

The Basic Helix-Loop-Helix Transcription Factor MYC2 Directly Represses *PLETHORA* Expression during Jasmonate-Mediated Modulation of the Root Stem Cell Niche in *Arabidopsis* ^{WIOA}

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The root stem cell niche, which in the *Arabidopsis thaliana* root meristem is an area of four mitotically inactive quiescent cells (QCs) and the surrounding mitotically active stem cells, is critical for root development and growth. We report here that during jasmonate-induced inhibition of primary root growth, jasmonate reduces root meristem activity and leads to irregular QC division and columella stem cell differentiation. Consistently, jasmonate reduces the expression levels of the AP2-domain transcription factors *PLETHORA1* (*PLT1*) and *PLT2*, which form a developmentally instructive protein gradient and mediate auxin-induced regulation of stem cell niche maintenance. Not surprisingly, the effects of jasmonate on root stem cell niche maintenance and *PLT* expression require the functioning of *MYC2/JASMONATE INSENSITIVE1*, a basic helix-loop-helix transcription factor that involves versatile aspects of jasmonate-regulated gene expression. Gel shift and chromatin immunoprecipitation experiments reveal that *MYC2* directly binds the promoters of *PLT1* and *PLT2* and represses their expression. We propose that *MYC2*-mediated repression of *PLT* expression integrates jasmonate action into the auxin pathway in regulating root meristem activity and stem cell niche maintenance. This study illustrates a molecular framework for jasmonate-induced inhibition of root growth through interaction with the growth regulator auxin.

INTRODUCTION

Postembryonic root growth of higher plants is maintained by the root meristem, in which stem cells, including the mitotically inactive quiescent center (QC) and its surrounding stem cells, reside in a specialized microenvironment called the stem cell niche (Dolan et al., 1993; Watt and Hogan, 2000; Weigel and Jürgens, 2002; Laux, 2003; Aida et al., 2004). In *Arabidopsis thaliana*, the stem cell niche serves as the source of all differentiated cell types during root development (van den Berg et al., 1997; Dello Iorio et al., 2008). Although the structure of the primary root meristem is preestablished during embryo development, the meristematic activity can be altered by versatile developmental and environmental cues during postembryonic growth.

Several hormonal pathways are involved in the regulation of root growth, with auxin being the key player (Benjamins and Scheres, 2008; Benková and Hejác̃ko, 2009; Santner et al., 2009; Vanneste and Friml, 2009). The whole process of root organogenesis, including initiation of the root pole (Friml et al., 2003), formation of the root stem cell niche (Sabatini et al., 1999; Billou et al., 2005), maintenance of mitotic activity of the root meristem (Beemster and Baskin, 2000; Dello Iorio et al., 2007; Stepanova et al., 2008), and elongation and differentiation of cells leaving the root meristem (Rahman et al., 2007), has been demonstrated to be under the control of auxin, especially its featured gradient distribution (Tanaka et al., 2006; Benková and Hejác̃ko, 2009; Petrác̃sek and Friml, 2009). The *PIN-FORMED* (*PIN*) genes, which encode components of the auxin efflux machinery mediating polar auxin transport, are critical for the formation of a proper auxin distribution gradient and therefore direct root pattern formation and outgrowth (Billou et al., 2005). It was proposed that the instructive auxin gradient should be translated into developmental information by molecular components. The auxin-inducible *PLETHORA* (*PLT*) genes, which encode the AP2 class of transcription factors that are essential for root stem cell niche patterning (Aida et al., 2004; Galinha et al., 2007), are good candidates for performing this translation (Benjamins and

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Scheres, 2008). Interestingly, the expression domain of *PLT* genes overlaps with the auxin maximum in the root. Recent work reveals an elegant interaction network of *PIN*s and *PLT*s in regulating auxin-mediated root patterning and outgrowth; *PIN* proteins restrict *PLT* expression in the basal embryo region to initiate the root primordium. In turn, *PLT* genes maintain *PIN* transcription, which stabilizes the position of the root stem cell niche (Blilou et al., 2005; Grieneisen et al., 2007; Dinneny and Benfey, 2008).

Significant progress in our understanding of auxin signaling was achieved by the demonstration that the F-box protein TIR1 is an auxin receptor (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). Auxin binds to TIR1 and promotes the interaction of TIR1 with its auxin/indole-3-acetic acid (IAA) substrates. This interaction leads to the degradation of the auxin/IAA repressors by the 26S proteasome and therefore releases the transcriptional regulation activity of the AUXIN RESPONSE FACTOR proteins (Mockaitis and Estelle, 2008).

The jasmonate family of oxylipins, including jasmonic acid (JA) and its metabolites, play a well-established role in mediating defense responses and a wide range of developmental processes (Creelman and Mullet, 1997; Turner et al., 2002; Browse, 2005; Wasternack, 2007; Howe and Jander, 2008; Kazan and Manners, 2008). Among the first characterized physiological effects of jasmonate is growth inhibition (Dathe et al., 1981). It is proposed that JA-induced growth inhibition involves phase-specific disruption of cell cycle progression. Indeed, application of JA arrests synchronized tobacco (*Nicotiana tabacum*) BY-2 cells in both G1- and G2-phases (Swiatek et al., 2004). Whole-genome gene expression profiling of *Arabidopsis* cell cultures indicates that MeJA, the methyl ester of JA, represses the activation of M-phase genes and arrests the cell cycle in the G2-phase (Pauwels et al., 2008). Consistently, recent evidence suggests that wound-induced jasmonates stunt plant growth by inhibiting mitosis (Zhang and Turner, 2008). Even though the action mechanism governing JA-induced inhibition of plant growth remains elusive, characterization of JA-related mutants defective in JA-induced inhibition of root growth has significantly advanced our understanding of the molecular mechanism of JA-regulated gene expression (Browse, 2009). The *coronatine insensitive1* (*coi1*) mutant is fully insensitive to JA both in terms of root growth inhibition and defense gene expression (Feys et al., 1994). *COI1* encodes an F-box protein that interacts with SKP1 and CULLIN1 to assemble a functional SCF^{COI1} ubiquitin ligase complex in vivo (Xie et al., 1998; Devoto et al., 2002; Xu et al., 2002). *COI1*, which shows high sequence homology with the auxin receptor TIR1, was shown recently to be the jasmonate receptor (Yan et al., 2009; Sheard et al., 2010). Compared with *coi1*, the *jasmonate insensitive1* (*jin1*) (Berger et al., 1996; Lorenzo et al., 2004) mutant exhibits a relatively weak phenotype in JA-induced inhibition of root growth. *JIN1* encodes a nuclear-localized basic helix-loop-helix-type transcription factor known as MYC2 (Boter et al., 2004; Lorenzo et al., 2004), which acts as both an activator and a repressor to regulate diverse aspects of JA-mediated gene expression (Dombrecht et al., 2007). A family of jasmonate ZIM domain (JAZ) proteins was identified as the target of the SCF^{COI1} ubiquitin ligase complex in JA signaling (Chini et al., 2007; Thines et al., 2007). Recent structure-function

studies indicated that the jasmonate receptor is a three-molecule complex consisting of *COI1*, JAZ transcriptional repressors, and inositol pentakisphosphate (Sheard et al., 2010). These studies together revealed that jasmonate and auxin show a similar signal perception and transduction paradigm in which F-box proteins (receptors) mediate the degradation of negative regulators (Mockaitis and Estelle, 2008). Jasmonoyl Ile, an active form of JA, promotes the degradation of JAZ proteins and, in turn, frees the transcriptional regulation activity of MYC2, the major transcription factor of jasmonate-mediated gene expression.

It has been shown that the jasmonate and auxin signaling pathways share important components in regulating root growth. For example, the first characterized auxin-resistant mutant, *axr1* (Lincoln et al., 1990; Leyser et al., 1993), was later shown to be also resistant to JA in root growth (Tiryaki and Staswick, 2002). *AXR1* encodes a subunit of the heterodimeric RUB-E1 enzyme, which is important for the modification of CUL1, a shared component of SCF^{TIR1} and SCF^{COI1}, demonstrating that the auxin and jasmonate proteasome pathways are directly connected through *AXR1* (Tiryaki and Staswick, 2002). Recently, it was reported that JAZs interact with Novel Interactor of JAZ to recruit TOPLESS as a corepressor to repress JAZ-targeted transcription factors (Pauwels et al., 2010). Because TOPLESS also mediates auxin-dependent transcriptional repression (Szemenyei et al., 2008), these results provide another line of evidence for jasmonate/auxin interaction at the level of transcriptional repression.

In this study, we investigate the cellular and molecular mechanisms of jasmonate action on the inhibition of primary root growth. We show that JA modulates the cellular organization of the stem cell niche by promoting QC division and columella stem cell (CSC) differentiation. We also show that JA represses the expression of *PLT1* and *PLT2*, and this effect of JA is executed by direct binding of MYC2 to the promoters of the *PLT* genes. Our results reveal that MYC2-mediated repression of *PLT* expression is involved in the long-standing observation that jasmonate inhibits primary root growth in *Arabidopsis*. *PLT1* and *PLT2* are key interaction nodes between jasmonate and auxin in the regulation of root stem cell niche maintenance and meristem activity.

RESULTS

JA-Induced Inhibition of Primary Root Growth Involves a Reduction of Root Meristem Activity

To understand how jasmonate inhibits root growth, we measured primary root length of *Arabidopsis* seedlings by germinating seeds in medium containing different concentrations of JA. As expected, root growth of wild-type plants was inhibited by JA in a dose-dependent manner, while root growth of the JA-insensitive *coi1-1* mutants was largely unaffected by JA (Figures 1A and 1B). We then investigated the JA-induced cellular changes in the three morphologically distinguishable developmental zones along the longitudinal axis of the root: the differentiation zone (DZ), the elongation zone (EZ), and the meristem zone (MZ). As shown in Figure 1C, JA decreased the final cell length of the DZ

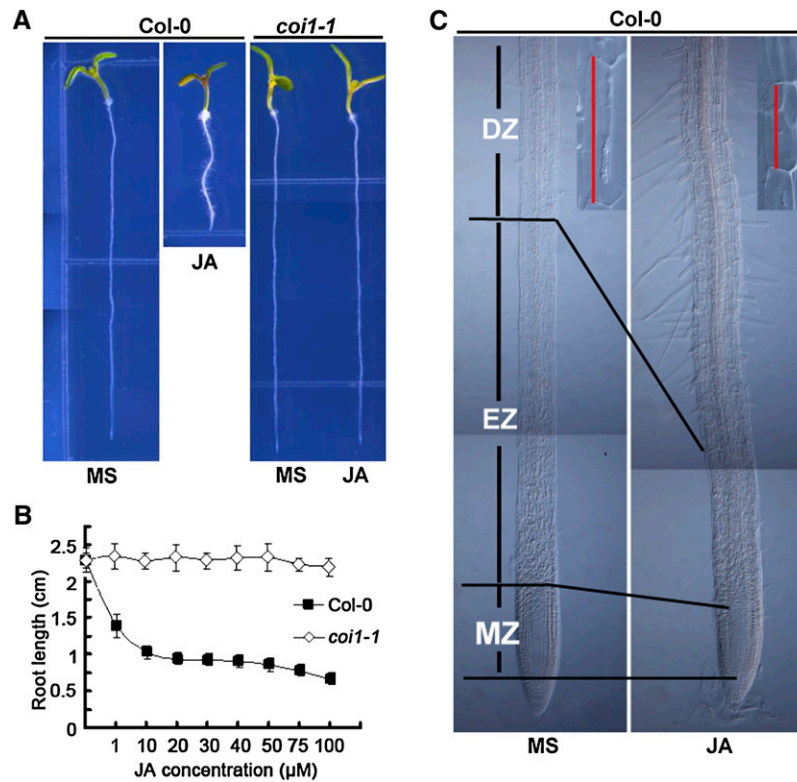


Figure 1. JA Represses Root Growth through Inhibition of both Cell Proliferation and Cell Elongation.

(A) Six-day-old seedlings of the wild type (Col-0) and *coi1-1* were grown on medium without (MS) or with 20 μM JA.
(B) JA-mediated inhibition of root growth in Col-0 and *coi1-1*. Col-0 and *coi1-1* seeds were germinated on medium containing different concentrations of JA, and seedling root length was measured at 6 d after germination. The effects of JA on Col-0 and *coi1-1* were significantly different. Data shown are average and SD ($n > 20$) and are representative of at least three independent experiments.
(C) JA reduces the MZ, the EZ, and epidermal cell length of the DZ. A representative image of 6-d-old Col-0 seedlings grown in the absence or presence of JA. Insets show that JA reduces cell length (marked with red lines) of Col-0 epidermal cells in the DZ. Bars = 50 μm.

and reduced the size of the EZ as well as the size of the MZ. Closer observation of the DZ and EZ of JA-treated roots indicated that JA reduced both cell number and cell length of these regions (see Supplemental Figure 1 online), indicating that JA reduces both cell proliferation and cell elongation, two basic cellular processes affecting primary root growth (Scheres et al., 2002).

We then focused on the cellular and molecular mechanisms underlying the JA-induced reduction of root meristem size. The effect of JA on root meristem size was evaluated by determining the number of cortical cells in the region from the QC to the first elongated cell (Casamitjana-Martínez et al., 2003; Dello Iorio et al., 2007). As shown in Figures 2A to 2G, JA effectively reduced the root meristem cell number in the wild type, and this effect was substantially reduced in *coi1-2* (Xu et al., 2002) and *myc2-2* (Boter et al., 2004), indicating that JA reduces the number of the transit amplifying cells in a COI1- and MYC2-dependent manner. The reduction of root meristem cell number by JA could result from its negative effect on cell division. To test this idea, we monitored the expression of the transcriptional fusion reporter *CYCB1;1_{pro}:GUS* (for β-glucuronidase), a widely used marker to indicate G2/M-phase of the cell cycle (Colón-Carmona et al.,

1999). Without JA treatment, *CYCB1;1_{pro}:GUS* was expressed in the actively dividing cells of the root meristem (Figures 2H and 2J). JA treatment markedly reduced the expression of *CYCB1;1_{pro}:GUS* in the wild type (Figure 2I) but not in *coi1-1* (Figure 2K), suggesting that the JA signaling pathway represses the cell division activity of the transit amplifying cells. It is noteworthy that the effect of JA on *CYCB1;1_{pro}:GUS* expression was different from that of ethylene, which exerted little effect on *CYCB1;1_{pro}:GUS* expression (Růžicka et al., 2007). Consistent with JA-induced reduction of *CYCB1;1_{pro}:GUS* expression, our quantitative RT-PCR (qRT-PCR) assays revealed that exogenous JA markedly reduces the expression levels of several cell cycle-related genes, including *CYCB1;1* (Fuerst et al., 1996), *CDC2A* (Martinez et al., 1992), *KRP1* (Wang et al., 1997), and *PCNA1* (Egelkrout et al., 2002) (Figure 2L). It is noteworthy that JA failed to repress the expression of cell cycle-related genes in *coi1-2* and *myc2-2* (Figure 2L), indicating that JA negatively regulates cell division activity of the root meristem through COI1 and MYC2. Consistently, using transgenic plants containing gene promoter and *GUS* fusions, we found that *COI1_{pro}:GUS* and *MYC2_{pro}:GUS* are richly expressed in the root meristem (see Supplemental Figures 2A to 2D online).

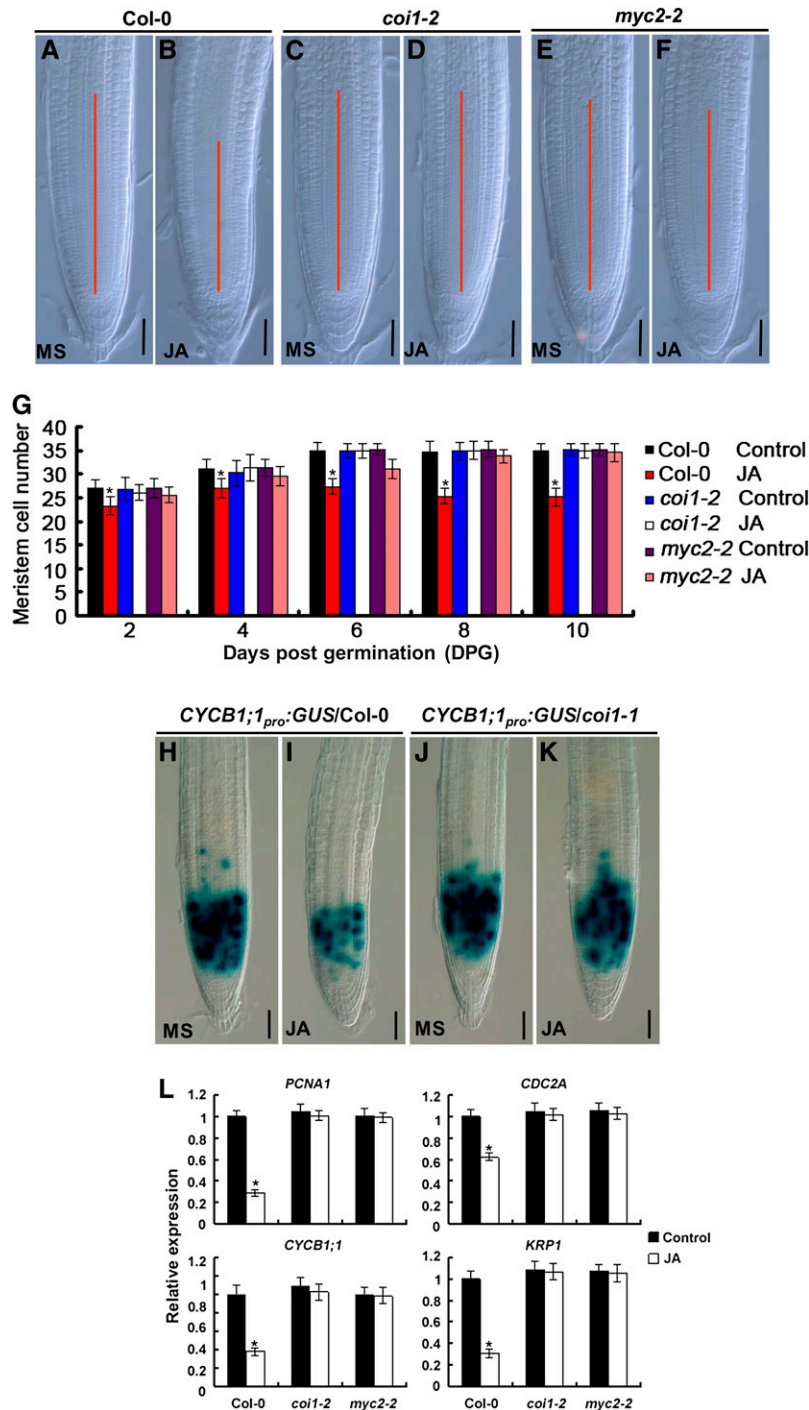


Figure 2. JA Reduces Cell Division Activity of the Root Meristem in the Wild Type but Not in *coi1-2* and *myc2-2*.

(A) to (F) Root meristems of 6-d-old Col-0, *coi1-2*, and *myc2-2* seedlings grown on medium without (MS) or with 20 μ M JA. The meristem zone was marked with red line. Bars = 50 μ m.

(G) JA-induced reduction of root meristem cell number in Col-0, *coi1-2*, and *myc2-2*. Seedlings of the indicated genotypes were grown on medium without (MS) or with 20 μ M JA, and cell number in the root meristem was examined at a 2-d interval. Data shown are average and SD ($n = 20$). Asterisks denote Student's *t* test significance compared with untreated plants: * $P < 0.05$.

(H) to (K) JA-induced reduction of *CYCB1;1_{pro}:GUS* expression in Col-0 and *coi1-1*. Five-day-old seedlings germinated on control medium were transferred to medium without (MS) or with 20 μ M JA for 1 d, and the *CYCB1;1_{pro}:GUS* expression was monitored. Bars = 50 μ m.

JA Promotes QC Division in a COI1- and MYC2-Dependent Manner

Our finding that JA alters root meristem activity prompted us to investigate its possible effect on the cellular organization of QC and its surrounding stem cells. In normal-grown wild-type roots, the well-defined QC cells rarely divide (Figures 3A, 3G, 3I, 3K, 3M, and 3O). However, obvious QC division was observed in a significant proportion of JA-treated roots (Figures 3B, 3H, 3J, 3L, 3N, and 3P). Using the QC-specific markers *QC25* and *WOX5_{pro}:GFP* (for green fluorescent protein; Bllilou et al., 2005; Sarkar et al., 2007), we confirmed that the dividing cells were QC cells (Figures 3G to 3J). To test whether JA induces QC differentiation, we performed Lugol staining experiments using the *QC25* marker line. As shown in Figure 3H, no starch accumulation was detected in the irregularly divided QC cells, suggesting that JA promotes QC division but does not induce QC differentiation. Considering that *WOX5* plays an important role in regulating the maintenance of the root stem cell niche (Sarkar et al., 2007; Ding and Friml, 2010), we examined whether JA treatment affects *WOX5* expression. RNA in situ assays indicated that JA treatment led to a slight expansion of the *WOX5* expression domain (cf. Figures 3L and 3K), which could have resulted from the irregular QC division. However, as revealed by our qRT-PCR assays, JA treatment did not substantially alter the *WOX5* transcript levels (Figure 3Q). To check whether cell cycle activity is altered in the QC of JA-treated roots, we cultured 2-d-old seedlings for 24 h in the presence of JA and EdU, a nucleoside analog of thymidine. EdU has been used to mark cell division in the root meristem because incorporation of this chemical in the nuclei is indicative of S-phase progression (Vanstraelen et al., 2009). In this assay, mitotically active cells show red fluorescent nuclei after coupling of the EdU with the Alexa Fluor 647 substrate. In untreated wild-type root meristems, most meristematic cells, including those QC-associated stem cells, incorporated EdU with red fluorescent nuclei, but only occasionally in the QC cells (marked with *WOX5_{pro}:GFP*), indicating their low mitotic activity (Figures 3M and 3O). In JA-treated root meristems, however, the QC cells were able to incorporate EdU (Figures 3N and 3P; 13/15 in JA-treated roots versus 2/15 in control roots, $n = 15$), indicating that JA application induces high mitotic activity of the QC cells.

Similar to JA-induced root growth inhibition, we found that JA-induced QC division was dose dependent (Figure 3R). We followed the time course of the onset of the JA-induced QC phenotype by quantifying extra QC cells for 10 d after germination on medium containing 20 μ M JA. Quantification of extra QC cells has been successfully used to characterize ethylene-induced QC division (Ortega-Martínez et al., 2007). In the wild type, obvious QC division was observed as early as 2 d after

germination, and the number of extra QC cells increased gradually with time (Figure 3T). Importantly, our parallel experiments revealed that the effect of JA on QC division was largely abolished in the JA signaling mutants, including *coi1-2* and *myc2-2* (Figures 3C to 3F, 3S, and 3T), indicating that the promotional effect of JA on QC division requires the function of COI1 and MYC2.

To test whether the above-described effect of JA on the promotion of QC division is independent of that of ethylene, which has also been shown to modulate stem cell division in *Arabidopsis* roots (Ortega-Martínez et al., 2007), we examined the effect of JA on QC division in the previously described ethylene signaling mutants *ein2-5* and *ein3-1 eil1-1* (Guo and Ecker, 2003). As shown in Supplemental Figure 3 online, the *ein2-5* and *ein3-1 eil1-1* mutants did not affect the promotional effect of JA on QC division. Similarly, the addition of AgNO₃, which effectively blocks the ethylene signaling pathway, did not alter the effect of JA on the promotion of QC division (see Supplemental Figure 3 online). These results confirmed that the effect of JA on the promotion of QC division is independent of that of ethylene.

JA Induces Deregulated Differentiation of CSCs

In the root stem cell niche, the mitotically inactive QC cells act to maintain the identity and function of the surrounding stem cells. To assess the possible effect of JA-induced QC division on stem cell maintenance, we examined the cell layer immediately below the QC, at the position of the CSCs. The *J2341* marker, which is specifically expressed in the single layer of CSCs (Figure 4A), expanded to more than one cell tier upon JA treatment (cf. Figures 4A and 4B). We then asked whether the distal stem cells might have undergone premature differentiation in JA-treated roots, which in the columella can be visualized by the accumulation of starch granule-containing organelles. In the absence of JA, 6-d-old wild-type roots do not show starch granule accumulation in the CSCs (Figure 4C, white arrow), which are distinct from the underlying well-organized four tiers of columella cells that accumulate starch granules (Figures 4C, white dashed line). In the presence of JA, however, irregular cells containing starch granules were observed immediately below the QC cells (Figure 4D, red arrow), indicating that the CSCs were in a state of differentiation. Our time-course study revealed that, in JA-treated roots, the appearance of QC division is generally earlier than that of CSC differentiation (Figure 4K), suggesting that the JA-induced irregular QC divisions may lead to a failure to maintain the stem cell fate of CSCs. In addition, JA treatment led to the formation of extra columella cell layers, and the general cellular organization of columella tiers was disturbed (Figures 4D, red dashed line, 4I, and 4J). The above-described effect of JA on

Figure 2. (continued).

(L) JA-regulated expression of cell cycle-related genes in Col-0, *coi1-2*, and *myc2-2*. Five-day-old Col-0 seedlings germinated on control medium were transferred to liquid medium without (control) or with 20 μ M JA for 6 h, and 2-mm root tips were harvested for RNA extraction and qRT-PCR analysis. Transcript levels of cell cycle-related genes were normalized to *ACTIN2* expression. The transcript levels of the indicated genes in Col-0 without JA treatment were arbitrarily set to 1. Error bars represent the SD of triplicate reactions of independent RNA samples prepared from three batches of *Arabidopsis* roots. Asterisks denote Student's *t* test significance compared with untreated plants: * $P < 0.05$.

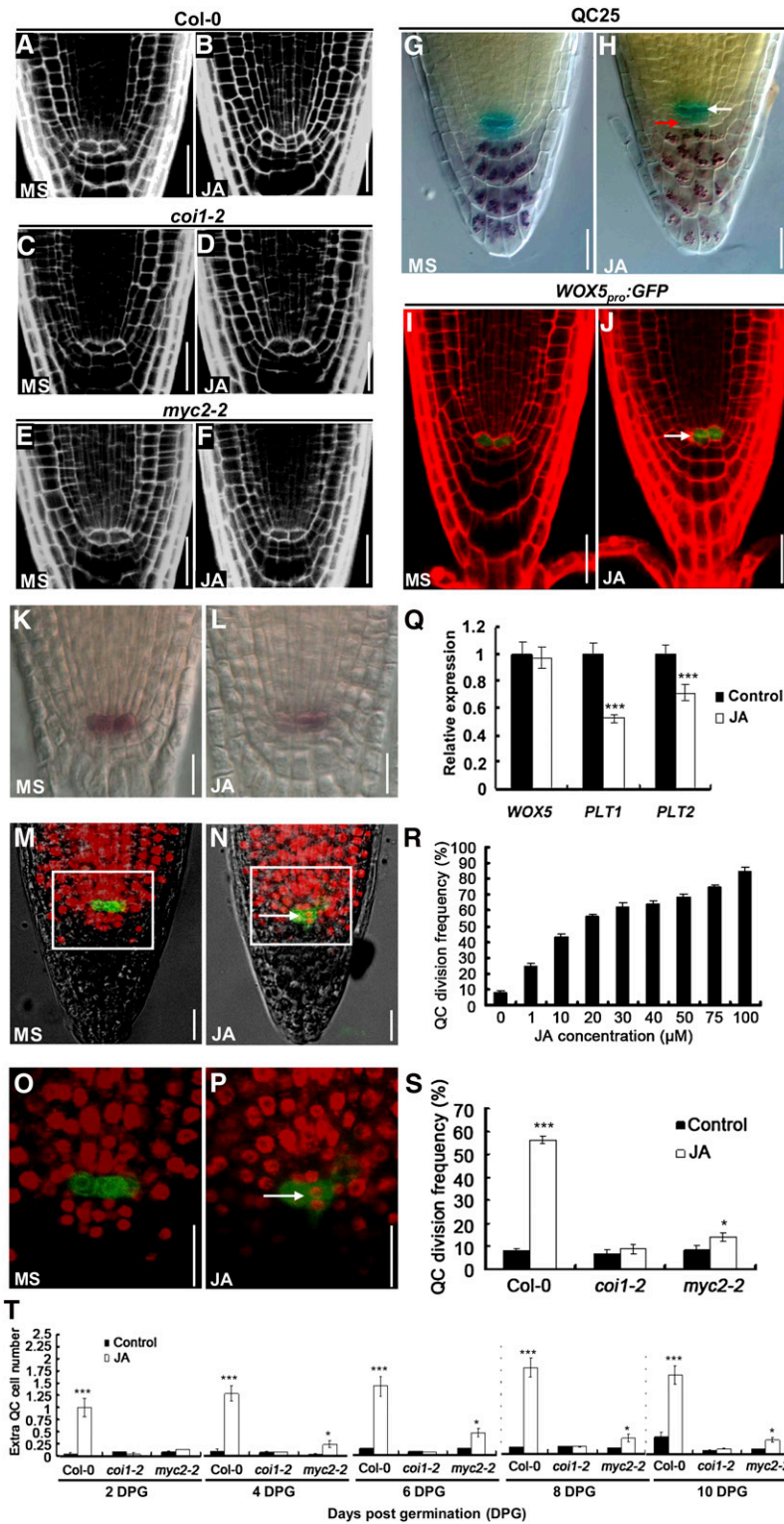


Figure 3. JA-mediated Promotion of QC Division in the Wild Type, *coi1-2*, and *myc2-2*.

(A) to (F) Representative confocal images showing that JA promotes QC division in Col-0 but not in *coi1-2* and *myc2-2*. Five-day-old seedlings were transferred to medium without (MS) or with 20 μM JA for another day before QC division was monitored. Bars = 20 μm.

(G) and (H) JA induces QC division and CSC differentiation as revealed by Lugol staining of the QC25 marker line. Five-day-old seedlings of the QC25

CSC differentiation and extra columella layer formation was substantially reduced in *coi1-2* (Figures 4E, 4F, 4I, and 4J) and *myc2-2* (Figures 4G to 4J), suggesting that the effect of JA on CSC differentiation and columella organization requires the function of COI1 and MYC2.

To test whether endogenous JA has a similar effect as exogenous JA (i.e., to reduce root meristem activity and to disturb the maintenance of the root stem cell niche), we sought to obtain mutants with elevated endogenous JA levels or with increased JA sensitivity. The *constitutive expression of VSP1 (cev1)* mutant has increased production of both JA and ethylene and exhibits a short root phenotype (Ellis and Turner, 2001; Ellis et al., 2002). When grown on control medium, *cev1* seedlings show reduced meristem cell number (see Supplemental Figure 4A online), increased QC division (see Supplemental Figures 4B and 4C online), and CSC differentiation (see Supplemental Figures 4D to 4I online) than wild-type seedlings. When grown on medium containing 2-aminoethoxyvinyl glycine or AgNO₃, which can effectively block ethylene biosynthesis and perception, respectively, *cev1* roots still exhibit reduced meristem size, exaggerated QC division, and CSC differentiation compared with wild-type roots (see Supplemental Figure 4 online). These results support that endogenous JA exerts a similar effect as exogenous JA on stem cell niche maintenance.

Given that JAZ proteins serve as negative regulators of JA signaling, it is reasonable to expect that loss-of-function mutants of JAZ genes exhibit increased sensitivity to JA in root growth inhibition. Indeed, it has been shown that RNA interference (*RNAi*) lines of *JAZ10* show a hypersensitive response to JA (Yan et al., 2007). We found that, like the *JAZ10 RNAi* lines, a T-DNA

insertion line (CS872819, SAIL_92_D08) that disrupts the expression of *JAZ10* also exhibits increased sensitivity to JA in root growth inhibition (see Supplemental Figure 5A online). Consistently, JA-induced meristem cell number reduction (see Supplemental Figure 5B online), QC division (see Supplemental Figure 5C online), CSC differentiation (see Supplemental Figures 5E to 5H online), and columella layer disorganization (see Supplemental Figure 5D online) are all increased in *jaz10* relative to the wild type. These results confirm that, like exogenous JA, endogenous JA also modulates the cellular organization of the root stem cell niche and reduces root meristem activity.

JA Reduces the Expression of *PLT1* and *PLT2* in a MYC2-Dependent Manner

The PLT family of transcription factors provides important patterning information for the root stem cell niche and determines the number of the transit-amplifying daughter cells that make up the meristem (Aida et al., 2004; Galinha et al., 2007). We show that *Arabidopsis* roots grown on JA-containing medium exhibit ectopic cell division in the QC, irregular differentiation of CSCs, and loss of transit-amplifying cells, defects that are also observed in the *plt1-4 plt2-2* double mutant roots (Aida et al., 2004). The root phenotype similarity between JA-treated roots and mutants of the *PLT* genes prompted us to test the possible link between JA and the *PLT* network in regulating root development. We first examined JA-induced expression of *PLT1* and *PLT2* at the transcription level. Using transgenic plants containing the promoters of *PLT1* and *PLT2* fused to the cyan fluorescent protein (*CFP*) gene (Galinha et al., 2007), we found that JA

Figure 3. (continued).

marker line were transferred to medium without (MS) or with 100 μ M JA for 1 d before GUS (blue) and Lugol (dark brown) double stainings were performed. White arrow indicates no starch accumulation in the irregular QC cells. Red arrow shows starch accumulation in the irregular CSCs. Bars = 20 μ m.

(I) and **(J)** JA-induced QC division in the QC marker line *WOX5_{pro}:GFP*. Five-day-old seedlings of *WOX5_{pro}:GFP* were transferred to medium without (MS) or with 20 μ M JA for another day before QC division was monitored. White arrows mark the cell divisions in the QC. Bars = 20 μ m.

(K) and **(L)** Whole-mount in situ hybridization with a *WOX5*-specific probe showing that JA causes supernumerary QC cells. Roots of seedlings 5 d after germination grown on medium without (MS) or with 20 μ M JA were used for whole-mount in situ hybridization. Bars = 20 μ m.

(M) to **(P)** EdU incorporation assays showing that JA promotes QC division. Two-day-old *WOX5_{pro}:GFP* seedlings grown on medium without (MS) (**(M)** and **(O)**) or with 100 μ M JA treatment (**(N)** and **(P)**) were cultured with EdU for 1 d before EdU incorporation in the QC was examined. **(O)** represents the outlined area in **(M)**, and **(P)** represents the outlined area in **(N)**. Red fluorescence of EdU-positive nuclei in QC cells of JA-treated roots indicates that QC is in a state of active division (white arrow). Bars = 20 μ m.

(Q) JA-induced *WOX5* expression revealed by qRT-PCR. Five-day-old Col-0 seedlings germinated on control medium were transferred to liquid medium without (control) or with 20 μ M JA for 6 h, and 2-mm root tips were harvested for RNA extraction and qRT-PCR analysis. *WOX5*, *PLT1*, and *PLT2* transcription levels without JA treatment were arbitrarily set to 1. Error bars represent the SD of triplicate reactions of independent RNA samples prepared from three batches of *Arabidopsis* roots. Asterisks denote Student's *t* test significance compared with untreated plants: ****P* < 0.001.

(R) Dose-dependent effect of JA on QC division in Col-0 roots. Col-0 seedlings were grown on medium containing indicated concentrations of JA for 6 d before QC division frequency (percentage of seedlings with obvious QC division) was determined. At least 50 seedlings were examined for each concentration for each biological repeat. Data shown are average and SD and are representative of at least three independent experiments.

(S) JA-induced QC division frequency in Col-0, *coi1-2*, and *myc2-2*. Seedlings were grown on medium without (Control) or with 20 μ M JA for 6 d before QC division frequency was determined. At least 50 seedlings were examined for each biological repeat. Data shown are average and SD and are representative of at least three independent experiments. Asterisks denote Student's *t* test significance compared with untreated plants: **P* < 0.05 and ****P* < 0.001.

(T) Time course of JA-induced extra QC cells in Col-0, *coi1-2*, and *myc2-2*. Seeds of the indicated genotypes were germinated on medium without (Control) or with 20 μ M JA for 10 d, and extra QC cells were quantified at a 2-d interval. The effects of JA on Col-0 compared with *coi1-2* and *myc2-2* were significantly different. At least 50 seedlings were examined for each biological repeat. Data shown are average and SD and are representative of at least three independent experiments. Asterisks denote Student's *t* test significance compared with untreated plants: **P* < 0.05 and ****P* < 0.001.

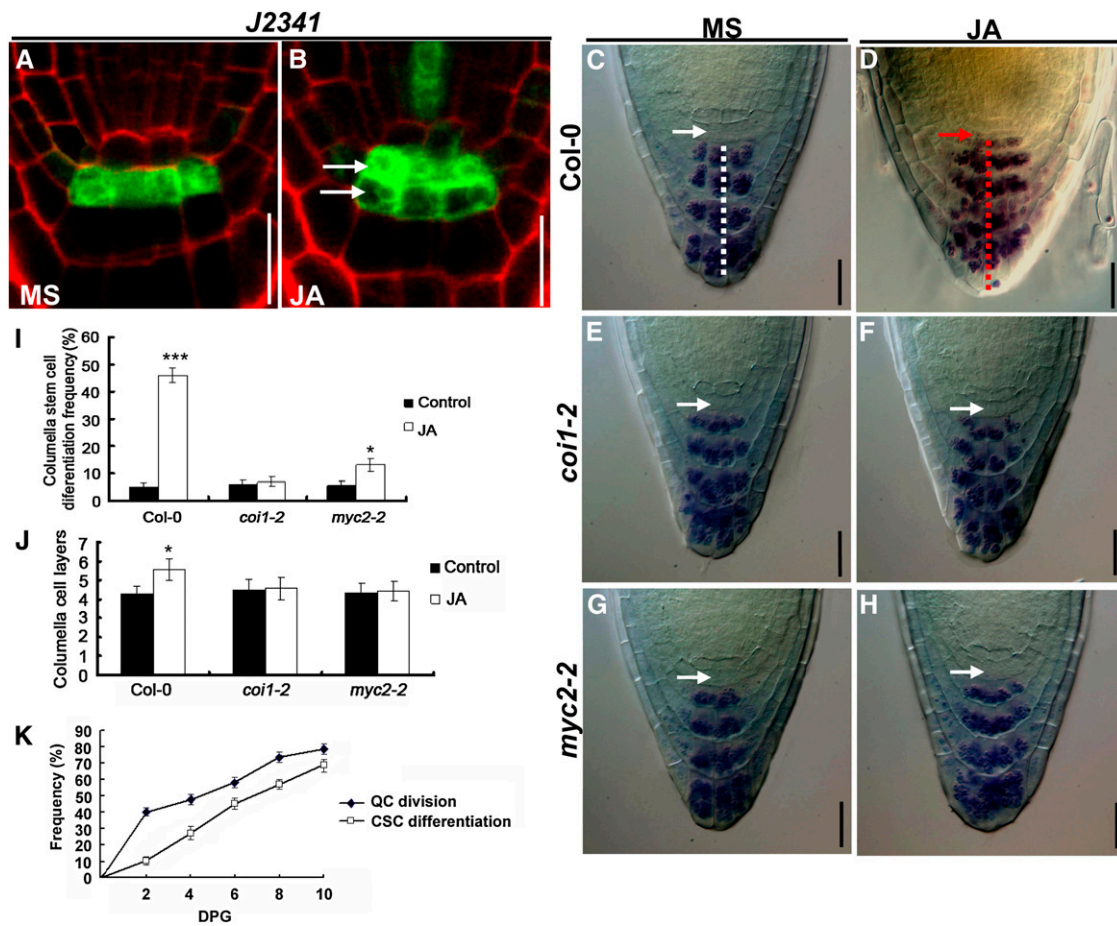


Figure 4. JA-Mediated Promotion of CSC Differentiation in the Wild Type, *coi1-2*, and *myc2-2*.

(A) and (B) JA induces deregulated division of CSCs, as revealed by the CSC-specific marker *J2341*. Five-day-old seedlings of the *J2341* marker line were transferred to medium without (MS) or with 20 μ M JA for 1 d before GFP expression in CSCs was examined. White arrows indicate the presence of two CSC layers in JA-treated roots. Bars = 20 μ m.

(C) to (H) Lugol staining showing that JA induces CSC differentiation in Col-0 [(C) and (D)] but not in *coi1-2* [(E) and (F)] and *myc2-2* [(G) and (H)]. Five-day-old seedlings were transferred to medium without (MS) or with 20 μ M JA for 1 d before Lugol staining was performed. Nondifferentiated CSCs (white arrows) below the QC are characterized by the absence of starch granules, whereas starch granules are visible in differentiated CSCs (red arrow). The white dashed line in (C) indicates the well-organized columella cell layers of untreated wild-type roots. The red dashed line in (D) indicates increased and disorganized columella cell layers of JA-treated wild-type roots. Bars = 20 μ m.

(I) JA-induced CSC differentiation frequency in Col-0, *coi1-2*, and *myc2-2*. Seedlings were grown on medium without (Control) or with 20 μ M JA for 6 d before CSC differentiation frequency was determined. At least 50 seedlings were examined for each genotype for each experiment. Data shown are average and SD and are representative of at least three independent experiments. Asterisks denote Student's *t* test significance compared with untreated plants: **P* < 0.05 and ****P* < 0.001.

(J) JA induces extra columella cell layers in Col-0 but not in *coi1-2* and *myc2-2*. Seedlings were grown on medium without (Control) or with 20 μ M JA for 6 d before columella cell layers were determined. At least 50 seedlings were examined for each genotype for each experiment. Data shown are average and SD and are representative of at least three independent experiments. Asterisks denote Student's *t* test significance compared with untreated plants: **P* < 0.05.

(K) Kinetics of JA-induced QC division and CSC differentiation. Col-0 seeds were germinated on medium containing 20 μ M JA (JA), and QC division frequency and CSC differentiation frequency were determined at a 2-d interval. At least 50 seedlings were examined for each biological repeat. Data shown are average and SD and are representative of at least three independent experiments.

significantly reduced the expression levels of *PLT1_{pro}:CFP* (Figures 5A and 5B) and *PLT2_{pro}:CFP* (Figures 5C and 5D) in the wild type but not in *coi1-2* and *myc2-2*, suggesting that JA represses *PLT1* and *PLT2* expression in a COI1- and MYC2-dependent manner. Next, using qRT-PCR assays, we compared the re-

pression effect of JA on *PLT* expression in the wild type, *coi1-2*, *myc2-2*, and the *MYC2* overexpression line *35S_{pro}:MYC2* (see Supplemental Figures 2E and 2F online). In line with previous reports (Boter et al., 2004; Lorenzo et al., 2004), our *35S_{pro}:MYC2* plants did not show constitutive activation of JA responses,

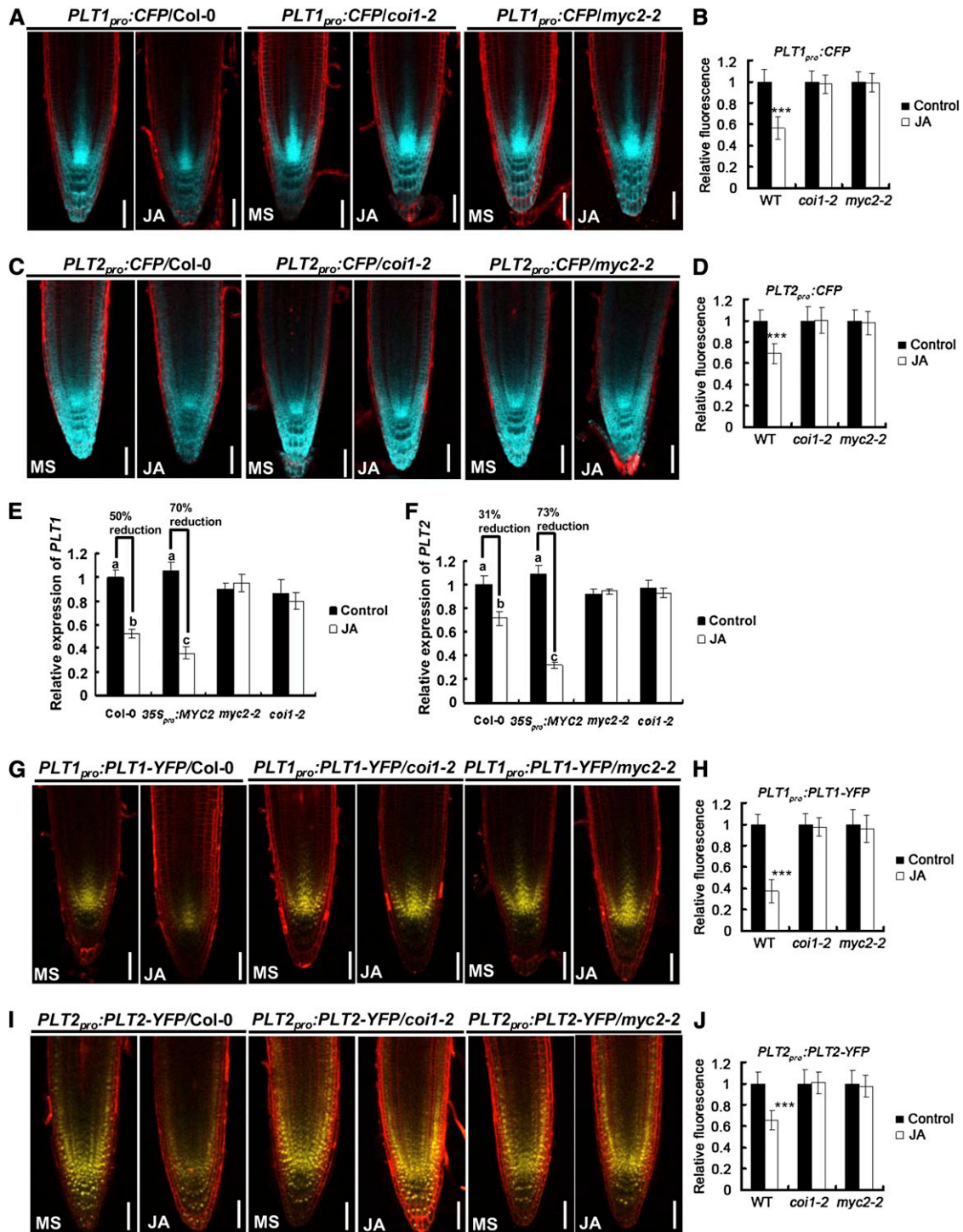


Figure 5. JA Reduces the Expression of *PLT1* and *PLT2* through *COI1* and *MYC2*.

(A) JA reduces *PLT1_{pro}:CFP* expression levels in Col-0, but not in *coi1-2* and *myc2-2*.

(B) Quantification of CFP fluorescence shown in (A).

(C) JA reduces *PLT2_{pro}:CFP* expression levels in Col-0, but not in *coi1-2* and *myc2-2*.

(D) Quantification of CFP fluorescence shown in (C).

For (A) to (D), 5-d-old seedlings were transferred to medium without (MS) or with 20 μ M JA for 1 d before CFP fluorescence was monitored. Data shown are average and SD ($n = 15$ to 20). Asterisks denote Student's *t* test significance compared with untreated plants: *** $P < 0.001$. Bars = 50 μ m.

although enhanced inhibition of root growth was observed in these $35S_{pro}:MYC2$ seedlings after treatment with JA (see Supplemental Figure 2F online). Consistently, we showed that, in the absence of JA, *PLT1* and *PLT2* expression was largely similar among the genotypes compared (Figures 5E and 5F). JA treatment markedly downregulated the transcript levels of *PLT1* and *PLT2* in the wild type but not in *coi1-2* and *myc2-2* (Figures 5E and 5F). Significantly, JA-induced downregulation of *PLT1* and *PLT2* expression was substantially exaggerated in $35S_{pro}:MYC2$ compared with the wild type (Figures 5E and 5F), indicating an important role of MYC2 in mediating JA-induced repression of *PLT1* and *PLT2* expression. Importantly, the observation that MYC2 represses *PLT* gene expression only in the presence of JA suggests that this action of MYC2 may require JA perception or other upstream signaling events.

Next, using transgenic plants containing the promoters of *PLT1* and *PLT2* combined with *PLT-YFP* (for yellow fluorescent protein) protein fusions (Galinha et al., 2007), we found that JA also reduced the expression levels of $PLT1_{pro}:PLT1-YFP$ (Figures 5G and 5H) and $PLT2_{pro}:PLT2-YFP$ (Figures 5I and 5J) in the wild type but not in *coi1-2* and *myc2-2*. Together, our results support the hypothesis that JA represses the transcriptional expression of *PLT1* and *PLT2* through the COI1- and MYC2-mediated jasmonate signaling pathway.

Given that JA promotes auxin biosynthesis (Dombrecht et al., 2007; Sun et al., 2009) and auxin itself regulates *PLT* expression (Aida et al., 2004), we designed experiments to test whether the above-described effect of JA on *PLT* expression is achieved through auxin. To this end, we compared JA-induced expression of *PLT1* and *PLT2* in the wild type and the auxin biosynthesis mutants *asa1-1* (Sun et al., 2009) and *yuc1D* (previously known as *yucca*; Zhao et al., 2001). Our recent work demonstrates that the *asa1-1* mutant contains a mutation in the *Anthranilate Synthase $\alpha 1$* (*ASA1/WEI2*) gene and therefore is defective in JA-induced auxin biosynthesis (Sun et al., 2009). The dominant *yuc1D* mutant contains elevated free IAA levels, resulting from the overexpression of a rate-limiting auxin biosynthesis gene *YUCCA1* (*YUC1*) (Zhao et al., 2001; Zhao, 2010). As revealed by our qRT-PCR assays, in wild-type roots, a slight reduction of *PLT1* and *PLT2* transcripts was observed 2 h after JA treatment and, a marked reduction of these transcripts was observed at 6 and 12 h after JA treatment (see Supplemental Figures 6A and 6B online). Importantly, JA-induced reduction levels and kinetics of

PLT1 and *PLT2* expression were largely similar among *asa1-1*, *yuc1D*, and the wild type (see Supplemental Figures 6A and 6B online), indicating that the effect of JA on *PLT* expression is not achieved through the *ASA1*- and *YUC1*-dependent auxin biosynthesis. Consistently, the *asa1-1* and *yuc1D* mutations did not affect JA-induced inhibition of root growth (see Supplemental Figure 6C online) and JA-induced reduction of root meristem cell number (see Supplemental Figure 6D online). Together, these data support the notion that the effect of JA on *PLT* expression is independent of the auxin pathway.

MYC2 Directly Regulates the Expression of *PLT1* and *PLT2*

The basic helix-loop-helix-type transcription factor MYC2 acts as a master regulator of JA-mediated gene transcription and regulates versatile aspects of JA responses, including defense gene expression and root growth inhibition (Dombrecht et al., 2007; Memelink, 2009). Our finding that JA represses the transcriptional expression of *PLT1* and *PLT2* in a MYC2-dependent manner suggests that MYC2 may associate with promoters of *PLT1* and *PLT2*. It is well known that MYC2 preferably binds to the G-box-related hexamer 5'-CACATG-3' of its target genes (Dombrecht et al., 2007). Our sequence analysis revealed one 5'-CACATG-3' motif in the P1 region (located -1609 to -1614 bp upstream of the translational start codon; Figure 6A) of the *PLT1* promoter. Sequence analysis also identified two 5'-CACATG-3' motifs in the P2 region (located -940 to -945 and -1098 to -1103 bp upstream of the translational start codon; Figure 6A) and one 5'-CACATG-3' motif close to the P3 region (located +288 to +293 bp downstream of the translational start codon; Figure 6A) of *PLT2*. To test the in vivo interaction between MYC2 and the chromatin regions of *PLT1* and *PLT2*, we performed chromatin immunoprecipitation (ChIP) assays using the $35S_{pro}:MYC2$ transgenic plants (containing the $35S_{pro}:MYC2-4Myc$ construct) and anti-Myc antibodies (Roche). As shown in Figure 6B, chromatin immunoprecipitated with anti-Myc antibodies was profoundly enriched in the P1 region of the *PLT1* promoter and in the P3 region of the *PLT2* promoter. These results indicate a specific association of MYC2 with the promoters of *PLT1* and *PLT2*.

A DNA electrophoretic mobility shift assay (EMSA) was conducted to confirm that MYC2 directly binds these G-box-related motifs presented in the upstream regions of *PLT1* and *PLT2*.

Figure 5. (continued).

(E) and (F) qRT-PCR assay showing that JA downregulates the transcription of *PLT1* (E) and *PLT2* (F) in a COI1- and MYC2-dependent manner. Five-day-old seedlings germinated on MS medium were transferred to medium without (Control) or with 20 μ M JA for 1 d, and 2-mm root tips were harvested for RNA extraction and qRT-PCR analysis. The transcript levels of *PLT1* and *PLT2* were normalized to the *ACTIN2* expression. *PLT1* and *PLT2* transcription levels of Col-0 without JA treatment were arbitrarily set to 1. Data presented are mean values of four biological repeats with SD. Samples with different letters are significantly different: $P < 0.01$.

(G) JA reduces $PLT1_{pro}:PLT1-YFP$ expression levels in Col-0, but not in *coi1-2* and *myc2-2*.

(H) Quantification of YFP fluorescence shown in (G). Data shown are average and SD ($n = 15$ to 20). Asterisks denote Student's *t* test significance compared with untreated plants: *** $P < 0.001$.

(I) JA reduces $PLT2_{pro}:PLT2-YFP$ expression levels in Col-0, but not in *coi1-2* and *myc2-2*.

(J) Quantification of YFP fluorescence shown in (I). Data shown are average and SD ($n = 15$ to 20). Asterisks denote Student's *t* test significance compared with untreated plants: *** $P < 0.001$.

For (G) to (J), 5-d-old seedlings were transferred to medium without (MS) or with 20 μ M JA for 1 d before YFP fluorescence was monitored. Bars = 50 μ m.

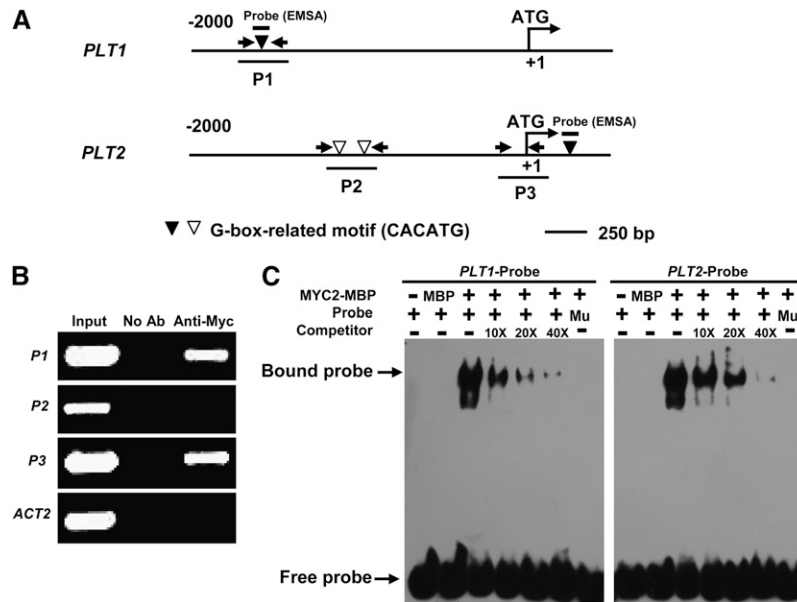


Figure 6. MYC2 Associates with *PLT1* and *PLT2* Promoters.

(A) Schematic diagram of potential MYC2 binding sites (white and black triangles), DNA fragments (P1, P2, and P3) used for ChIP, and probes used for EMSA. The sequence 2 kb upstream of the start site and part of the coding sequence for *PLT1* and *PLT2* are shown. The translational start site (ATG) is shown at position +1.

(B) Enrichment of the indicated DNA fragments (P1 and P3) following ChIP using anti-Myc antibodies. Chromatin of transgenic plants expressing *35S_{pro}:MYC2-4Myc* was immunoprecipitated with anti-Myc antibodies, and the presence of the indicated DNA in the immune complex was determined by RT-PCR. The *Actin2* promoter fragment was used as a negative control. The experiment was repeated three times with similar results.

(C) EMSA showing that the MYC2-MBP fusion protein binds to the DNA probes of *PLT1* and *PLT2* in vitro. Biotin-labeled probes were incubated with MYC2-MBP protein, and the free and bound DNAs (arrows) were separated in an acrylamide gel. As indicated, unlabeled probes were used as competitors. Mu, mutated probe in which the 5'-CACATG-3' motif was deleted.

Full-length MYC2 protein was expressed as a maltose binding protein (MBP) fusion protein in *Escherichia coli* and affinity purified. As shown in Figure 6C, the MYC2-MBP fusion proteins were able to bind DNA probes containing the 5'-CACATG-3' motif located in the P1 region of the *PLT1* promoter and in the P3 region of the *PLT2* gene. Furthermore, additional unlabeled DNA probes competed for binding in a dose-dependent manner (Figure 6C). Parallel experiments indicated that MYC2-MBP failed to bind DNA probes containing the mutant form of the 5'-CACATG-3' motif (Figure 6C). Together, these results reveal that MYC2 regulates *PLT1* and *PLT2* expression through direct association with their promoters.

Next, using the well-established transient expression assay of *Nicotiana benthamiana* leaves, we verified the repression effect of MYC2 on the expression of a reporter containing the *PLT1* promoter fused with the firefly luciferase gene (*LUC*). When the *PLT1_{pro}:LUC* reporter was infiltrated into *N. benthamiana*, a substantial amount of LUC activity could be detected (Figures 7A and 7B), indicating that endogenous factors of *N. benthamiana* may activate the expression of the *PLT1_{pro}:LUC* reporter. Coexpression of *PLT1_{pro}:LUC* with the *35S_{pro}:MYC2* construct led to an obvious reduction in luminescence intensity (Figure 7), suggesting that the manipulation process of the assay could have simulated the JA signaling of the infiltrated *N. benthamiana* leaf area, which, in turn, activated the repression effect *35S_{pro}:MYC2* on *PLT1_{pro}:LUC* expression. Significantly, exogenous

application of coronatine (COR), a jasmonoyl Ile mimic molecule that can potently activate JA signaling (Katsir et al., 2008; Fonseca et al., 2009; Sheard et al., 2010), onto the *N. benthamiana* leaf area coexpressing *PLT1_{pro}:LUC* and *35S_{pro}:MYC2*, led to a more dramatic reduction effect of *35S_{pro}:MYC2* on *PLT1_{pro}:LUC* expression (Figure 7). In a parallel experiment, *PLT1-m_{pro}:LUC*, in which the G-box of the *PLT1* promoter was deleted and fused with *LUC*, was coexpressed with *35S_{pro}:MYC2* in *N. benthamiana* leaves. As shown in Figure 7, in the absence or presence of COR, the repression effect of *35S_{pro}:MYC2* on *PLT1-m_{pro}:LUC* expression was largely attenuated. The observation that the *PLT1-m_{pro}:LUC* reporter was still responsive to the repression of *35S_{pro}:MYC2* could be explained by the presence of the 5'-GAGTA-3' motif in the *PLT1* promoter (see Supplemental Figure 7 online), which has been shown to be responsive to JA (Boter et al., 2004). Together, our transient assays of *N. benthamiana* leaves confirmed that MYC2 represses *PLT1* expression in vivo.

The above-described results suggest that MYC2-mediated repression of *PLT1* and *PLT2* expression is involved in JA-induced modulation of root meristem activity and stem cell niche maintenance. To determine the genetic relationship between JA and PLT proteins, we assessed the effect of JA on root meristems of the wild type and the *plt1-4 plt2-2* double mutant. As shown in Figures 8A and 8B, JA reduced the root meristem cell number by 33% in the wild type but only by 19.7% in *plt1-4 plt2-2*, indicating that the

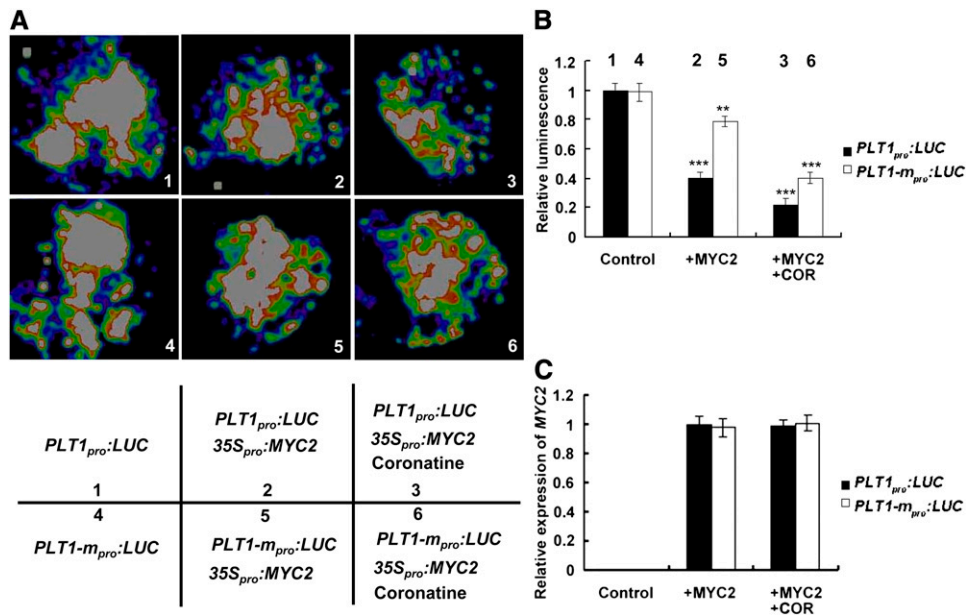


Figure 7. MYC2 Represses *PLT1* Expression, as Revealed by Transient Assays of *N. benthamiana* leaves.

(A) Transient expression assays showing that MYC2 represses the expression of *PLT1*. Representative images of *N. benthamiana* leaves 72 h after infiltration are shown. The bottom panel indicates the infiltrated constructs and treatments.

(B) Quantitative analysis of luminescence intensity in **(A)**. Five independent determinations were assessed. Error bars represent SD. Asterisks denote Student's *t* test significance compared with control plants: ***P* < 0.01 and ****P* < 0.001.

(C) qRT-PCR analysis of *MYC2* expression in the infiltrated leaf areas shown in **(A)**. Total RNAs were extracted from leaves of *N. benthamiana* coinfiltrated with the constructs. Five independent determinations were assessed. Error bars represent SD.

double mutations significantly attenuated the reduction effect of JA on the root meristem cell number. To test whether overexpression of *PLT* genes could somehow rescue the reduction effect of JA on root meristem cell number, we used transgenic plants containing the inducible construct *35S_{pro}:PLT2-GR* (Galinha et al., 2007). Figures 8C and 8D indicated that, without dexamethasone (DEX) induction (i.e., without *PLT2* overexpression), JA reduced the root meristem cell number of *35S_{pro}:PLT2-GR* plants by 31.4%. In the presence of DEX induction; however, JA reduced the root meristem cell number of the *PLT2* overexpressor by 20% (Figures 8C and 8D). The finding that the JA-induced reduction of root meristem cell number is reduced in *plt1-4 plt2-2* and *PLT2* overexpression plants supports the scenario that PLT proteins act genetically downstream of JA in regulating root meristem maintenance. The fact that *plt1-4 plt2-2* double mutants and *PLT2* overexpression plants are still responsive to JA could be explained by at least two possibilities: first, the existence of PLT family members other than *PLT1* and *PLT2* (i.e., *PLT3* and *BABY BOOM*) (Galinha et al., 2007); second, in addition to the PLT family of transcription factors, JA could employ other mechanisms to regulate root meristem maintenance.

DISCUSSION

Postembryonic root growth of higher plants exhibits remarkable plasticity to adapt to the ever-changing environmental conditions. The adaptation ability of plant root growth is determined by

the postembryonic activity of cells within the root meristem that is coordinated by several phytohormones, including jasmonate (Benková and Hejátko, 2009; Petrásek and Friml, 2009; Wolters and Jürgens, 2009). Here, we address the cellular and molecular mechanisms underlying JA-induced inhibition of primary root growth of *Arabidopsis* plants. We show that, at the cellular level, JA reduces cell number and cell elongation of the DZ and EZ, inhibits cell division activity of the root meristem, and alters the cellular organization of the stem cell niche. We also show that, at the molecular level, MYC2 directly represses the expression of *PLT1* and *PLT2* upon JA treatment, suggesting that MYC2-mediated repression of *PLT* expression is important for JA-induced modulation of root meristem activity and stem cell niche maintenance. This study demonstrates a mode of jasmonate-auxin crosstalk, in which a major transcription factor of the JA signaling pathway directly represses master transcription factors known to act in the auxin pathway. Our results extend the view of how plants can translate stress cues into growth response and thus retain growth plasticity during postembryonic development.

JA Modulates Maintenance of the Root Stem Cell Niche

Although jasmonate-induced root growth inhibition has been employed as the most prominent genetic assay to identify jasmonate-related mutants in *Arabidopsis* (Wasternack, 2007; Browse, 2009), the cellular and molecular basis of jasmonate action on root growth is hitherto not clear. We show here that JA reduces

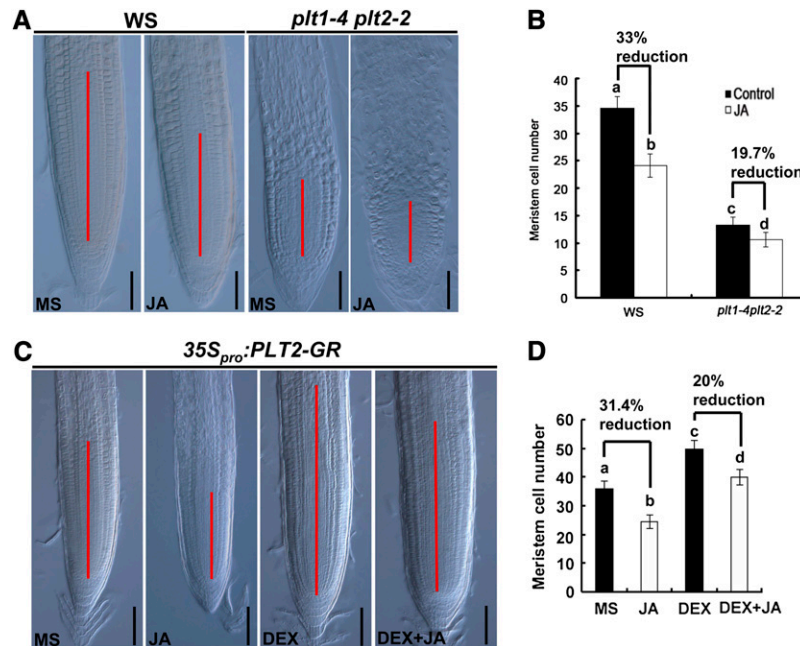


Figure 8. JA-induced Root Meristem Cell Number in *plt1-4 plt2-2* and *PLT2*-overexpression Plants.

(A) JA-induced reduction of meristem size in wild-type (Wassilewskija [WS]) and *plt1-4 plt2-2* plants. Seedlings were grown on medium without (MS) or with 20 μ M JA for 4 d before meristem cell number was determined. The MZ is marked with a red line. Bars = 50 μ m.

(B) Quantification of meristem cell number in **(A)**. Data shown are average and SD ($n = 20$). Samples with different letters are significantly different: $P < 0.05$. These experiments were repeated at least three times with similar results.

(C) JA-induced reduction of root meristem size in transgenic plants expressing *35S_{pro}:PLT2-GR*. Bars = 50 μ m.

(D) Statistics of the meristem cell number shown in **(C)**. Data shown are average and SD ($n = 15$ to 20). Samples with different letters are significantly different: $P < 0.01$. These experiments were repeated at least three times with similar results.

For **(C)** and **(D)**, 4-d-old seedlings germinated on MS medium were transferred to control medium (MS) or medium containing 2 μ M DEX for another 2 d before root meristem cell number was determined. Four-day-old seedlings germinated on medium with 20 μ M JA were transferred to medium with 20 μ M JA + 2 μ M DEX (DEX + JA) or 20 μ M JA only (JA) for another 2 d before root meristem cell number was determined.

both cell number and cell length of the root, indicating that JA-induced inhibition of primary root growth is a complex process that involves multiple cellular processes in distinct root tissues.

JA-induced reduction of root meristem activity is evidenced by reduced meristem size and decreased expression levels of the *CYCB1;1_{pro}:GUS* reporter gene and a group of cell cycle-related genes. The reduced cell proliferation rate in the JA-treated root meristem could contribute to the observed reduction of cell number in the EZ. Our observations reveal that the mechanism by which JA inhibits primary root growth is distinct from that of ethylene, an important stress hormone showing extensive cross-talk with JA (Lorenzo et al., 2003). Several groups showed that ethylene does not affect cell cycle activity of the root meristem but strongly reduces cell elongation in the EZ (Růzicka et al., 2007; Stepanova et al., 2007; Swarup et al., 2007).

Consistent with a reduced root meristem activity by JA, we found that JA disturbs the cellular organization of the root stem cell niche. For example, JA triggers the division of QC cells and differentiation of CSCs. Our time-course study revealed that, upon JA treatment, the occurrence of QC division is generally earlier than that of CSC differentiation. In the context that proper expression of *WOX5* in the QC is required to maintain CSCs as undiffer-

entiated (Sarkar et al., 2007), we examined the possible effect of JA on *WOX5* expression and found that JA treatment has little, if any, effect on *WOX5* expression. Given that ethylene has been shown to be able to induce QC division (Ortega-Martínez et al., 2007), it is important to determine whether this hormone and JA might affect the mitotic activity of QC through similar mechanisms. Two lines of evidence favor that JA and ethylene modulate the balance between proliferation and quiescence of QC cells through distinct mechanisms. First, whereas the supernumerary QC cells produced by ethylene-induced QC division still display their QC function to maintain the stem cell identity of CSCs (Ortega-Martínez et al., 2007), JA-induced supernumerary QC cells partially lose their ability to maintain the stem cell identity of CSCs. Second, blocking of the ethylene pathway by genetic or chemical manipulation does not affect JA-induced QC division.

Interestingly, our results reveal that, depending on the tissue context, JA exerts opposite effects on cell division. For example, JA represses cell division in the proximal root meristem, as evidenced by reduced meristem cell number, reduced expression of the mitotic cell cycle marker *CYCB1;1_{pro}:GUS*, and reduced expression levels of several cell cycle-related genes in JA-treated roots. These results are consistent with previous

observations showing that, as a growth regulator, JA generally inhibits cell division and therefore represses growth (Dathe et al., 1981; Swiatek et al., 2004; Pauwels et al., 2008; Zhang and Turner, 2008). On the other hand, JA promotes cell division in the QC, as evidenced by the presence of supernumerary QC cells in JA-treated roots, QC-specific marker gene expression, and EdU staining assays. It will be interesting in future studies to unravel the molecular mechanisms underlying these distinct aspects of JA action. In the context that JA plays an important role in transmitting environmental cues into developmental responses, our finding that JA impacts QC division presents an attractive scenario on how plants adapt their growth to environments by modulating stem cell division and activity.

MYC2-Mediated Repression of *PLT* Expression Integrates Jasmonate and Auxin Pathways in Regulating Maintenance of the Root Stem Cell Niche

It is noteworthy that the above-described effects of JA on the root meristem and stem cell niche require the function of MYC2, a major transcription factor that positively or negatively regulates different aspects of jasmonate responses, including defense gene expression and root growth inhibition. Therefore, our investigation hints at the involvement of MYC2-mediated transcriptional regulation in JA-induced root growth inhibition.

PLT genes encode proteins that act as dose-dependent regulators for root development (Aida et al., 2004; Galinha et al., 2007). For example, high levels of *PLT2* maintain the stem cells, and intermediate levels facilitate transit amplifying cell divisions that define the meristem region (Galinha et al., 2007). In line with the JA-induced reduction of root meristem activity and disturbance of stem cell niche maintenance, we found that JA downregulates the expression levels of *PLT1* and *PLT2* in a MYC2-dependent manner. In the context that JA affects auxin biosynthesis (Dombrecht et al., 2007; Sun et al., 2009) and that auxin itself regulates the transcriptional expression of *PLT* genes (Aida et al., 2004), we provided several lines of evidence that the effect of JA on *PLT* expression and, therefore, on root stem cell niche maintenance is independent of the effect of JA on auxin biosynthesis. First, our previous work revealed that JA promotes auxin biosynthesis through transcriptional activation of the auxin biosynthetic gene *ASA1/WEI2* (Sun et al., 2009; Stepanova et al., 2005). We show here that the *asa1-1* mutant, which harbors a loss-of-function mutation of *ASA1/WEI2* and is therefore defective in JA-induced auxin biosynthesis, shows normal response to JA in terms of root growth inhibition and meristem cell number reduction. Second, the *asa1-1* (Stepanova et al., 2005; Sun et al., 2009) and *yuc1D* (Zhao et al., 2001) mutations, which are defective in auxin biosynthesis, do not affect JA-induced repression levels and kinetics of *PLT1* and *PLT2* expression. Third, the action mode of JA on *PLT* expression is distinct from that of auxin. Whereas auxin positively regulates *PLT1* and *PLT2* expression (Aida et al., 2004), we show here that JA negatively regulates their expression. These, together with other data described in this work, support that JA-mediated regulation of *PLT1* and *PLT2* expression is independent of the auxin pathway.

Our finding that JA represses *PLT1* and *PLT2* expression in a MYC2-dependent manner raises the interesting possibility that MYC2 may directly regulate *PLT* expression. Indeed, our EMSA

and ChIP assays indicate that MYC2 associates with the promoters of both *PLT1* and *PLT2*. Using the well-established transient assay of *N. benthamiana* leaves, we show that MYC2 indeed represses *PLT1_{pro}:LUC* expression and, importantly, that the G-box-related motif CACATG in the promoter of *PLT1* is important for this MYC2 action. Consistently, the reduction effect of JA on root meristem cell number was substantially reduced in the *plt1-4 plt2-2* double mutant and transgenic plants overexpressing *PLT2*. Collectively, these results support the notion that the *PLT1* and *PLT2* transcription factors act downstream of MYC2 in the JA-mediated regulation of stem cell niche maintenance and root meristem activity. Considering the established role of the *PLT* transcription factors in mediating developmental response to auxin in the root meristem (Aida et al., 2004; Dinneny and Benfey, 2008), we propose that MYC2-induced repression of *PLT1* and *PLT2* expression integrates JA action into the auxin signaling pathway in regulating postembryonic root growth. It has been shown that auxin upregulates *PLT1* and *PLT2* transcripts and positively regulates stem cell niche maintenance and meristem activity (Aida et al., 2004). In contrast with auxin, this study reveals that JA downregulates *PLT1* and *PLT2* expression and negatively regulates stem cell niche maintenance and meristem activity. *PLT1* and *PLT2* therefore represent a key junction for crosstalk between the JA and auxin signaling pathways in the regulation of root stem cell niche maintenance.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotypes Columbia-0 (Col-0), C24, and Wassilewskija were used. Some of the plant materials used in this study were previously described: *J2341* and *QC25* (Sabatini et al., 1999), *PLT1_{pro}:PLT1-YFP*, *PLT2_{pro}:PLT2-YFP*, *PLT1_{pro}:CFP*, and *PLT2_{pro}:CFP* (Galinha et al., 2007), *coi1-1* (Xie et al., 1998), *coi1-2* (Xu et al., 2002), *cev1* (Ellis and Turner, 2001), *ein3-1 eil1-1* (Alonso et al., 2003a), *ein2-5* (Alonso et al., 1999), *CYCB1;1_{pro}:GUS* (Colón-Carmona et al., 1999), *WOX5_{pro}:GFP* (Bililou et al., 2005), *myc2-2* (Boter et al., 2004), *plt1-4 plt2-2* (Aida et al., 2004), and *35S_{pro}:PLT2-GR* (Galinha et al., 2007). *jaz10* (CS872819, SAIL_92_D08) was identified from the SIGnAL T-DNA collection (Alonso et al., 2003b).

MYC2_{pro}:GUS and *COI1_{pro}:GUS* plants were obtained from transformation of Col-0 plants with the *MYC2_{pro}:GUS* or *COI1_{pro}:GUS* constructs. *35S_{pro}:MYC2* plants were obtained from transformation of Col-0 with the *35S_{pro}:MYC2-4Myc* constructs.

Arabidopsis seeds were surface sterilized for 15 min in 10% bleach, washed four times with sterile water, and plated on half-strength Murashige and Skoog (MS) medium. Plants were stratified at 4°C for 2 d in darkness and then transferred to a phytotron set at 22°C with a 16-h-light/8-h-dark photoperiod (light intensity 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$). *Nicotiana benthamiana* was grown under a 16-h-light (28°C)/8-h-dark (22°C) photoperiod. All experiments were repeated at least three times.

JA (Sigma-Aldrich) was dissolved with ethanol and prepared as a 200 mM stock solution. The 1-amino-1-cyclopropane carboxylic acid (Sigma-Aldrich) was dissolved with water and prepared as a 10 mM stock solution.

DNA Constructs and Plant Transformation

The *COI1* promoter region was PCR amplified with the primers *COI1_{pro}-F*, 5'-CCGCTGCAGGAGTCAACACCAAGTACAATGAC-3', and *COI1_{pro}-R*, 5'-CACCTCTTGATATCAGGATCTCCATCGGATCG-3'. The PCR product was cloned into the *Pst*I-*Bam*HI sites of the binary vector

pCAMBIA1391-Z (CAMBIA) to generate the *COI1_{pro}:GUS* construct. The *MYC2* promoter region was PCR amplified with the primers *MYC2_{pro}-F*, 5'-CACCGACCCTTCCTAAAAAGTAAG-3', and *MYC2_{pro}-R*, 5'-CATTCCATAAACCGGTGACCGGT-3'. The PCR product was cloned using a pENTR Directional TOPO cloning kit (Invitrogen) and then recombined with the plant binary vector pGWB3 (Nakagawa et al., 2007) to generate the *MYC2_{pro}:GUS* construct. *MYC2* cDNA was PCR amplified from the reverse transcription product with primers 5'-CACCATGACTGAT-TACCGGCTACA-3' and 5'-ACCGATTTTTGAAATCAAACCTTGC-3'. The PCR product was cloned using the pENTR Directional TOPO cloning kit and then recombined with the binary vector pGWB17 (35S promoter, *C-4Myc*) to generate the *35S_{pro}:MYC2-4Myc* construct.

The above constructs were then transformed into *Agrobacterium tumefaciens* strain GV3101 (pMP90), which was used for transformation of *Arabidopsis* plants by vacuum infiltration (Bechtold and Pelletier, 1998).

Phenotypic Analysis, Statistics, and Microscopy

Seedlings were photographed and root length was measured (Image J; National Institutes of Health; <http://rsb.info.nih.gov/ij/>). Root meristem size and QC division were analyzed on seedlings mounted in HCG solution (chloroacetaldehyde:water:glycerol = 8:3:1). Microscopy was performed on a Leica Microsystems DM5000B microscope and DFC490 charge-coupled device (CCD) camera. Root meristem size was assessed as the cell number between the QC and the first elongating cell in the cortex cell file (Casamitjana-Martínez et al., 2003; Dello Ioio et al., 2007). The statistical significance was evaluated by Student's *t* test analysis. For multiple comparisons, an analysis of variance followed by Fisher's LSD mean separation test (SPSS) was performed on the data. Samples with different letters are significantly different at $P < 0.01$ or $P < 0.05$. Data presented are mean values of at least three biological repeats with SD. Images were processed with Adobe Photoshop CS and Spot Flex software.

GFP, YFP, CFP, and propidium iodide fluorescence was imaged under a Leica confocal laser scanning microscope (Leica Microsystems). For imaging GFP, YFP, CFP, and propidium iodide, the 488-, 514-, 458-, and 543-nm lines of the laser were used for excitation, and emission was detected at 510, 530, 480, and 620 nm, respectively. Fluorescence was quantified with the LAS AF Lite program on confocal sections acquired with the same microscope settings (Růzicka et al., 2007; Zhou et al., 2010). Approximately 10 seedlings/images were examined, and at least three independent experiments were performed, giving the same statistically significant results.

Whole seedlings or different tissues were cleared in HCG solution for several minutes before microscopy analysis. For Lugol staining of roots, tissues were incubated in Lugol solution (Sigma-Aldrich) for 3 to 5 min, washed in water once, and mounted in HCG solution for microscopy analysis (Zhou et al., 2010). Histochemical staining for GUS activity in transgenic plants was performed as described previously (Jefferson et al., 1987).

An EdU incorporation assay was performed as previously described (Vanstraelen et al., 2009; Zhou et al., 2010). Seedlings were grown in liquid MS containing 10 μ M EdU (Click-iT EdU Alexa Fluor 647 imaging kit; Invitrogen). After growth, seedlings were fixed in 3.7% formaldehyde in PBS, pH 7.4, for 15 min. Samples were then washed in 3% BSA in PBS, treated with 0.5% Triton in PBS for 20 min, and washed again. Coupling of EdU to the Alexa fluor substrate occurred in the dark in the Click-iT reaction mixture, prepared according to the manufacturer's instructions, and observations were made using confocal microscopy.

Whole-Mount in Situ Hybridization

Whole-mount in situ hybridization was performed according to the reported protocols (Weigel and Glazebrook, 2002; Hejácíko et al., 2006). Antisense and sense probes were synthesized with digoxigenin-11-UTP (Roche Diagnostics) using T7 and SP6 RNA polymerases, respectively. The following primers

were used to amplify the DNA template for the probe synthesis: *WOX5* primers 5'-AAACTCGAGAGGCAGAAACGTCGTAATCT-3' and 5'-TCAG-GATCCTTAAAGAAAGCTTAATCGAAGATCTA-3'.

Gene Expression Analysis

For qRT-PCR analysis, seedling roots were harvested and frozen in liquid nitrogen for RNA extraction. RNA was extracted with the RNeasy kit (Qiagen). Poly(dT) cDNA was prepared from 10 μ g of total RNA with Superscript III reverse transcriptase (Invitrogen) and quantified with a cyclor apparatus (Bio-Rad) with the Real Master Mix kit (SYBR Green; Tiangen) according to the manufacturer's instructions. PCR was performed in 96-well optical reaction plates heated for 5 min at 95°C to activate hot start Taq DNA polymerase, followed by 40 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 59°C, and extension for 20 s at 68°C. Expression levels of target genes were normalized to those of *ACTIN2* or *ACTIN7*. The statistical significance was evaluated by Student's *t* test. For multiple comparisons, an analysis of variance followed by Fisher's LSD mean separation test (SPSS) was performed on the data. Samples with different letters are significantly different at $P < 0.01$ or $P < 0.05$. Primers used to quantify gene expression levels are listed in Supplemental Table 1 online.

ChIP-PCR Assay

One gram of 8-d-old *35S_{pro}:MYC2* (*35S_{pro}:MYC2-4Myc*) seedlings was used for ChIP experiments. ChIP was performed as previously described (Gendrel et al., 2005). The enrichment of DNA fragments was determined by semiquantitative PCR using the following primer pairs: *ACT2_{pro} F*, 5'-CGTTTCGCTTCCCTTAGTGTAGCT-3'; *ACT2_{pro} R*, 5'-CACACCGCATGCTAAACAGATCTAG-3'; *PLT1_{pro}(P1)F*, 5'-GCCCCCTTATTGAAT-TGGCTCTT-3'; *PLT1_{pro}(P1)R*, 5'-GCATACTTGATCCAGTATATGCA-3'; *PLT2_{pro}(P2)F*, 5'-CACATGCAATGAACCTCGGGGATC-3'; *PLT2_{pro}(P2)R*, 5'-CCCTCTGATCTCTACATACTAAC-3'; *PLT2_{pro}(P3)F*, 5'-CAGTCCCT-CCTTGATGAGATCAT-3'; and *PLT2_{pro}(P3)R*, 5'-CATTTCCCTTTCTTG-GAATCAA-3'.

EMSA Assay

To construct a plasmid for the expression of recombinant *MYC2* protein in *Escheichia coli*, the full-length cDNA fragment was amplified by PCR using primers 5'-GCTAGCATGACTGATTACCGGCTACAAC-3' and 5'-CTGCAGGACCGATTTTTGAAATCAAACCTTGC-3' and cloned into the pMAL-c2 vector via *Xba*I and *Pst*I restriction sites. Oligonucleotide probes were synthesized and labeled with biotin at the 3' end (Invitrogen). EMSA was performed using a LightShift Chemiluminescent EMSA kit (Thermo Scientific). Briefly, biotin-labeled probes were incubated in 1 \times binding buffer, 2.5% glycerol, 50 mM KCl, 5 mM MgCl₂, and 10 mM EDTA with or without proteins at room temperature for 20 min. For nonlabeled probe competition, nonlabeled probes were added to the reactions. The probe sequences were as follows: *PLT1* probe sequence, 5'-GAAT-TGGCTCTTATAATATTGCATGAACGTACACATGCAGAAGTGAGAGAA-TTCGGGGATCTACAATAA-3'; *PLT2* probe sequence, 5'-AAGACATG-TAAAATCGAAAGCATCATTGCACATGTTTTAAGATTGTTATGTTTCATAT-ATTTTCTTG-3'; *PLT1* probe without the CACATG motif (Mu), 5'-GAA-TTGGCTCTTATAATATTGCATGAACGTACAGAAGTGAGAGAAATTCGG-GGATCTACAATAA-3'; *PLT2* probe without the CACATG motif (Mu), 5'-AAGACATG-TAAAATCGAAAGCATCATTGTTTTAAGATTGTTATGTTTC-ATATATTTTCTTG-3'.

Transinhibition of *PLT1* Promoter Activity by *MYC2* in *N. benthamiana* Leaves

The transient expression assays were performed in *N. benthamiana* leaves as previously described (Matsui et al., 2008; Shang et al., 2010; Qi

et al., 2011). The *PLT1* promoter was amplified with the primer pairs 5'-CACCCCTAGCACAAATACAATGCAAAGGAC-3' and 5'-CTACCACTT-TGGTATGATCAATATACC-3' and cloned into pENTR using the pENTR Directional TOPO cloning kit (Invitrogen). To generate *PLT1* promoter with mutations, site-directed mutagenesis was used to delete the CACATG motif in the P1 region of the *PLT1* promoter (Figure 6A) using the TaKaRa MutanBEST kit. Then, both *PLT1* promoter versions were fused with the luciferase reporter gene *LUC* through the Gateway reactions into the plant binary vector pGWB35 (Nakagawa et al., 2007) to generate the reporter constructs *PLT1_{pro}:LUC* and *PLT1-m_{pro}:LUC*. The *MYC2* effector construct was the above-described *35S_{pro}:MYC2-4Myc* (*35S_{pro}:MYC2*).

Agrobacterium-mediated infiltration of *N. benthamiana* leaves was performed as described (Shang et al., 2010; Qi et al., 2011). Infiltrated plants were incubated at 22°C for 72 h before CCD imaging. For COR treatment, the infiltration buffer containing 5 μM COR (+COR) (or solvent buffer as control) was infiltrated into the *N. benthamiana* leaf area coexpressing the indicated constructs 10 h before CCD imaging.

We used a low-light cooled CCD imaging apparatus (NightOWL II LB983 with indigo software) to capture the LUC image and to count luminescence intensity. The leaves were sprayed with 100 mM luciferin and were placed in darkness for 3 min before luminescence detection. Five independent determinations were assessed. Error bars represent SD. The experiments were repeated at least five times with similar results.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative under the following accession numbers: At3g18780 (*ACTIN2*), At5g09810 (*ACTIN7*), At4g37490 (*CYCB1;1*), At3g48750 (*CDC2A*), At2g23430 (*KRP1*), At1g07370 (*PCNA1*), At3g20840 (*PLT1*), At1g51190 (*PLT2*), At3g11260 (*WOX5*), At1g32640 (*MYC2*), and At2g39940 (*COI1*).

Supplemental Data

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

Supplemental Figure 1. JA Reduces Both Cell Number and Cell Length of the DZ and EZ.

Supplemental Figure 2. COI1 and MYC2 Are Involved in JA-Induced Inhibition of Root Growth.

Supplemental Figure 3. JA-Mediated Promotion of QC Division in the Ethylene Signaling Mutants *ein2-5* and *ein3-1 eil1-1*.

Supplemental Figure 4. The JA Overproduction Mutant *cev1* Is Defective in Maintenance of the Root Stem Cell Niche.

Supplemental Figure 5. JA-Induced Modulation of Root Meristem Size and Stem Cell Niche Maintenance in the JA-Hypersensitive Mutant *jaz10*.

Supplemental Figure 6. JA-Induced Repression of *PLT* Expression in the Auxin Biosynthesis Mutants *asa1-1* and *yuc1D*.

Supplemental Figure 7. Presence of the JA-Responsive 5'-GAGTA-3' Motif in Promoter Regions of *PLT1* and *PLT2*.

Supplemental Table 1. DNA Primers Used for qRT-PCR Assays in This Study.

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AUTHOR CONTRIBUTIONS

C.L. designed the research, analyzed the data, and wrote the article. Q.C. and J.S. designed and performed the research and analyzed the data. Q.Z., W.Z., L.Q., L.X., B.W., R.C., H.J., J.Q., and X.L. performed the research and analyzed the data. K.P. analyzed the data.

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