh17* mutant lines were generated via standard crossing. Plants were grown in an artificial growth chamber at 22°C under a 16-h light (100 mE m^{-2} s^{-1}, white fluorescent bulbs, full light wavelength range)/8-h dark long-day photoperiod or an 8-h light/16-h dark short-day photoperiod. MeJA was dissolved in 10% (v/v) ethanol as a 10-mM stock solution. In the mock treatment, an equal volume of 10% (v/v) ethanol was added to the medium.

GUS staining

The putative *CO* promoter sequence (*proCO*; 3,576 bp) was amplified from Col-0 genomic DNA using gene-specific primers (Takada and Goto, 2003; Supplemental Data Set S1). The *proCO:GUS* construct was cloned into the pOCA28 binary vector and introduced into wild-type (Col-0) plants using the floral dip method (Clough and Bent, 1998). T_5 seedlings from *proCO:GUS* transgenic lines were treated with 50- or 100-μM MeJA for 4 h and then samples were harvested at ZT 16 for staining. The histochemical detection of GUS activity was performed as previously described (Chen et al., 2010). The primers used for cloning are listed in Supplemental Data Set S1.

RNA extraction and RT-qPCR

Total RNA was extracted from seedlings of the indicated age that had been treated with or without MeJA using Trizol reagent (Invitrogen) for RT-qPCR analysis, which was performed as previously described (Han et al., 2020). Briefly, 1.0-μg DNase-treated total RNA was reverse transcribed in a 20-μL reaction volume using the oligo-(dT)18 primer and Moloney murine leukemia virus reverse transcriptase (Fermentas, Hanover, MD, USA). The cDNA was diluted 1:1 prior to use. Each qPCR analysis was completed using 1.0-μL cDNA, a SYBR Premix Ex Taq kit (Takara Biotechnology), and a LightCycler 480 real-time PCR instrument (Roche) according to the manufacturer's instructions. *ACTIN2* (At3g18780) was used as the internal control. The gene-specific qPCR primers are listed in Supplemental Table S1.

Anthocyanin content measurement

Arabidopsis seeds were surface-sterilized with 5% [v/v] NaClO solution for 15 min and washed with sterile water three times, then incubated in the dark at 4°C for 2 days for stratification. Arabidopsis seedlings were grown on MS medium with or without 30-μM MeJA for 10 days before measuring their anthocyanin content as previously described (Qi et al., 2011). The anthocyanin content is expressed as $(A_{535}-A_{650})$ per gram fresh weight. All experiments were performed at least eight times with similar results by analyzing seedlings of different batches.

Root measurements

For jasmonate-mediated root-growth inhibition assays, the root length of 8-day-old seedlings grown on MS medium with or without 30- μM MeJA (Sigma-Aldrich) was measured. All experiments were performed at least five times with similar results by analyzing seedlings of different batches. For each sample, the root length of 20 representative seedlings was measured ($n = 20$). Values represent means ± SD. Comparisons between different lines and the wild type were done by a two-way analysis of variance (ANOVA).

Yeast two-hybrid (Y2H) assays

The coding sequence encoding the C-terminal half of CO (amino acids 176–373) was cloned into pGBKT7 (Clontech) to generate the bait vector (BD-$CO^{176-373}$) containing the GAL4 DNA-BD sequence. The full-length coding sequence of each target (*JAZ*, *MYC2*, *MYC3*, *MYC4*, *bHLH3*, *bHLH13*, *bHLH14*, or *bHLH17*) was individually cloned into pGADT7 vector (Clontech) to produce the prey vectors (AD-JAZ, AD-MYC, or AD-bHLH) containing the sequence encoding the GAL4 activation domain (AD). To identify specific regions critical for protein interactions, multiple truncated *JAZ1* and *bHLH17* sequences were cloned into pGADT7. The Y2H assays were performed as previously described (Hu et al., 2013). Yeast strain AH109 was co-transformed with the bait and prey vectors and then protein interactions were evaluated based on the ability of the cells to grow on synthetic defined (SD) medium lacking Leu, Trp, His, and Ade after 4 days of growth at 28°C. The primers used for cloning are listed in Supplemental Data Set S1.

BiFC assays

The coding sequences encoding the YFP C-terminal (64 amino acids) region (cYFP) and the YFP N-terminal (173 amino acids) region (nYFP) were inserted into separate pFGC5941 plasmids behind the FLAG or MYC tag sequences under the control of the CaMV 35S promoter to generate pFGC-cYFP and pFGC-nYFP, respectively (Kim et al., 2008).

The full-length or truncated *CO* coding sequence was cloned into pFGC-cYFP to generate *CO-cYFP* or CO^{1-105}*-cYFP*. Similarly, the full-length coding sequences or truncated forms of *JAZ1*, *JAZ5*, *JAZ9*, or *bHLH17* were cloned upstream of the *nYFP* sequence to generate *JAZ1-nYFP*, *JAZ5-nYFP*, *JAZ9-nYFP*, $JAZ1^{1-200}$*-nYFP*, $JAZ1^{155-201}$*-nYFP*, $bHLH17^{1-360}$*-nYFP*, and $bHLH17^{350-566}$*-nYFP*. The resulting plasmids were introduced into Agrobacterium (*Agrobacterium tumefaciens*) strain EHA105 for transient infiltration of *N. benthamiana* leaves as previously described (Han et al., 2020). The experiments were conducted at least three times using different batches of *N. benthamiana* plants. For each replicate, more than 12 *N. benthamiana* plants were infiltrated and more than 600 cells were analyzed. The fluorescence of YFP in DAPI-stained leaves was examined 48 h after infiltration using a confocal laser-scanning microscope (Olympus, Tokyo, Japan). The primers used for cloning are listed in Supplemental Data Set S1.

Protein extraction, immunoblots, and Co-IP assays

Eight-day-old Arabidopsis seedlings were ground in liquid nitrogen and homogenized in extraction buffer containing 50-mM Tris–HCl (pH 7.4), 1-mM EDTA, 150-mM NaCl, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, 1-mM PMSF, and 1× Roche Protease Inhibitor Cocktail. The extracts were incubated for 3 h at 4°C and the supernatant was collected by centrifugation (12,000*g* for 10 min at 4°C). For immunoblotting analysis, seedlings were harvested, frozen in liquid nitrogen, and homogenized in 100 µL of 1× Laemmli SDS-PAGE protein loading buffer. The extracts were boiled at 95°C for 5 min. After centrifugation (12,000*g* for 10 min at room temperature), the supernatant was collected. For immunoblotting, a 20-µL volume of each sample was run on 10% SDS-PAGE gels, transferred to nitrocellulose membrane (Bio-Rad), blocked for 1 h in 5% (w/v) reconstituted nonfat skim milk powder in TBST at room temperature, and incubated with an anti-Myc antibody (1:10,000; catalog no. M4439, mouse, monoclonal antibody, Sigma-Aldrich) or an anti-Flag antibody (1:10,000; catalog no. F3165, mouse, monoclonal antibody, Sigma-Aldrich). HRP-conjugated secondary antibody (goat anti-mouse, 1:10,000; D110087) was from Sangon Biotech (Shanghai, China). The luminescence was detected with Luminata Forte Western HRP substrate (Millipore).

For Co-IP assays, total proteins were extracted from *35S:2Flag-CO 35S:3Myc-JAZ1* transgenic Arabidopsis seedlings or from *35S:3Myc-CO 35S:2Flag-bHLH17* seedlings. Immunoprecipitation was performed with protein A/G Plus-agarose beads following the manufacturer's protocol. In brief, cell lysates were precleared with protein A/G Plus-agarose beads and incubated with an anti-Myc antibody (1:250) or an anti-Flag antibody (1:250) and protein A/G Plus-agarose beads at 4°C overnight in extraction buffer. The beads were washed twice extensively with extraction buffer and the co-immunoprecipitated protein was then detected by immunoblotting using an anti-Flag antibody (1:10,000) or anti-Myc antibody (1:10,000).

ChIP analyses

The ChIP assay was performed essentially as previously described (Mukhopadhyay et al., 2008; Hu et al., 2019). Eight-day-old seedlings (with or without 100-µM MeJA treatment for 1 h; harvested at ZT0 or ZT16) of the wild type, *35S:2Flag-bHLH17*, or *35S:Myc-CO 35S:2Flag-bHLH17* were crosslinked in 1% (w/v) formaldehyde and their chromatin was isolated. The anti-Flag antibody or IgG antibody (the negative control) was used to immunoprecipitate the protein–DNA (target promoter) complex, and the precipitated DNA was purified using a PCR purification kit (Qiagen) for qPCR analysis. To quantitatively assess bHLH17–DNA binding, qPCR analysis was performed as described previously (Mukhopadhyay et al., 2008) with the *ACTIN2* untranslated region sequence (*pACTIN2*) as an endogenous control. The relative quantity value was calculated by the 2 ($^{-DDCt}$) method (Mukhopadhyay et al., 2008) and presented as the DNA-binding ratio. The results presented were obtained from at least eight independent experiments. The primers used for ChIP assays are listed in Supplemental Document S1.

Transient transactivation assays

The putative promoter *JAZ2* sequence (proJAZ2, 2,000 bp) was amplified from Col-0 genomic DNA and cloned into the pGreenII 0800-LUC vector to generate the *ProJAZ2:LUC* reporter construct (Hellens et al., 2005). The full-length coding sequences of *CO*, *JAZ1*, *JAZ9*, *bHLH17*, and *GFP* were individually amplified and cloned into separate pGreenII 62-SK vectors under the control of the CaMV 35S promoter as effectors (Hellens et al., 2005). Different combinations of plasmids were transfected into Arabidopsis leaf mesophyll protoplasts as previously described (Sheen, 2001). Transfected cells were cultured for 16 h in the light and then relative LUC activity was detected using a Dual-Luciferase Reporter Assay system (Promega), which measured the activities of firefly LUC and that of the internal control *Renilla reniformis* LUC (REN). The primers used for constructs are listed in Supplemental Data Set S1.

Statistical analysis

Statistical analyses were performed as ANOVA using Tukey's honest significant difference as a post-hoc test. Statistically significant differences were defined as those with $P < 0.05$. Different lowercase letters indicate significant differences ($P < 0.05$). Asterisks in Figures 1 and 4 also represent differences that are statistically significant ($^{*}P < 0.05$) or highly significant ($^{**}P < 0.01$) at the indicated times., All statistical analyses were performed using GraphPad Prism version 8.0. The results are shown in Supplemental Data Set S2.

Accession numbers

Arabidopsis Genome Initiative numbers for the genes discussed in this article are as follows: *CO* (At5g15840); *COI1*

(At2g39940); *JAZ1* (At1g19180); *JAZ2* (At1g74950); *JAZ3* (At3g17860); *JAZ4* (At1g48500); *JAZ5* (At1g17380); *JAZ6* (At1g72450); *JAZ7* (At2g34600); *JAZ8* (At1g30135); *JAZ9* (At1g70700); *JAZ10* (At5g13220); *JAZ11* (At3g43440); *JAZ12* (At5g20900); *bHLH3* (At4g16430); *bHLH13* (At1G01260); *bHLH14* (At4G00870); *bHLH17* (At2g46510); *MYC2* (At1g32640); and *ACTIN2* (At3g18780).

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. RT-qPCR analyses of CO expression in 35S:3Myc-CO and 35S:2Flag-CO transgenic plants.

Supplemental Figure S2. RT-qPCR analysis of relative JAZ2, LOX2, and LDOX expression levels in co-4 and co-6 mutants.

Supplemental Figure S3. Jasmonate responses of co mutant plants under short-day conditions.

Supplemental Figure S4. Negative controls for the interactions between CO and JAZ proteins in yeast and *N. benthamiana*.

Supplemental Figure S5. Negative controls for the interactions between CO and bHLH transcription factors in yeast and *N. benthamiana*.

Supplemental Figure S6. Schematic diagrams of the effectors and reporters used in the transient transactivation assays.

Supplemental Figure S7. Accumulation of CO and bHLH17 in Arabidopsis leaf mesophyll protoplasts.

Supplemental Figure S8. Induced expression of bHLH3 and bHLH17 in response to MeJA.

Supplemental Document S1. Information for bHLH17-binding sequences in the *JAZ1* and *JAZ2* promoters (for ChIP assays).

Supplemental Table S1. Primers used for RT-qPCR analyses.

Supplemental Data Set S1. Primers used for cloning.

Supplemental Data Set S2. ANOVA tables.

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