Jasmonic Acid Inhibits Auxin-Induced Lateral Rooting Independently of the CORONATINE INSENSITIVE1 Receptor[1]

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Plant root systems are indispensable for water uptake, nutrient acquisition, and anchoring plants in the soil. Previous studies using auxin inhibitors definitively established that auxin plays a central role regulating root growth and development. Most auxin inhibitors affect all auxin signaling at the same time, which obscures an understanding of individual events. Here, we report that jasmonic acid (JA) functions as a lateral root (LR)-preferential auxin inhibitor in Arabidopsis (*Arabidopsis thaliana*) in a manner that is independent of the JA receptor, CORONATINE INSENSITIVE1 (COI1). Treatment of wild-type Arabidopsis with either (−)-JA or (+)-JA reduced primary root length and LR number; the reduction of LR number was also observed in *coi1* mutants. Treatment of seedlings with (−)-JA or (+)-JA suppressed auxin-inducible genes related to LR formation, diminished accumulation of the auxin reporter *DR5::GUS*, and inhibited auxin-dependent DII-VENUS degradation. A structural mimic of (−)-JA and (+)-coronafacic acid also inhibited LR formation and stabilized DII-VENUS protein. COI1-independent activity was retained in the double mutant of *transport inhibitor response1* and *auxin signaling f-box protein2* (*tir1 afb2*) but reduced in the *afb5* single mutant. These results reveal JAs and (+)-coronafacic acid to be selective counter-auxins, a finding that could lead to new approaches for studying the mechanisms of LR formation.

Plant root systems adapt rapidly to environmental changes and enable the plant to survive adverse conditions (Fitter, 1996). Lateral roots extend horizontally from the primary root, contributing to the architecture of the root system. They also help anchor the plant in the soil and facilitate water, micronutrient, and macronutrient uptake (Lynch, 1995). Auxin is a multifunctional plant factor essential to root development, driving gravitropism (Chen et al., 1998; Rashotte et al., 2000; Sukumar et al., 2009), root hair formation (Masucci and Schiefelbein, 1994, 1996; Pitts et al., 1998), elongation and differentiation of root cells (Rahman et al., 2007), and lateral root (LR) formation (Reed et al., 1998; Casimiro et al., 2001; Bhalerao et al., 2002).

Auxin signaling is derived from the interaction between endogenous auxin indole-3-acetic acid (IAA), a TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX PROTEIN (TIR1/AFB) auxin receptor and a family of transcriptional repressors known as AUXIN/IAAs (Aux/IAAs; Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Salehin et al., 2015). In the presence of IAA, the SKP1-CULLIN1-F-BOX (SCF) TIR1/AFB ubiquitin ligase complex binds to Aux/IAAs, which triggers their ubiquitin-mediated degradation of Aux/IAA (Calderon-Villalobos et al., 2010). Most Aux/IAAs contain four conserved domains that contribute to their functional properties. Domain I is a repressor domain (Long et al., 2006;

Szemenyei et al., 2008; Causier et al., 2012); domain II is involved in protein stability and is the site of interaction with the TIR1/AFBs and auxin (Gray et al., 2001; Ouellet et al., 2001; Ramos et al., 2001; Dreher et al., 2006); and domains III and IV are responsible for dimerization with other Aux/IAAs and heterodimerization with AUXIN RESPONSE FACTORs (ARFs; Ulmasov et al., 1997). Aux/IAAs themselves bind to and inhibit ARFs, which are DNA-binding transcription factors capable of directing the expression of auxin-responsive genes.

Inhibitors of auxin transport, biosynthesis, and signaling are widely used to block auxin bioactivities. The identification and functional characterization of the membrane proteins involved in polar auxin transport have been greatly facilitated by the use of auxin transport inhibitors such as 1-N-naphthylphthalamic acid (NPA), 2,3,5-triiodobenzoic acid, and gravacin (Casimiro et al., 2001; De Rybel et al., 2009). It was recently reported that L-amino-oxyphenylpropionic acid, L-kynurenine, yucasin, and aminooxy-naphthylpropionic acid were auxin biosynthesis inhibitors (Soeno et al., 2010; He et al., 2011; Nishimura et al., 2014; Narukawa-Nara et al., 2016). In auxin signaling, p-chlorophenoxyisobutyric acid (PCIB), PEO-IAA, and auxinole were reported (Oono et al., 2003; Hayashi et al., 2012). These signaling inhibitors competitively disturb diverse auxin responses, including primary root elongation, root gravitropism, root hair formation, and LR formation.

In Arabidopsis (*Arabidopsis thaliana*), perception of jasmonic acid (JA) takes place in a similar manner to that of auxin and involves analogous components, such as jasmonoyl-L-Ile (JA-L-Ile) as the ligand, COI1 (CORONATINE INSENSITIVE1) as the receptor, and jasmonate ZIM-domain (JAZ proteins) as coreceptors (Xie et al., 1998; Devoto et al., 2002; Xu et al., 2002; Chini et al., 2007; Thines et al., 2007; Browse, 2009; Chung and Howe, 2009; Yan et al., 2009; Sheard et al., 2010; Pauwels and Goossens, 2011). In the presence of the JA-L-Ile, JAZ repressor proteins are degraded by the action of the SCF^{COI1}-E3 ubiquitin ligase complex, releasing MYC2/3/4 transcription factors to trigger the expression of JA-dependent genes (Chini et al., 2007; Fernández-Calvo et al., 2011; Niu et al., 2011). It was recently reported that some jasmonates have bioactivities distinct from the COI1-dependent pathway. For example, 12-oxo-phytodienoic acid and JA-Trp competitively inhibit auxin signaling (Ribot et al., 2008; Staswick, 2009). In this work, we shed new light on the regulation of COI1-independent jasmonate signaling. We show that (−)-JA, (+)-JA and (+)-CFA have a peculiar bioactivity in the inhibition of LR formation, independent of the COI1 signaling pathway.

RESULTS

LR Number Was Reduced by (−)-JA and (+)-JA Independent of COI1

Arabidopsis plants separately treated with both enantiomers of JA exhibited similarly reduced primary root growth compared to mock (Fig. 1, A and B; Asamitsu et al., 2006). Although both (−)-JA and (+)-JA reduced the number of LR, the effect was greater for (+)-JA (Fig. 1C). To investigate whether (+)-JA also induced the JA responses, JA responsive gene expression, JAZ1-GUS degradation, and anthocyanin accumulation were analyzed. Expression of the enzyme ALLENE OXIDE SYNTHASE (AOS) from a COI1-dependent JA biosynthetic gene was induced by both (−)-JA or (+)-JA (Supplemental Fig. S1A; Park et al., 2002a). JAZ1-GUS degradation by either (−)-JA or (+)-JA treatment was rescued by either the proteasome inhibitor MG132, or jarin-1, a selective inhibitor of the JA-amino acid conjugating enzyme JASMONATE RESISTANT1 (Supplemental Fig. S1B; Meesters et al., 2014). Moreover, (−)-JA and (+)-JA both induced anthocyanin accumulation (Supplemental Fig. S1C), but less JA-L-Ile accumulation was observed after (+)-JA treatment compared to (−)-JA treatment, consistent with the low activity of JASMONATE RESISTANT1 for (+)-JA and the stronger binding activity between COI1-JAZ and (+)-JA-L-Ile (Guranowski et al., 2007; Fonseca et al., 2009; Supplemental Fig. S1D).

Low concentrations of JA induce COI1-dependent LR formation (Sun et al., 2009, 2011). We also observed a small stimulation of COI1-dependent LR formation upon treatment with a low concentration (1 µM) of (−)-JA, (±)-methyl-JA (MeJA), or (±)-JA, but treatments with either 50 µM (−)-JA or 20 or 50 µM (+)-JA reduced LR numbers in the temperature-dependent COI1 single mutant, *coi1-16s*, and the COI1 loss-of-function mutant *coi1-1* (Fig. 1, D–F; Supplemental Figs. S2 and S3; Reymond et al., 2000; Chen et al., 2013). Slight reductions in primary root length were observed in *coi1-16s* at higher concentrations of (−)-JA or (±)-JA (Fig. 1, G and H).

ANTHRANILATE SYNTHASE α1 (ASA1), a JA-inducible biosynthetic enzyme of IAA that is associated with LR formation, is induced as a consequence of COI1-JAZ signaling (Sun et al., 2009). Treatment with 50 µM (−)-JA or (+)-JA decreased LR numbers despite induction of *ASA1* and the accumulation of IAA in wild type and also decreased LR number with slight *ASA1* induction and the accumulation of IAA in *coi1-16s* (Supplemental Fig. S4). These results suggest that inhibition of LR formation by (−)-JA and (+)-JA is independent of the induction of *ASA1*, and hence of COI1 regulation.

The Reduction of LR Number Was Not Induced by JA Derivatives

To identify the active form of JA responsible for inhibiting LR formation, we examined the effects of known bioactive JA metabolites in plants, such as (−)-JA-L-Ile for JA response and (−)-12-OH-JA just for tuberization in the potato (*Solanum tuberosum*; Fonseca et al., 2009; Seto et al., 2009). In contrast to the JA isomers, (−)-JA-L-Ile, (+)-JA-L-Ile, (−)-12-OH-JA, and (+)-12-OH-JA did not inhibit LR formation at
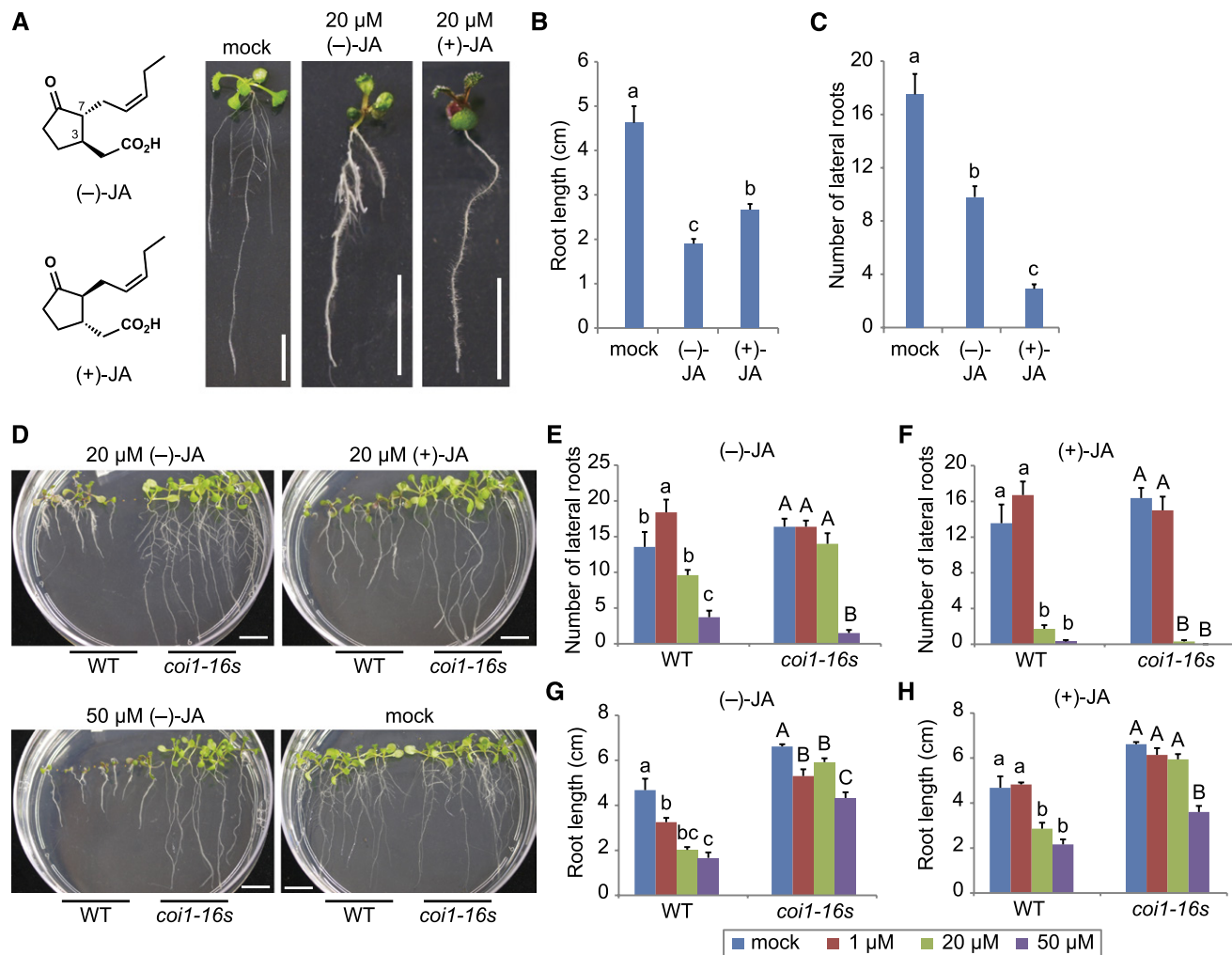
Figure 1. Reduction in LR numbers induced by JAs is independent of COI1. A to C, Phenotype (A), the primary root length (B), and LR number (C) of 9-d-old wild-type (Col-0) seedlings grown on 1/2 MS plate containing 20 µM (−)-JA, 20 µM (+)-JA, or water (mock). Values are the means with se (*n* = 8–10). Different letters indicate significant differences between means (*P* < 0.05). D, Phenotypes of 10-d-old wild-type (Col-0) and *coi1-16s* seedlings grown on 1/2 MS plate containing 20 µM (−)-JA, 20 µM (+)-JA, 50 µM (−)-JA, or water (mock). E and F, Number of LR of 10-d-old wild-type (Col-0) and *coi1-16s* seedlings grown on 1/2 MS plate containing 1, 20, or 50 µM (−)-JA (E), 1, 20, or 50 µM (+)-JA (F), or mock. G and H, The primary root length of 10-d-old wild-type (Col-0) and *coi1-16s* seedlings grown on 1/2 MS plate containing 1, 20, or 50 µM (−)-JA (G), 1, 20, or 50 µM (+)-JA (H), or mock. Values are the means with se (*n* = 15–20). Different letters indicate significant differences between means (*P* < 0.05). Scale bars, 1 cm.

50 µM (Fig. 2, A and B; Supplemental Fig. S5, A and B). It should be emphasized that after treatments with (±)-JA-Trp (JA-Trp), which was reported to be an auxin inhibitor, even at 100 µM, root architectures were distinctly different from those induced by JAs in both wild type and the *coi1* mutant (Fig. 2C; Supplemental Fig. S5C; Staswick, 2009).

JA Suppressed the Genes Related to Auxin-Inducible LR Formation

To reveal the molecular mechanism for JA-mediated inhibition of LR formation, (−)-JA- and (+)-JA-induced gene expression profiles were analyzed using a 44K microarray. We identified different classes of JA-responsive genes by two criteria: first, expression was unaffected by 50 µM MeJA, which is converted to JA and then to bioactive jasmonoyl-Ile in plants (independent of COI1-JAZ signaling; Browse, 2009); and second, expression level was changed >2.5-fold by (+)-JA compared to (−)-JA treatment. Three transcription factor genes, *LATERAL ORGAN BOUNDARIES DOMAIN* (*LBD*)*29*, *PUCHI*, and *GATA23*, all known to be auxin-inducible genes and related to PIN-FORMEDs regulation leading to LR formation, were dramatically down-regulated in response to JA treatment (Supplemental Table S1; Hirota et al., 2007;
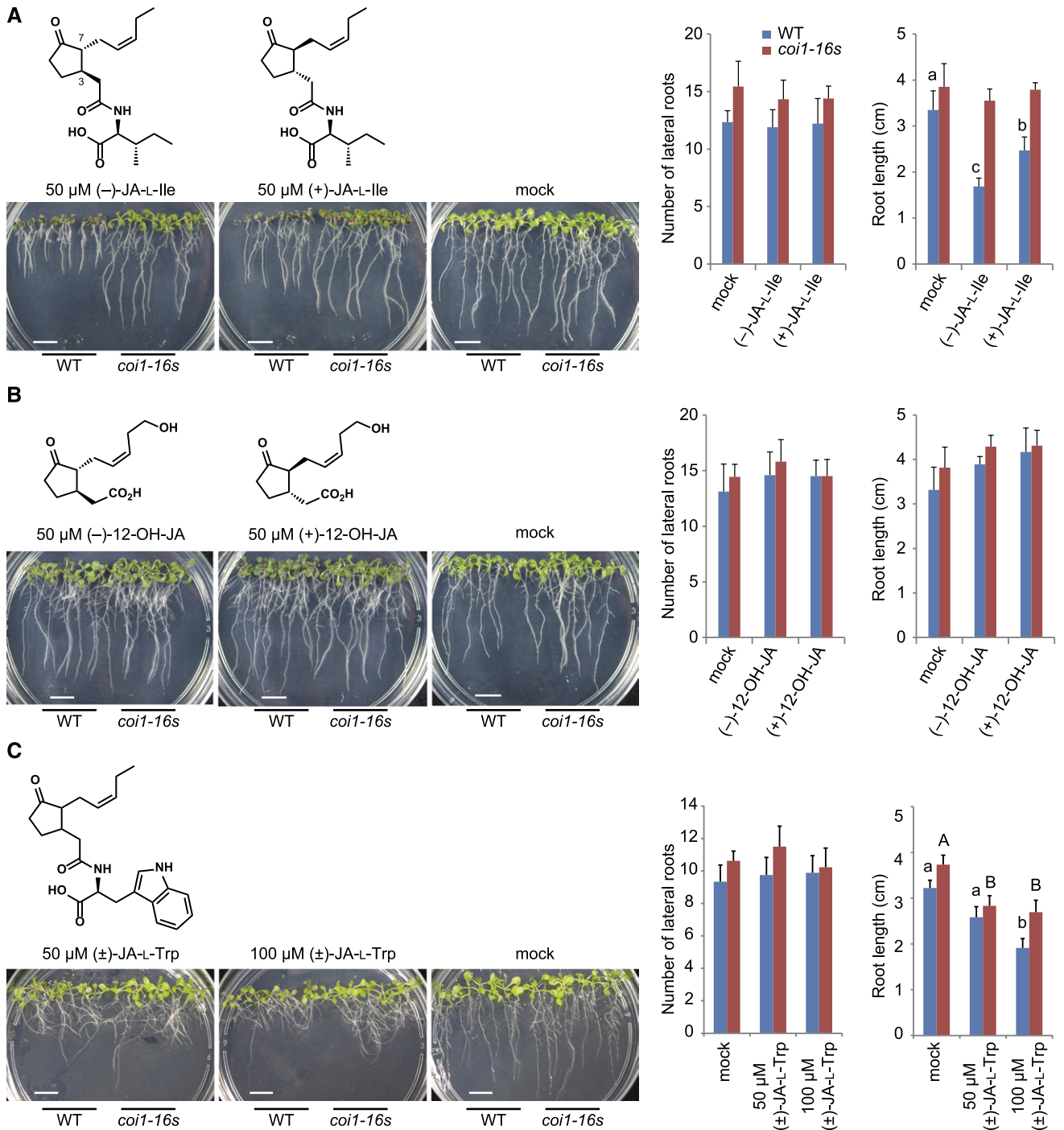
Figure 2. Effects of known bioactive JA metabolites on LR number and the primary root length. A, Phenotype, LR number, and the primary root length of 9-d-old wild-type (Col-0) and *coi1-16s* seedlings grown on 1/2 MS plate containing 50 μM (−)-JA-ʟ-Ile, 50 μM (+)-JA-ʟ-Ile, or 0.05% ethanol (mock). B, Phenotype, LR number, and the primary root length of 9-d-old wild-type (Col-0) and *coi1-16s* seedlings grown on 1/2 MS plate containing 50 μM (−)-12-OH-JA, 50 μM (+)-12-OH-JA, or water (mock). C, Phenotype, LR number, and the primary root length of 9-d-old wild-type (Col-0) and *coi1-16s* seedlings grown on 1/2 MS plate containing 50 or 100 μM (±)-JA-ʟ-Trp or 0.1% DMSO (mock). Scale bars, 1 cm. Values are the means with sᴇ (*n* = 9–10). Different letters indicate significant differences between means (*P* < 0.05).
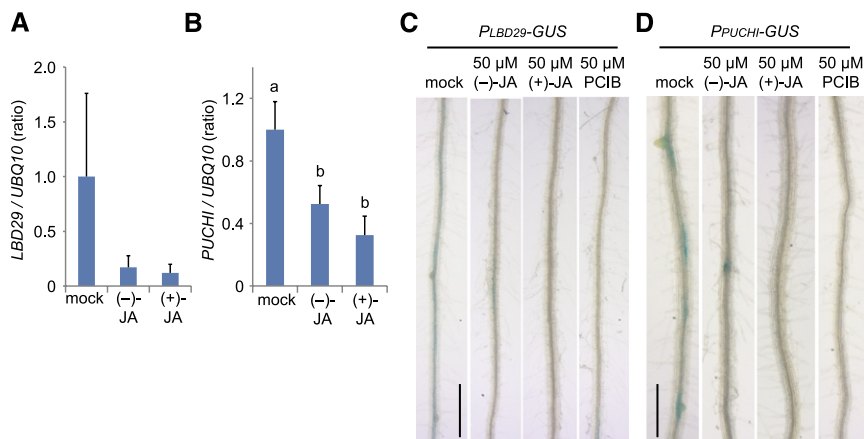
Figure 3. Expression of *LBD29* and *PUCHI* in seedlings treated with JAs. A and B, Quantitative RT-PCR data of *LBD29* (A) and *PUCHI* (B) in 4-d-old wild type (Col-0) roots grown on 1/2 MS plate containing 50 μM (−)-JA, 50 μM (+)-JA, or water (mock). Values are the means with sd (*n* = 3). Different letters indicate significant differences between means (*P* < 0.05). C and D, GUS localizations in *P_LBD29_-GUS* (C) and *P_PUCHI_-GUS* (D) in roots. Four-day-old seedlings were transferred to 1/2 MS plate containing 50 μM (−)-JA, 50 μM (+)-JA, 50 μM PCIB, or 0.04% dimethyl sulfoxide (mock) in the absence or presence of 0.1 μM IAA and grown for an additional 3 d. Scale bars, 0.5 mm.

Okushima et al., 2007; De Rybel et al., 2010). Quantitative reverse transcription (RT)-PCR revealed that only *PUCHI* was downregulated by 50 μM (−)-JA or (+)-JA in the roots, although the expression levels of *PUCHI* and *LBD29* were considerably reduced (Fig. 3, A and B). Consistent with the microarray data, GUS reporter expression around LR primordia in *P_LBD29_-GUS* and *P_PUCHI_-GUS* was suppressed by 50 μM (−)-JA or (+)-JA, as well as by the antiauxin-like compound, *p*-chlorophenoxyisobutyric acid (PCIB; Fig. 3, C and D; Oono et al., 2003; Biswas et al., 2007).

These expression changes may explain phenotypes of suppression of LR formation by the treatment of (−)-JA and (+)-JA under LR promotion by auxin signaling. Therefore, the effects for LR number were analyzed by the cotreatment with IAA and JAs. Cotreatment with various concentrations of IAA reduced the inhibitory effects of (−)-JA and (+)-JA on LR formation in a concentration-dependent manner in wild type, *coi1-16s*, and *coi1-1* mutants, with a slight reduction of the primary root length (Fig. 4, A–D; Supplemental Fig. S6).

To assess the effects of JA on auxin signaling directly, auxin-responsive gene expression was analyzed using the *DR5::GUS* reporter line (Ulmasov et al., 1997). (−)-JA at a concentration of 50 μM inhibited IAA-induced *DR5::GUS* expression, but 50 μM (+)-JA completely eliminated the response, as did 50 μM PCIB and 500 μM (−)-JA treatments (Fig. 5). Polar auxin transport is essential for LR formation, and the auxin transport inhibitor NPA inhibits LR formation (Casimiro et al., 2001). In contrast to the inhibition of *DR5::GUS* expression by JA and PCIB, NPA enhanced *DR5::GUS* expression (Fig. 5). This result suggests that (−)-JA might reduce auxin signaling, but not auxin transport.
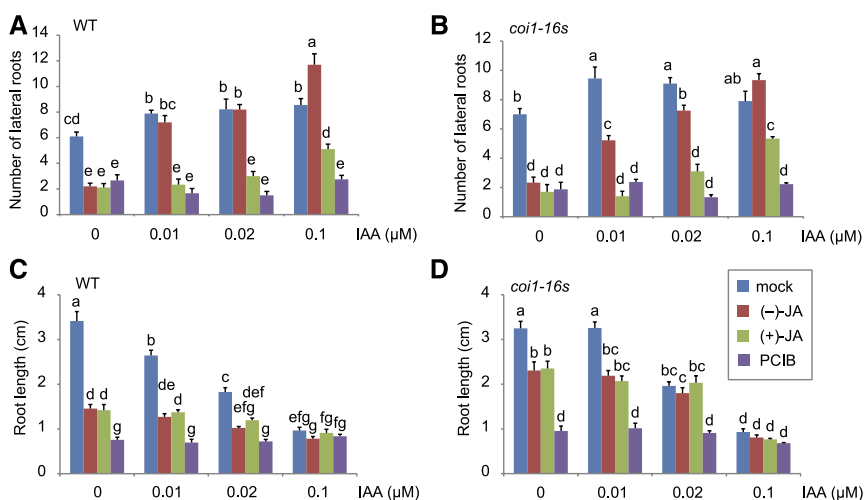
Figure 4. Effects by the cotreatment of JAs and IAA on LR number and the primary root length. A and B, LR number of wild-type (Col-0; A) and *coi1-16s* (B). C and D, The primary root length of wild type (Col-0; C) and *coi1-16s* (D). Four-day-old seedlings were transferred to 1/2 MS plate containing 0.04% DMSO (mock), 50 μM (−)-JA, 50 μM (+)-JA, or 50 μM PCIB in the absence or presence of 0.1 μM IAA and grown for an additional 3 d. Values are the means with se (*n* = 9–10). Different letters indicate significant differences between means (*P* < 0.05).
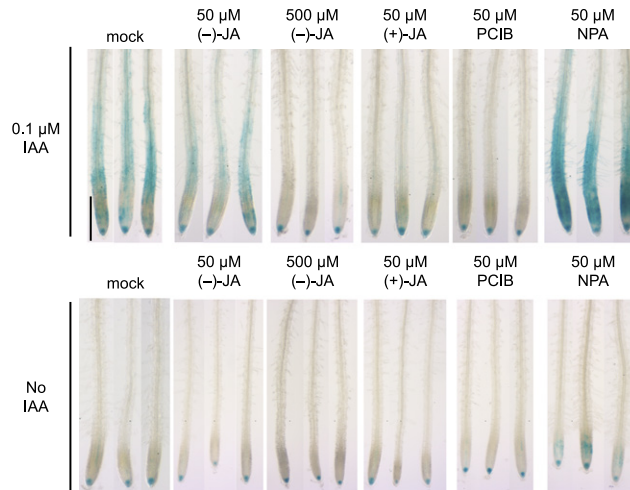
Figure 5. Expression patterns of the *DR5::GUS* reporter line in roots of seedlings treated with JAs. Expression patterns of *DR5::GUS* in roots. Five-day-old seedlings were incubated in 1/2 MS liquid medium with 0.04% DMSO (mock) or indicated compounds for 6 h before development. Scale bar, 0.5 mm.

JAs Stabilized Aux/IAA

To reveal whether (−)-JA targets the auxin-signaling cascade, further analysis was performed using the DII-VENUS reporter line as an auxin sensor (Brunoud et al., 2012). Degradation of DII-VENUS fusion protein is accelerated by IAA via the SCF$^{TIR1/AFB}$ pathway. The auxin signal is repressed by PCIB, indicated by the accumulation of DII-VENUS in the root maturation zone and the root tip (Fig. 6; Supplemental Fig. S7). Similarly, 50 μM (−)-JA or (+)-JA treatment caused the accumulation of DII-VENUS fluorescence in the root maturation zone (Fig. 6). This fluorescence was enhanced by cotreatment with jarin-1, which suppresses the conversion of JA to JA-Ile and increased JA concentration, and diminished by cotreatment with IAA in both the maturation zone and the root tip (Fig. 6; Supplemental Figure S7; Meesters et al., 2014). These data are consistent with the loss of SCF$^{TIR1/AFB}$ activity and, hence, auxin signaling in the root maturation zone where LRs elongate (Fig. 1).

To elucidate the relationships between TIR1/AFB1–5 receptors and JA, we analyzed *tir1afb2-3* and *afb5-5* mutants. Their treatment with (−)-JA or (+)-JA caused
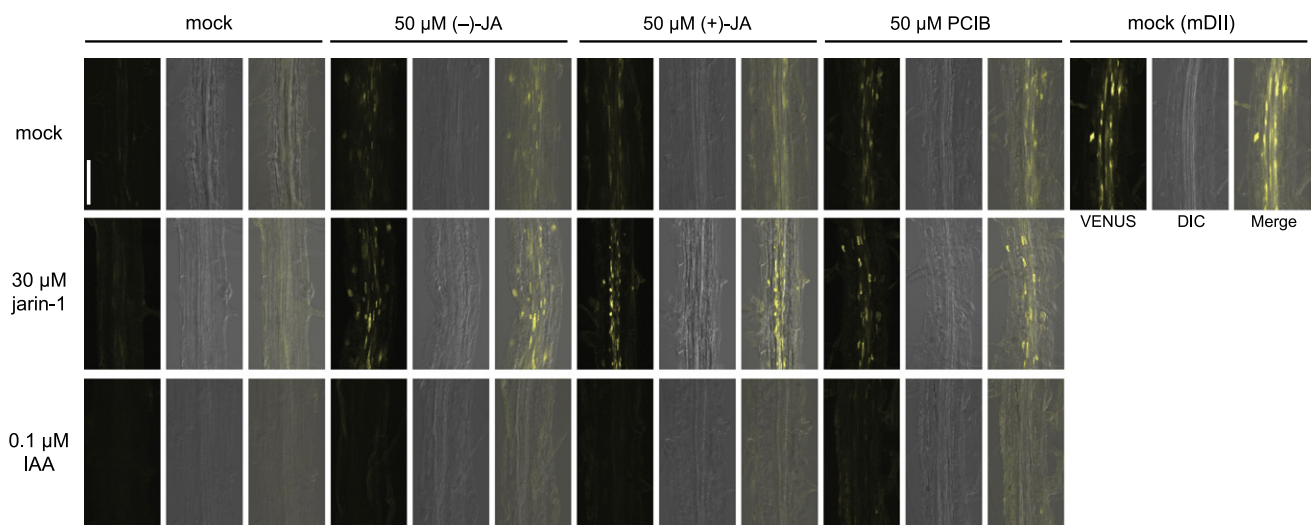
Figure 6. DII-VENUS fluorescence in seedlings treated with JAs. Laser scanning confocal micrographs of DII-/mDII-VENUS at the root maturation zone. Left, fluorescent image of DII-VENUS (false-colored yellow). Center, DIC image. Right, merge of DII-VENUS and DIC. Seedlings were incubated in 1/2 MS liquid medium with 0.05% DMSO (mock), 50 μM (−)-JA, 50 μM (+)-JA, or 50 μM PCIB in mock, 30 μM jarin-1, or 0.1 μM IAA for 3 h. Scale bar, 0.1 mm.
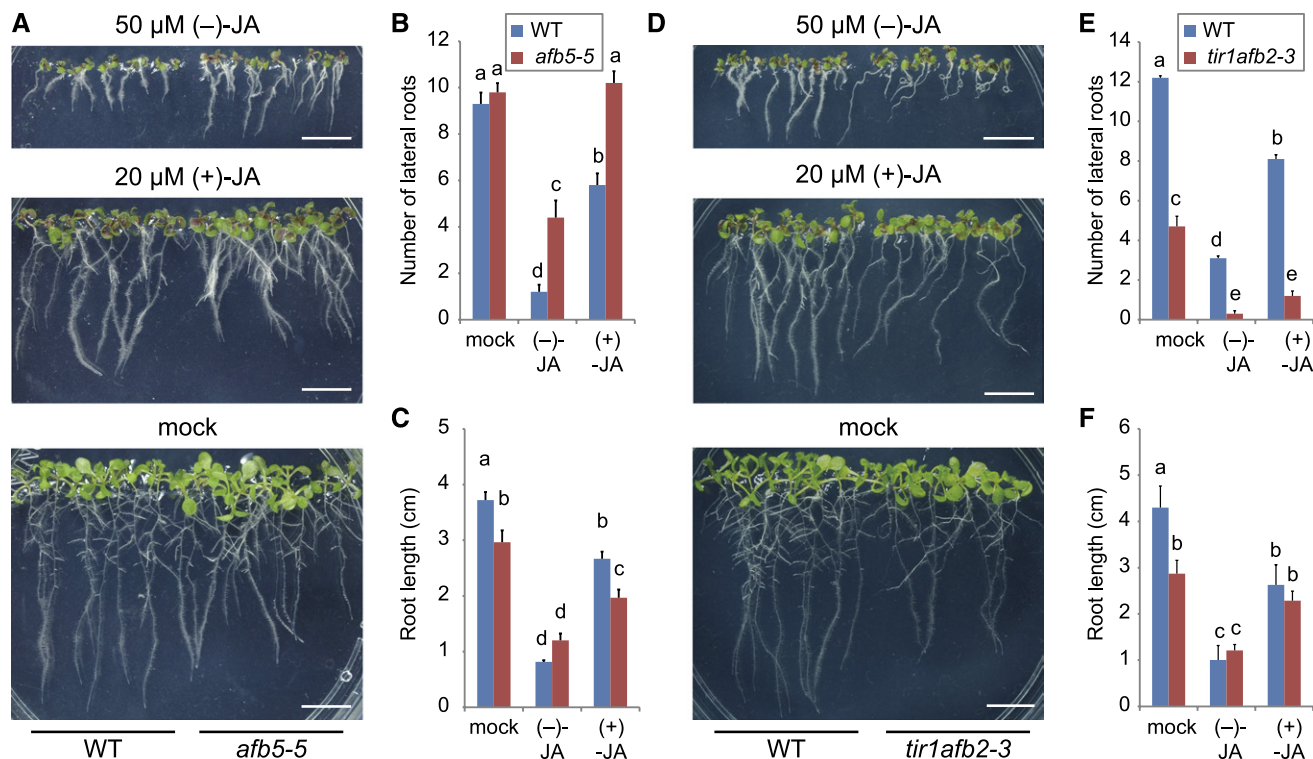
Figure 7. Effects of JAs on *afb5* and *tir1afb2* mutant lines compared to wild type. A, Phenotype of 9-d-old wild-type (WT; Col-0) or *afb5-5* seedlings grown on 1/2 MS plate containing 50 μM (−)-JA, 20 μM (+)-JA, or water (mock). B and C, LR number (B) and the primary root length (C) in 9-d-old wild-type (Col-0) or *afb5-5* seedlings grown on 1/2 MS plate containing 50 μM (−)-JA, 20 μM (+)-JA, or water (mock). D, Phenotype of 9-d-old wild-type (Col-0) or *tir1afb2-3* seedlings grown on 1/2 MS plate containing 50 μM (−)-JA, 20 μM (+)-JA, or water (mock). E and F, LR number (E) and the primary root length (F) in 9-d-old wild-type (Col-0) or *tir1afb2-3* seedlings grown on 1/2 MS plate containing 50 μM (−)-JA, 20 μM (+)-JA, or water (mock). Values are the means with SE (n = 9–10). Different letters indicate significant differences between means ($P < 0.05$). Scale bars, 1 cm.

a decrease in the number of LR in *tir1afb2-3* compared with wild type, whereas *afb5-5* resisted (−)-JA and (+)-JA and no reduction in LR formation was observed (Fig. 7).

Coronafacic Acid Was an LR-Preferential Auxin Inhibitor

Jasmonic acids are converted to JA-ʟ-Iles, which induce COI1-dependent responses, and so *coi1* mutants are necessary to analyze JA treatment-induced LR formation (Supplemental Fig. S1). Accordingly, we sought an LR-preferential auxin inhibitor that does not induce COI1-dependent JA responses. Coronatine is a phytotoxin produced by *Pseudomonas syringae* and known to function as a structural mimic of (+)-7-*iso*-JA-ʟ-Ile in planta. The structure of coronatine consists of coronafacic acid (+)-CFA (an analog of MeJA) and coronamic acid, joined by an amide bond between the acid group of CFA and the amino group of coronamic acid, and so (+)-CFA could be considered as a structural mimic of (+)-7-*iso*-JA (Supplemental Fig. S8; Egoshi et al., 2016). Treatment of wild type with (+)-CFA dramatically suppressed LR formation, with only slight effects on primary root growth (Fig. 8, A–C). Enantiomeric (−)-CFA did not noticeably affect LR numbers (Fig. 8,

A–C). Gene expression of *AOS* was not observed after treatment with either (+)-CFA or (−)-CFA, suggesting that (+)-CFA can indeed mimic JA, without inducing a COI1-dependent JA response (Fig. 8D). (+)-CFA treatment did not affect root gravitropism or auxin-inducible root hair formation (Fig. 8, E and F). Treatment with 10 μM (+)-CFA induced accumulation of DII-VENUS fluorescence (Fig. 8G), but treatment with (−)-CFA did not. As was found for the JA treatments, (+)-CFA decreased LR numbers in *tir1afb2-3* compared with wild type, whereas *afb5-5* resisted (+)-CFA-induced reductions in LR formation (Fig. 8, H and I). We also tested whether (+)-CFA cross-reacted with auxin receptors using surface plasmon resonance (SPR) assays; however, (+)-CFA exhibited neither auxin-like nor antiauxin-like activity, when binding to either AFB5-Aux/IAA7 or 28 (Fig. 8J; Supplemental Fig. S9).

DISCUSSION

(−)-JA and (+)-JA have been implicated in the inhibition of LR formation in Arabidopsis, independent of COI1 signaling (Fig. 1). Both (−)-JA and (+)-JA stabilize DII-VENUS, indicating that their activity stabilizes
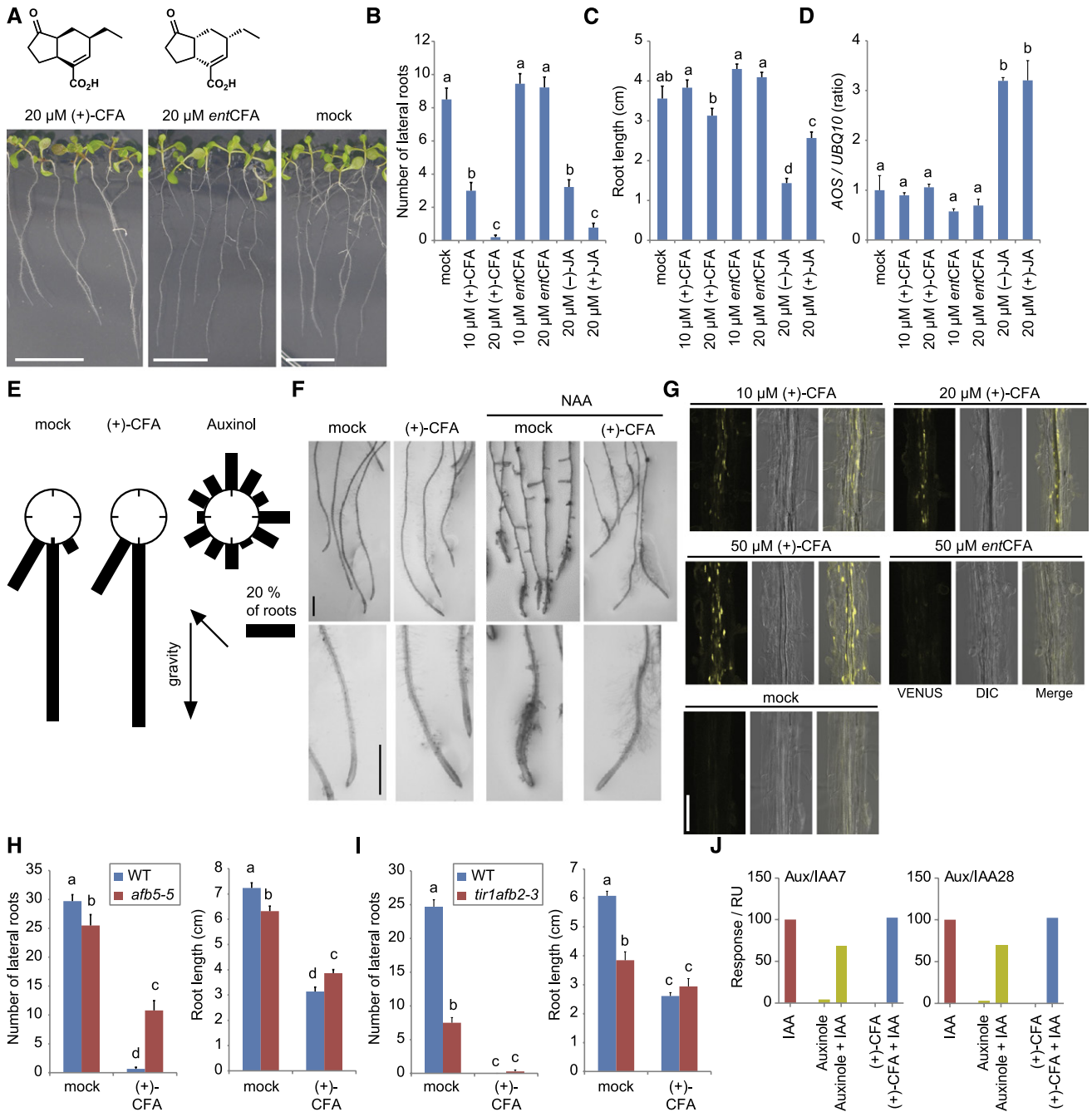
Figure 8. The effects of CFA on the wild type. A–C, Phenotype of 8-d-old wild-type (Col-0) seedlings grown on 1/2 MS plate containing 20 μM (+)-/(−)-CFA or 0.05% ethanol (mock). LR number (B), the primary root length (C), and expression patterns of *AOS* (D) in 8-d-old wild-type (Col-0) seedlings grown on 1/2 MS plate containing 10 μM or 20 μM (+)-CFA, 10 μM or 20 μM (−)-CFA, 20 μM (−)-JA, 20 μM (+)-JA, or 0.05% ethanol (mock). Values are the means with SE (*n* = 18–20). Different letters indicate significant differences between means (*P* < 0.05). Scale bars, 1 cm. E, Effects of (+)-CFA on root gravitropism. Five-day-old seedlings were vertically grown in the first gravity direction (first g) with 50 μM (+)-CFA or 5 μM auxinole and then rotated at an angle of 135° (second g) and cultured for an additional 24 h. The angles were grouped into 30° classes and plotted as circular histograms (*n* = 40). F, Root hair growth of 5-d-old Col-0 with 50 μM (+)-CFA or 0.05% ethanol (mock) with/without 0.5 μM naphthalene1-acetic acid (NAA) for 2 d. Scale bars, 1 mm. G, Laser scanning confocal micrographs of DII-VENUS at root maturation zone. Five-day-old seedlings were incubated in 1/2 MS liquid medium with 10, 20, or 50 μM (+)-CFA or 50 μM (−)-CFA or 0.05% ethanol (mock) for 3 h. Scale bars, 0.1 mm. H, LR number and primary root length in 12-d-old wild-type (Col-0) or *afb5-5* seedlings grown on 1/2 MS plate containing 50 μM (+)-CFA or 0.05% ethanol (mock). I, LR number and primary root length in 11-d-old wild-type (Col-0) or *tir1afb2-3* seedlings grown on 1/2 MS plate containing 50 μM (+)-CFA or 0.05% ethanol (mock). Values are the means with SE (*n* = 9–10). Different letters indicate significant differences between means (*P* < 0.05). J,

domain II of Aux/IAAs and therefore suppresses the expression of auxin-inducible genes related to LR formation, such as *LBD29* and *PUCHI* (Figs. 3, 6, and 7). Furthermore, (+)-CFA was found to be an active structural mimic of (−)-JA, establishing it as a preferential-auxin inhibitor for LR formation (Fig. 8).

Bioactivities of JA

JAZ degradation, induction of JA-responsive genes, anthocyanin accumulation, chlorophyll accumulation, and growth defects are also observed by the treatment of high JA concentration, as well as low JA concentration and wounding stress (Lorenzo et al., 2004; Chini et al., 2007; Fonseca et al., 2009; Fernández-Calvo et al., 2011; Boter et al., 2015). Moreover, the COI1-independent LR inhibition dominated at higher concentrations, despite *ASA1* induction and the accumulation of IAA both in wild type and *coi1-16s* (Supplemental Fig. S4). Conversely, a low concentration of JA has been reported to stimulate LR formation and to inhibit primary root elongation in a COI1-dependent manner (Sun et al., 2009, 2011; Chen et al., 2011). Small stimulation of COI1-dependent LR formation and inhibition of primary root elongation upon treatment with a lower concentration of either (−)-JA, (+)-JA, (±)-methyl-JA, or (±)-JA was also observed (Fig. 1; Supplemental Fig. S3). These results suggest that JA modulates LR formation via regulatory pathways transduced both by COI1-dependent and COI1-independent signaling. The positive/negative effect on LR formation depends on the concentration of JA.

Stabilization of Aux/IAAs by JAs and (+)-CFA

There are many known mutations in domain II of the Aux/IAA protein that confer improved functional activity and increase the stability of the protein, such as *iaa1/axr5* (Park et al., 2002b; Yang et al., 2004), *iaa3/shy2* (Tian and Reed, 1999), *iaa6/shy1* (Reed, 2001), *iaa7/axr2* (Nagpal et al., 2000), *iaa12/bdl* (Hamann et al., 2002), *iaa14/slr* (Fukaki et al., 2002), *iaa17/axr3* (Rouse et al., 1998), *iaa18/crane* (Reed, 2001; Uehara et al., 2008; Ploense et al., 2009), *iaa19/msg2* (Tatematsu et al., 2004), and *iaa28* (Rogg et al., 2001). These mutants exhibit various abnormal phenotypes involved in auxin-mediated growth and development, as well as altered gene expression in response to auxin. The phenotypes are distinct in each *Aux/IAA* mutant, although some persistent similarities suggest that each Aux/IAA has both similar and unique roles in the auxin response. The phenotypes of preferential LR inhibition by JAs and (+)-CFA was most similar to those of *iaa3/shy2*, *iaa14/slr*, *iaa18/crane*, and *iaa28* gain-of-function mutants (Figs. 1 and 8, A–D). The DII-VENUS reporter line was designed on IAA28 (Brunoud et al., 2012), and the stabilization of DII-VENUS by JAs and (+)-CFA would at least explain similar phenotypes in the inhibition of LR formation observed in both *iaa28* gain-of-function mutants, and JA- and (+)-CFA-treated plants (Figs. 6 and 8E).

During LR formation, degradation of IAA14 and IAA28 activates the transcriptional activity of ARF5, 6, 7, 8, and 19 (De Rybel et al., 2010). Of these ARFs, ARF7 and ARF19 directly activate *LBD16* and *LBD29*, leading to the induction of *PUCHI* and LR primordium development (Hirota et al., 2007; Okushima et al., 2007; Lavenus et al., 2015). *LBD29* and *PUCHI* were found to be suppressed by JA treatments (Fig. 3), suggesting that JAs might also stabilize IAA14.

Relationships between JAs or (+)-CFA and TIR1/AFB Receptors

The degradation of Aux/IAAs by ubiquitination is initiated by auxin perception at the TIR1/AFB receptors (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). Previous phylogenetic studies revealed that TIR1/AFBs can be classified into three clades, represented by TIR1, AFB2, and AFB5 in Arabidopsis. The functionality of these receptors was found to overlap, but a functional difference was clearly demonstrated in planta; the *tir1afb2* double mutant is deficient in a variety of auxin-regulated growth processes, including hypocotyl elongation and LR formation, and the *afb5* mutant was tolerant to the piclolinate family of auxinic herbicides (Walsh et al., 2006; Parry et al., 2009; Prigge et al., 2016). Compared with wild type, *afb5-5* showed resistance to the reductions in LR formation by JAs and (+)-CFA treatments, but *tir1afb2-3* did not (Figs. 7 and 8, F–K). On the other hand, suppression of auxin-induced *DR5::GUS* and accumulation of DII-VENUS were observed after treatments of JA and (+)-CFA in wild-type plants (Figs. 5 and 6). Both TIR1 and AFB5 seem to be involved in the inhibition of Aux/IAA degradation by JAs and (+)-CFA, but it seems that AFB5 might have a dominant role, leading to reduced Aux/IAA breakdown and inhibition of LR formation.

While DII-VENUS accumulated in the wild type after treatments of (+)-CFA, SPR assays revealed that (+)-CFA neither agonized nor antagonized binding of the AFB5-Aux/IAA complex. This suggests that (+)-CFA may not interact with the auxin-binding pocket in AFB5 (Figs. 6 and 8). The mutant of SGT1B, which is required for the degradation of Aux/IAA proteins, also showed resistance to picolinate auxins, as well as

Figure 8. (*Continued.*)
Surface plasmon resonance assays of AFB5 auxin and antiauxin activities using (+)-CFA. All sensorgram data are normalized to the signal from 5 μM IAA (red, 100%). Auxinole is used as a control for antiauxin activity, using 50 μM auxinole. (+)-CFA were added at 50 μM, and with 5 μM IAA for the antiauxin assay. The SPR chip was coated with biotinylated Aux/IAA7-DII or Aux/IAA28-DII peptide and binding was recorded at the end of the association phase.

afb5 mutant (Walsh et al., 2006). The LR-preferential phenotype and the accumulation of DII-VENUS after treatments with JA and (+)-CFA suggest that their target might be proteins related to the Aux/IAAs degradation system (Figs. 1, 6, and 8).

Structural Requirements for JA-Mediated Inhibition of LR Formation

In our study, (−)-JA, (+)-JA, and (+)-CFA induced inhibition of LR formation. In Arabidopsis, (+)-7-*iso*-JA (with the *7S* configuration) was biosynthesized and converted into (+)-7-*iso*- JA-ʟ-Ile (Supplemental Fig. S8; Fonseca et al., 2009). However, (+)-7-*iso*-JA is easily epimerized to thermodynamically stable (−)-JA to give a mixture of 95% (−)-JA and 5% (+)-7-*iso*-JA (Mueller and Brodschelm, 1994). The decrease in LR number after treatment with (+)-*7S*-JA was more pronounced than that of (−)-*7R*-JA (Fig. 1, E and G). Considering the strong bioactivity of (+)-CFA, which is conformationally fixed in the *7S* configuration, the *S* configuration at the C-7 position of JA seems likely to contribute to bioactivity in COI1-independent LR formation (Fig. 8).

CONCLUSION

Both enantiomers of JA and (+)-CFA have been confirmed to inhibit LR formation by Aux/IAA stabilization, independent of COI1 signaling. The study has also identified (+)-CFA as a chemical, specialized, and conditional LR auxin inhibitor, showing similar LR phenotypes to those observed in several *aux/iaa* mutants. This inhibitor could prove a useful tool for a deeper understanding of the molecular basis of LR formation.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Chemical Treatment

Arabidopsis (*Arabidopsis thaliana*) accession Col-0 was used as the wild type in all experiments. Seeds of P_{35S}-*JAZ1:GUS* reporter line (Meesters et al., 2014), *DR5::GUS* (Ulmasov et al., 1997), DII-VENUS (Brunoud et al., 2012), P_{PUCHI}-*GUS* (Hirota et al., 2007), and P_{LBD29}-*GUS* (Okushima et al., 2007) were obtained as previously described. Seeds of *coi1-1* and *coi1-30* mutants were provided by Dr. R. Solano and Dr. A. Chini (National Center for Biotechnology, Madrid, Spain) and those of *coi1-16s* by Dr. H. Ohta (Tokyo Institute of Technology; Chen et al., 2013). The lines *tir1 afb2-3* and *afb5-5* were gifts from Dr. Mark Estelle (University of California at San Diego, CA). Sterilized seeds were sown on half-length Murashige-Skoog (1/2 MS) medium (2% [w/v] Suc, pH 5.8) hardened with 0.4% (w/v) gellan gum (Wako) and then vernalized for 3 d at 4°C. Seedlings were grown at 22°C under 12 h light (100–118 μmol·m^{-2}·s^{-1}; cool-white fluorescent light)/12 h dark in a BIOTRON LPH-240SP growth chamber (Nippon Medical and Chemical Instruments). For jasmonate quantification and expression analysis of JA responsive genes, seedlings were grown on 48-well plates containing 1/2 MS liquid medium (0.5% [w/v] Suc, pH 5.8) in the same chamber. The number of lateral roots and the primary root length were determined using a dissecting microscope and ImageJ software (https://imagej.nih.gov/ij/). Chemicals were stored as stock solutions, then directly added to the 50°C–60°C medium after autoclaving. For preparation of stock solutions, JA and (−)-12-OH-JA were dissolved in water and then filter sterilized (0.22 μm). Ethanol was used as the solvent for MeJA, JA-ʟ-Ile, and CFA, and DMSO was used for jarin-1 (Meesters et al., 2014), JA-Trp, IAA, PCIB (Oono et al., 2003), and NPA.

Chemicals

(±)-JA, prepared by hydrolysis of (±)-MeJA (Nihon-Zeon, Japan), was condensed with ʟ-Trp-Me ester (Wako), followed by hydrolysis to yield the product as a yellow solid (Ueda et al., 2017). (−)-TA, (+)-TA, (+)-CFA, (−)-CFA, and Trp conjugate of (±)-JA were prepared as previously described (Nakamura et al., 2008, 2011). IAA (Wako), NPA (Tokyo Chemical Industry), and PCIB (Sigma-Aldrich) were purchased as fine chemicals and used as supplied.

Jasmonate Quantification

Jasmonates were extracted from plants and quantified by UPLC (Agilent)-TOFMS (Bruker) as described (Kurotani et al., 2015). In brief, 50–100 mg fresh weight samples with 100 ng/g JA-d_6-Ile were homogenized in ethanol. After removing the debris by centrifugation, the supernatant was dried with N$_2$ gas, then dissolved in water. The aqueous solution (recovery rate of JA-d_6-Ile, 12.5%) was used for the following UPLC-TOF-MS measurement.

Anthocyanin Quantification

Anthocyanin was extracted from plants and quantified as described (Neff and Chory, 1998). Seedlings were snap-frozen with liquid N$_2$, then homogenized in methanol acidified with 1% (w/v) HCl and allowed to stand overnight at 4°C in the dark for efficient extraction. Sterile water was added to the lysate to give 60% (w/w) methanol, followed by addition of equal amount of chloroform. After vigorous mixing and centrifugation, the aqueous layer was carefully collected, and absorbance measured at 530 nm and 657 nm using GeneQuant1300 (GE Healthcare).

IAA Quantification

IAAs were extracted from plants and quantified with a UPLC (Agilent)-TOFMS (Bruker) system as described (Barkawi et al., 2008; Kurotani et al., 2015). In brief, 10 to 50 mg fresh weight samples with 10 ng/g *d5*-IAA were homogenized in extraction buffer (65% [v/v] isopropanol and 35% [w/v] imidazole, pH 7.0). After removal of the debris by centrifugation, the supernatant was dried with N$_2$ gas, then dissolved in 28% (v/v) aqueous acetonitrile containing 0.05% (w/v) acetic acid. The solution was used for the following UPLC-TOF-MS measurement.

Histochemical Staining of GUS

Transgenic plants expressing GUS were grown on 1/2 MS plates for 4 d, then transferred to plates containing the indicated compounds and grown for an additional 3 d. For histochemical staining, samples were immersed in GUS staining solution (40 mм Na-phosphate buffer [Wako] containing 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-ᴅ-glucuronide [Wako], 3 mм K$_4$[Fe(CN)$_6$] [Wako], 0.5 mм K$_3$[Fe(CN)$_6$] [Wako]) in 20% [v/v] methanol). After 30 min staining, samples were incubated in 70% [v/v] ethanol. Stemi 2000-C (Carl Zeiss), equipped with AxioCam ERc 5s (Carl Zeiss), was used to observe and photograph the samples, following the manufacturer's instructions.

Quantitative RT-PCR

Immediately after sampling, seedlings were frozen with liquid N$_2$. Extraction and purification of total RNA from plant samples were performed with RNeasy Plant Mini Kit (Qiagen), and the reverse transcription was achieved by ReverTra Ace (Toyobo). Quantitative RT-PCR was conducted with StepOnePlus real-time PCR system (Applied Biosystems) with primers (Supplemental Table S2). The sizes and sequences of the amplified fragments were confirmed by agarose gel electrophoresis and sequencing. *AOS*, *ASA1*, *LBD29*, and *PUCHI* were quantified with *UBQ10*.

Microarray Analysis

Total RNA was extracted from roots from 4-d-old seedlings using RNeasy Plant Mini Kit (QIAGEN). The microarray analyses, hybridization, data analyses, and data mining were carried out as previously described (Bashir et al., 2011). In brief, cDNAs were synthesized using 500 ng of total RNA and labeled with one color (Cy3) using a Quick Amp labeling kit (Agilent Technologies),

followed by fragmentation and hybridization to the Arabidopsis Oligo 44K DNA microarray (Ver. 4.0; Agilent Technologies). Three biological replicates were performed for each treatment, making a total of nine hybridizations. All arrays were scanned with a microarray scanner (G2505B; Agilent Technologies) and analyzed using GeneSpring Ver. 11 (Agilent Technologies). The Student's t test (P value) was used as a parametric test and the Benjamini and Hochberg false-discovery rate (q value) procedure was used to control the certainty level. Genes with at least a 2-fold difference in their expression levels and a q value < 0.05 were considered to be differentially expressed.

DII-VENUS Analysis

Six-day-old DII-VENUS seedlings were incubated with indicated compounds for 3 h. For jarin-1 treatment, seedlings were treated with jarin-1 for 1 h before the application of indicated compounds. Half-strength MS liquid medium (0.5% [w/v] Suc, pH 5.8) was used as an incubation buffer. After the treatment, roots were cut off and mounted on slide grasses with 1:1 phosphate-buffered saline:glycerol solution. Photographic and fluorescent images of DII-VENUS seedlings were taken with an LSM-710 laser scanning confocal microscope (Carl Zeiss), following the manufacturer's instructions. Acquisition parameters were as follows: master gain was always set for 726 with a digital gain of 1, a digital offset of 3, excitation at 488 nm (2%), and emission at 490 to 555 nm.

Microscopy

Photographic and fluorescent images were taken with LSM-710 laser scanning confocal microscope (Carl Zeiss). For histochemical analysis using transgenic plants carrying GUS, a Stemi 2000-C equipped with AxioCam ERc 5s (Carl Zeiss) was used to observe and photograph the samples, following the manufacturer's instructions.

Surface Plasmon Resonance

Surface plasmon resonance assays were done as described (Lee et al., 2014). Jasmonates were dissolved as stocks at 10 mM in DMSO and diluted to 50 μM for the assay with controls run in the equivalent DMSO concentration. Auxin-like activity and antiauxin activity were compared in each run against signals for IAA, with auxin-like activity recorded if the test compound acted to assemble the respective coreceptor complex (acting as "molecular glue"), and antiauxin activity recorded as a reduction in IAA-supported coreceptor complex. In the antiauxin assay, IAA was present at 5 μM, the test compound at 50 μM.

Statistical Analysis

The data were analyzed with one-way ANOVA followed by SNK post-hoc test among all means. Statistical analyses were conducted using CoStat Version 6.400 (CoHort Software).

Accession Numbers

COI1, AT2G39940; JAZ1, AT1G19180; TIR1, AT3G62980; AFB2, AT3G26810; AFB5, AT5G49980; Aux/IAA7, AT3G23050; Aux/IAA28, AT5G25890; AOS, AT5G42650; ASA1, AT5G05730; LBD29, AT3G58190; and PUCHI, AT5G18560.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Effects of (+)-JA on wild type.

Supplemental Figure S2. Effects of (−)-JA or (+)-JA on *coi1-1* null mutant.

Supplemental Figure S3. Effects of (±)-MeJA or (±)-JA treatment on LR number and primary root length.

Supplemental Figure S4. Expressions of *ASA1* and IAA concentration treated with JAs.

Supplemental Figure S5. Effects of known bioactive JA metabolites on LR number and the primary root length on *coi1-1* null mutant.

Supplemental Figure S6. Effects by the cotreatment of JAs and IAA on LR number and the primary root length on *coi1-1* null mutant.

Supplemental Figure S7. DII-VENUS fluorescence at the root tip.

Supplemental Figure S8. Structures of JAs and CFA.

Supplemental Figure S9. AFB5 antiauxin activity sensorgram.

Supplemental Table S1. Summary of the microarray analysis.

Supplemental Table S2. Primers for quantitative RT-PCR.

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