

The Tryptophan Conjugates of Jasmonic and Indole-3-Acetic Acids Are Endogenous Auxin Inhibitors^{1[W][OA]}

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Most conjugates of plant hormones are inactive, and some function to reduce the active hormone pool. This study characterized the activity of the tryptophan (Trp) conjugate of jasmonic acid (JA-Trp) in *Arabidopsis* (*Arabidopsis thaliana*). Unexpectedly, JA-Trp caused agravitropic root growth in seedlings, unlike JA or nine other JA-amino acid conjugates. The response was dose dependent from 1 to 100 μM , was independent of the *COI1* jasmonate signaling locus, and unlike the jasmonate signal JA-isoleucine, JA-Trp minimally inhibited root growth. The Trp conjugate with indole-3-acetic acid (IAA-Trp) produced a similar response, while Trp alone and conjugates with benzoic and cinnamic acids did not. JA-Trp and IAA-Trp at 25 μM nearly eliminated seedling root inhibition caused by 2 μM IAA. The TIR1 auxin receptor is required for activity because roots of *tir1-1* grew only approximately 60% of wild-type length on IAA plus JA-Trp, even though *tir1-1* is auxin resistant. However, neither JA-Trp nor IAA-Trp interfered with IAA-dependent interaction between TIR1 and Aux/IAA7 in cell-free assays. Trp conjugates inhibited IAA-stimulated lateral root production and DR5- β -glucuronidase gene expression. JA-deficient mutants were hypersensitive to IAA and a Trp-overaccumulating mutant was less sensitive, suggesting endogenous conjugates affect auxin sensitivity. Conjugates were present at 5.8 pmol g⁻¹ fresh weight or less in roots, seedlings, leaves, and flowers, and the values increased approximately 10-fold in roots incubated in 25 μM Trp and IAA or JA at 2 μM . These results show that JA-Trp and IAA-Trp constitute a previously unrecognized mechanism to regulate auxin action.

Throughout their life, plants use a variety of hormonal signals to adjust growth in response to developmental and external cues. Central among the growth regulating hormones is the auxin indole-3-acetic acid (IAA), which is involved in nearly all aspects of plant development (for review, see Woodward and Bartel, 2005). Optimal growth requires tight control of IAA activity, which is accomplished by diverse mechanisms that include regulating IAA biosynthesis, its transport among tissues, cycling between active and inactive forms of auxin, and hormone degradation through various oxidative pathways (see Ljung et al., 2002; Leyser, 2006; Scheres and Xu, 2006). Regulatory control is also accomplished at the level of IAA interaction with auxin receptors and at numerous steps downstream of this signaling interaction (Mockaitis and Estelle, 2008).

While jasmonates are best known for their role in defense against herbivores and certain pathogens, they also control growth (for recent overviews, see Westernack, 2007; Howe and Jander, 2008; Browse, 2009). Exogenous jasmonic acid (JA) inhibits growth (Yamane et al., 1980; Dathe et al., 1981; Ueda and Kato, 1982; Staswick et al., 1992), and both JA biosynthesis and jasmonate signaling mutants display growth abnormalities, particularly in reproductive organs and in vegetative tissues during defense responses (Mandaokar et al., 2006; Zavala and Baldwin, 2006; Yan et al., 2007; Zhang and Turner, 2008). It makes sense that jasmonate defense signaling would be tied to growth regulation because under stress plants must adjust their development and reallocate resources toward defense (Herms and Mattson, 1992). Although the full mechanism of jasmonate-mediated growth regulation is unclear, it may involve cell cycle transitions, cell division, and IAA-induced cell elongation (Miyamoto et al., 1997; Swiatek et al., 2002, 2004; Zhang and Turner, 2008).

Plants synthesize conjugated forms of both JA and IAA. Indeed, most IAA in *Arabidopsis* (*Arabidopsis thaliana*) is bound through ester or amide linkages to various constituents, including sugars, amino acids, and peptides (Ljung et al., 2002). Conjugates are generally considered inactive metabolites of the hormone. For example, IAA-Asp and IAA-Glu are catabolized, while IAA-Ala and IAA-Leu are stored forms of IAA that can be accessed at a later time by hydrolysis of the amide linkage (Rampey et al., 2004). Early physiolog-

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ical studies suggested that some JA conjugates might have biological activity, but like the IAA conjugates, most were considered inactive or of minor importance compared with JA and its methyl ester (JA-Me; Kramell et al., 1997; Miersch et al., 1999). That perspective changed with the discovery that the Ile conjugate of JA (JA-Ile) is a key jasmonate signal (Staswick and Tiriyaki, 2004).

JA-Ile accumulation is strongly but transiently induced in plant defense responses in both *Arabidopsis* and tobacco (*Nicotiana tabacum*), and this accumulation is tied to productive defense reactions (Kang et al., 2006; Wang et al., 2007; Suza and Staswick, 2008). Furthermore, JA-Ile promotes interaction between the COI1 F-box protein, the presumed jasmonate receptor, and its ubiquitination targets, the JAZ transcriptional repressors (Chini et al., 2007; Thines et al., 2007; Katsir et al., 2008a; Staswick, 2008). In contrast, JA and JA-Me have essentially no activity in this interaction. Instead, they are precursors that become effective signals when added exogenously to plants because they are converted to JA-Ile by the JAR1 conjugating enzyme (Staswick and Tiriyaki, 2004; Tamogami et al., 2008).

JA-Ile signaling closely parallels the mechanism previously established for auxin, although in the latter case, free rather than conjugated IAA is the active signal. IAA binds in a pocket of the TIR1 auxin receptor, which is an F-box component of the E3 ubiquitin ligase complex called SCF^{TIR1}. Auxin binding strengthens the interaction between TIR1 and the Aux/IAA protein targets for ubiquitination (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005; Tan et al., 2007). Degradation of the Aux/IAA transcriptional repressors by the 26 S proteasome then leads to auxin-dependent gene transcription and auxin response. Five TIR1-related proteins, AFB1 through AFB5, have been identified, and they have partially overlapping functions with TIR1 (Dharmasiri et al., 2005b; Mockaitis and Estelle, 2008). In contrast, COI1 is the primary, if not the sole, F-box protein in jasmonate signaling.

Critical tools that have shaped our understanding of auxin activity are auxin response inhibitors of two general classes: those that alter auxin transport and those that perturb auxin signaling. Although a few endogenous auxin inhibitors have been reported, little is known about their *in vivo* roles. Most studies have employed synthetic inhibitors, such as 1-naphthylphthalamic acid (NPA), *p*-chlorophenoxyisobutyric acid (PCIB), 4,4,4-trifluoro-3-indole-3-butyric acid, triiodobenzoic acid, and 1-naphthoxyacetic acid (MacRae and Bonner, 1953; Jönsson, 1961; Katayama et al., 1995; Tomic et al., 1998; Parry et al., 2001; Rahman et al., 2002). The TIR1 auxin receptor was discovered as a mutant (*tir1*) resistant to the NPA transport inhibitor, and new antagonists and agonists have been identified in recent chemical genetic screens (Ruegger et al., 1997; Armstrong et al., 2004; Surpin et al., 2005; Yamazoe et al., 2005; Hayashi et al., 2008).

While synthetic auxin inhibitors have been immensely informative, there are potential drawbacks

to using these unnatural compounds. They may work in ways that differ from endogenous inhibitors, and potential xenobiotic responses to the foreign compounds can complicate analysis of their mechanism of action (Armstrong et al., 2004). Naturally occurring auxin inhibitors, on the other hand, may refine our understanding of how auxin activity is controlled in planta, and they may illuminate new components of the auxin regulatory pathway. This study was initiated to evaluate possible jasmonate activity for a series of JA-amino acid conjugates. Surprisingly, the Trp conjugates of both JA and IAA are naturally occurring auxin antagonists that interfere with a broad range of IAA-mediated processes. Trp conjugates require TIR1 for full activity, and determining how they work may shed new light on the control of auxin signaling in plants.

RESULTS

JA-Trp Interferes with Root Gravitropic Response

During experiments to examine possible jasmonate-like properties of various JA-amino acid conjugates, it was noted that JA-Trp caused roots to grow agravitropically. Figure 1 illustrates the response, and root deviation from the gravity vector was quantified as described. Although roots were grown within the agar medium and not on the agar surface, only one of the two dimensions of vertical growth was quantified here. On 25 μM JA-Trp, roots had a mean deviation of 11.8° (SE = 1.5) compared with 3.8° (SE = 0.7) for the control (Fig. 1C). The response was specific to JA-Trp, as results for JA itself and JA conjugates with nine other amino acids did not differ from the control.

The sensitivity of roots to JA-Trp was determined in a dose-response assay shown in Figure 2A. For this experiment, the naturally occurring (–) and the synthetic (+) enantiomers were first separated by HPLC from JA-Trp synthesized using the commercially available enantiomer mixture of JA. The mean divergence from vertical for (–) JA-Trp was significantly different from the control at as little as 1 μM (*t* test, one-tail, *P* = 0.008, *n* = 27), while that of (+) JA-Trp was significant at 10 μM and higher. (+) JA-Trp was modestly more active than (–) JA-Trp at the higher concentrations. As jasmonates inhibit root growth, the length of roots after 6 d growth on JA-Trp was also determined. Figure 2B shows that this conjugate slowed root growth only modestly. Even at 100 μM , growth inhibition was <50% of the control value. By comparison, typical jasmonates like JA and JA-Ile produce 50% inhibition at concentrations at least two orders of magnitude lower than this (Staswick et al., 1992; Staswick and Tiriyaki, 2004). Further experiments showed that growth inhibition by JA-Trp was partially dependent on the JAR1 enzyme that conjugates JA to Ile (Supplemental Fig. S1). This suggests that some JA-Trp is hydrolyzed, releasing JA, which is then conju-

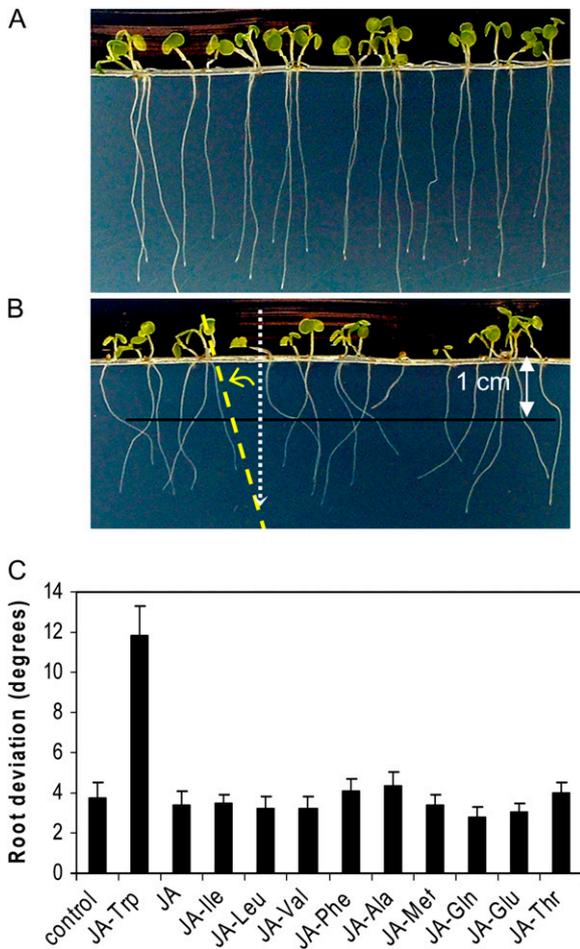


Figure 1. Arabidopsis root response to JA-Trp. A, Growth on MS medium without JA-Trp. B, Growth on a 50 μM mixture of (+)- and (-)-JA-Trp. Agravitropism was quantified by measuring the angle (curved yellow arrow) that roots deviated from the gravity vector (dotted vertical arrow) at the point each root passed through a plane 1 cm from the plane of germination. C, Effect of JA and several JA conjugates (25 μM) on gravitropic response. Vertical axis is root deviation from vertical. Values are the means of 16 to 24 seedlings with SE . JA and all of the conjugates synthesized from it were a mixture of the (+) and (-) enantiomers obtained from the commercial source of JA.

gated to Ile. Thus, JA-Trp does not appear to be a typical jasmonate signal.

Another measure of agravitropic response is the ability of seedling roots grown on control agar medium to reorient after transfer to test media and then rotating seedlings out of the original gravity vector. Figure 3 summarizes data for seedlings on four different concentrations of (\pm)-JA-Trp. Bar lengths on the circular histograms represent the proportion of seedlings growing in the indicated direction after 2 d in the dark. Relative to the control, JA-Trp at 2.5 μM interfered with the ability of seedlings to reorient to vertical, and the effect was markedly increased as the concentration of JA-Trp was increased. Together with the near normal root elongation on JA-Trp (Fig. 2B),

the evidence suggests that JA-Trp disrupts normal gravitropism and is not merely a toxin avoidance response.

Most jasmonate responses require the *COI1* jasmonate locus for activity (Devoto et al., 2005). To determine whether the agravitropic response to JA-Trp was *COI1* dependent, the *coil-17* allele was tested (Suza and Staswick, 2008). Figure 4A shows that JA-Trp at 10, 25, and 50 μM produced an agravitropic response in the mutant that was equal to or greater than that of wild-type seedlings. This is further evidence that JA-Trp is not a typical jasmonate signal and is consistent with the inability of JA-Trp to function in the molecular interaction between *COI1* and JAZ proteins (Katsir et al., 2008b). In contrast, the modest suppression of root elongation by JA-Trp was *COI1* dependent, consistent with conversion of small quantities of JA derived from this conjugate to a true jasmonate signal (Supplemental Fig. S1)

To examine whether the agravitropic activity was unique to JA-Trp, the Trp conjugates with IAA, benzoic acid, and trans-cinnamic acid (CA) were also tested. As shown in Figure 4B, Trp alone and BA-Trp had essentially no activity, while trans-CA-Trp produced only a slight increase in root deviation at 50 μM and higher. On the other hand, the response to IAA-Trp was similar to JA-Trp. The inactivity of Trp alone

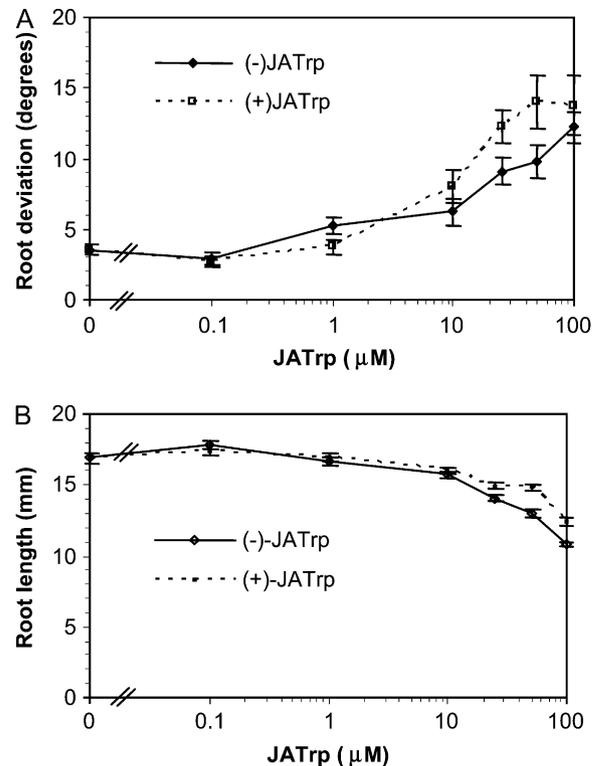


Figure 2. Root growth in the presence of (+)- and (-)-JA-Trp. A, Root deviation from the gravity vector was measured as described in Figure 1. B, Mean root length after 7 d growth of the same seedlings used for A. Values are the means with SE ($n = 26\text{--}31$ seedlings).

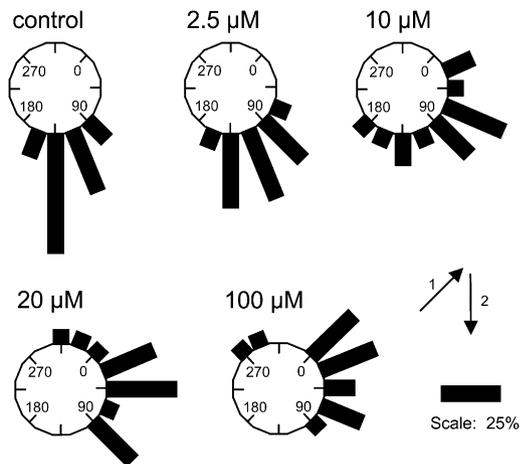


Figure 3. Effect of JA-Trp on gravitropic response. Seedlings were grown 5 d on the surface of MS agar plates in the absence of JA-Trp and then transferred to plates containing the indicated concentrations of the conjugate. Plates were rotated so that the orientation of seedlings was 135° (arrow 1) from the new gravity vector (arrow 2). Bar lengths represent the percentage of seedlings ($n = 13\text{--}15$ for each treatment) growing at the indicated orientation after 2 d growth in the dark.

establishes that the agravitropic activity is not simply due to Trp released by conjugate metabolism.

JA-Trp and IAA-Trp Antagonize Auxin-Inhibited Root Growth

Several synthetic inhibitors of gravitropic response interfere with auxin activity, and some mutants affected in auxin sensitivity are also compromised in gravitropic response (Estelle and Somerville, 1987; Hobbie and Estelle, 1995; Oono et al., 2003; Rojas-Pierce et al., 2007; Hayashi et al., 2008). To test whether JA-Trp and IAA-Trp impair auxin sensitivity, the ability of these conjugates to block IAA-inhibited root growth was evaluated. Figure 5A shows that both conjugates interfered with root inhibition by $2\ \mu\text{M}$ IAA. As little as $0.5\ \mu\text{M}$ of each conjugate significantly increased root growth (t test of means, $P < 0.01$, $n = 19$) compared with IAA alone. The maximal effect was seen at $25\ \mu\text{M}$, where roots on IAA-Trp grew to about 78% of those in the absence of auxin, while the conjugates at $50\ \mu\text{M}$ were slightly less effective. This reduction may result from hydrolysis of the respective conjugates to release small quantities of free JA and IAA, both of which inhibit root growth.

In contrast to the gravitropic response, Trp alone was active in suppressing IAA-inhibited growth (Fig. 5A). At 10 and $25\ \mu\text{M}$, the effect was modestly less than for the conjugates, but at $50\ \mu\text{M}$, Trp alone was more effective. Trp did not affect root growth in the absence of exogenous auxin (data not shown), and none of the other 19 protein amino acids diminished the response to IAA (Supplemental Fig. S2).

The ability of JA- and IAA-Trp to antagonize root inhibition by other auxins was examined. Figure 5B

shows that in addition to IAA, both conjugates suppressed inhibition by indole-3-butyric acid and 2,4-dichlorophenoxyacetic acid, but they had no effect on root inhibition by 1-naphthalenetic acid (NAA). In the absence of auxin, both JA-Trp and IAA-Trp modestly inhibited growth (possibly due to hydrolysis of the conjugates), so the antagonistic effect on auxin activity is likely underestimated in this assay.

Trp Conjugates Block IAA-Induced Lateral Root Growth and Gene Expression

The efficacy of the conjugates to inhibit IAA-stimulated lateral root growth was examined at 2, 3, and 4 d after transferring 4-d-old seedlings to media containing the compounds indicated in Figure 6A. Both conjugates at $25\ \mu\text{M}$ suppressed root production stimulated by IAA at 0.2 and $1.0\ \mu\text{M}$. Reduction in root number was also seen with conjugates at $10\ \mu\text{M}$, except that no significant difference occurred for IAA-Trp with $0.2\ \mu\text{M}$ IAA (t test, single-tailed, $P < 0.05$). Trp also inhibited root production stimulated by $1\ \mu\text{M}$ IAA, albeit less effectively than JA-Trp. When Trp was combined with $0.2\ \mu\text{M}$ IAA, there was no significant

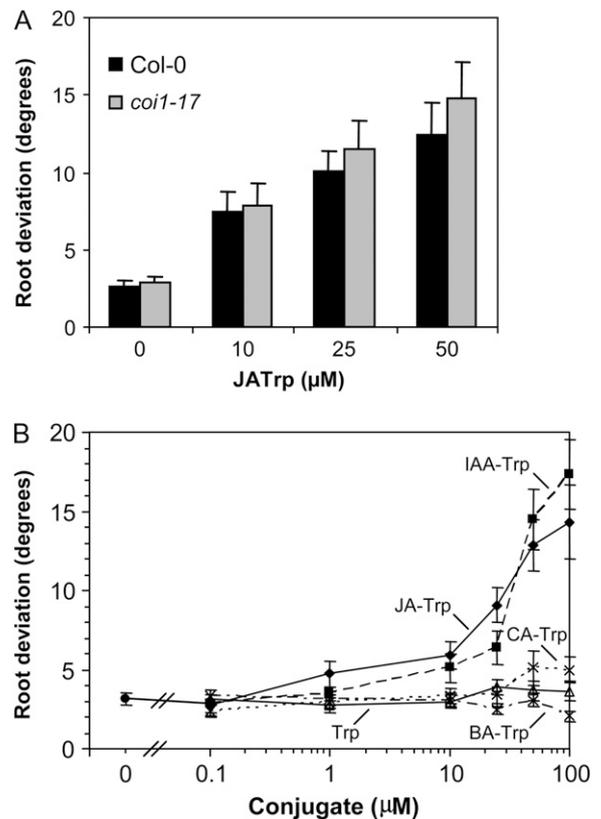


Figure 4. Gravitropic response in *coi1-17* and effect of other Trp conjugates. A, Assay of *coi1-17* and Col-0 was as described in Figure 1. Bars are the means of 16 to 21 seedlings with SE indicated. B, Induction of agravitropism by various Trp conjugates or Trp at indicated concentrations. Values are means ($n = 22$, SE indicated).

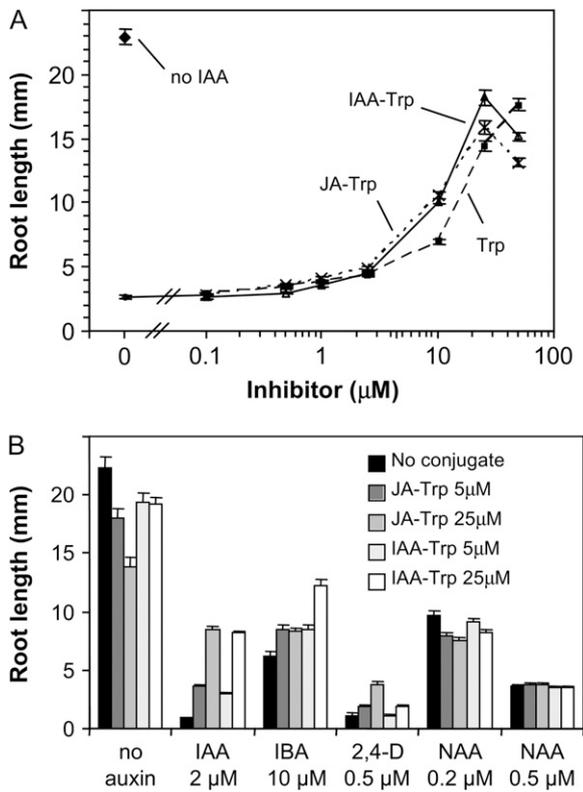


Figure 5. Suppression of auxin-inhibited root growth by JA-Trp and IAA-Trp. A, Seedling root length was measured after 7 d of growth on MS agar plates with 2 μM IAA plus the indicated amount of conjugate or Trp. Values are the means ($n = 19$) with se. B, Efficacy of Trp conjugates with other auxins was tested as above with auxins at the indicated concentrations with the indicated conjugates. Root lengths were measured after 8 d of growth, and values are the means ($n = 21$ seedlings) with se. IBA, Indole-3-butyric acid; 2,4-D, 2,4-dichlorophenoxyacetic acid.

effect compared with IAA alone. However, at 25 μM , this amino acid alone actually stimulated root production to about the same level as did 0.2 μM IAA.

Conjugates were also tested for their ability to inhibit IAA-induced expression in the Arabidopsis DR5-GUS reporter line (Ulmasov et al., 1997). Conjugates were added at 25 μM along with the indicated concentrations of IAA. Figure 6B shows that after 5 h of induction, modest suppression of IAA-stimulated GUS activity was seen for both the 0.2 and 1.0 μM IAA treatments (t test, single-tailed, $P < 0.05$). Trp produced no significant effect with 0.2 μM IAA but did reduce the induction seen with 1.0 μM IAA. Altogether, the results establish that exogenous JA-Trp and IAA-Trp interfere with a wide spectrum of auxin-mediated physiological responses.

JA-Trp and IAA-Trp Are Synthesized in Arabidopsis

Addition of Trp to media containing IAA (Fig. 5A) strongly suppressed the root-inhibiting effect of IAA, suggesting that IAA-Trp may be synthesized in these

roots. To test this possibility, roots supplied with IAA or JA along with Trp for 16 h were extracted and the conjugates quantified by gas chromatography/mass spectrometry (GC/MS). Table I shows that control roots contained 1.7 pmol g^{-1} fresh weight (FW) of both JA-Trp and IAA-Trp. Although considerably lower than the amount of free IAA and JA, these values were comparable to the basal level of the jasmonate signal JA-Ile. The level of JA-Trp and IAA-Trp in roots incubated in either JA or IAA along with Trp rose about 10-fold to 17.2 and 23.5 pmol g^{-1} FW, respectively. Interestingly, IAA-Trp also increased about 5-fold in the JA + Trp treatment, indicating that endogenous IAA was conjugated when additional Trp was available. This confirms that roots contain both conjugates, and they have the capacity to synthesize

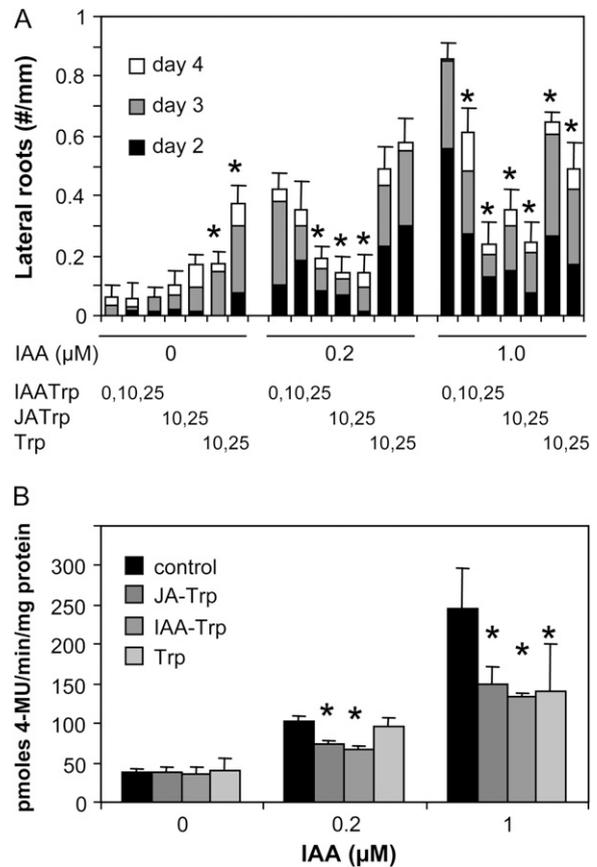


Figure 6. Suppression of IAA-promoted lateral root growth and gene expression by inhibitors. A, Bar segments indicate the incremental increase in lateral roots on days 2, 3, and 4 after transfer of 4-d-old seedlings to agar plates containing the indicated concentrations (μM) of IAA, Trp, and conjugate (means, $n = 10-12$ seedlings). se bars are for the total root numbers on day 4, and asterisks indicate significant difference from the control after 4 d for each IAA treatment (t test, single-tailed, $P < 0.05$). B, Five-day-old DR5-GUS reporter line seedlings were transferred to liquid MS medium for 6 h containing indicated concentrations of IAA with Trp, JA-Trp, or IAA-Trp at 25 μM . Values are the means of three replicates of 12 to 15 seedlings each with se. Asterisks indicate means significantly different from the control for each IAA concentration (t test, single-tailed, $P < 0.05$).

Table 1. Quantitation of jasmonates and auxins in *Arabidopsis* tissues

nd, Not detected. Values are the means of three or four independent tissue extractions with SE in parentheses.

Treatment ^a	JA-Trp	IAA-Trp	IAA	JA	JA-Ile
<i>pmol/g FW</i>					
Root					
Control	1.7 (0.3)	1.7 (0.5)	46.6 (8.5)	23.0 (1.5)	3.7 (1.2)
IAA + Trp	0.8 (0.4)	23.5 (3.1)	–	26.8 (3.7)	2.7 (1.1)
JA + Trp	17.2 (1.5)	10.5 (2.2)	60.3 (4.2)	–	6.9 (0.8)
Seedling					
Light	1.2 (0.3)	nd	2.9 (0.3)	2.9 (0.6)	5.4 (1.5)
Dark	1.0 (0.3)	nd	4.7 (0.9)	4.0 (0.8)	3.0 (1.5)
Leaf					
Mature	0.2 (0.02)	nd	3.8 (1.4)	78.6 (40.5)	3.8 (1.4)
Mature wound	0.7 (0.02)	nd	3.4 (0.5)	2,064.2 (429)	348.5 (19.7)
Expanding	nd	5.8 (2.8)	16.3 (0.9)	18.3 (3.6)	7.2 (0.7)
Flower	nd	2.8 (0.6)	62.1 (2.5)	47.4 (10.1)	8.1 (1.8)

^aRoots were grown 3 to 4 weeks in MS liquid media followed by 16-h incubation in the same or with IAA or JA at 2 μM and Trp at 25 μM added, as indicated. Seedlings were grown on filter paper with MS liquid media for 5 d in continuous light or continuous dark. Leaves and flowers were from plants grown in soil. Mature leaves were wounded with a hemostat and harvested 1 h later. Expanding leaves were ≤ 1 cm in length. Detection limit was 0.1 and 0.5 pmol/g FW for JA-Trp and IAA-Trp, respectively.

higher levels when the appropriate substrates are provided.

The amount of Trp conjugates was also examined for several other tissues. Seedlings grown 5 d in either light or dark had low levels of JA-Trp similar to control roots but no detectable IAA-Trp (Table I). In contrast, expanding leaves and flowers had small amounts of IAA-Trp but no detectable JA-Trp. To test whether the amount of JA-Trp might be limited by the availability of endogenous JA, mature leaves were wounded and analyzed after 60 min. As expected, wounding markedly increased both JA and JA-Ile, while JA-Trp increased from only 0.2 to 0.7 pmol g⁻¹ FW. Although this increase was significant (*t* test, two-tailed, $P = 0.0001$, $n = 4$), the low level of JA-Trp even when a large amount of JA is present suggests that JA availability is not the sole limitation to its accumulation in leaves. Analysis of leaves 10 and 240 min after wounding did not yield altered Trp conjugate levels (data not shown).

JA Biosynthesis and Trp-Hyperaccumulating Mutants Have Altered IAA Sensitivity

If endogenous JA-Trp helps regulate growth, then mutants defective in the production of JA might be more sensitive to IAA. The JA biosynthesis triple mutant *fad3-2 fad7-2 fad8* (McConn and Browse, 1996) and *opr3*, which is defective in conversion of the JA intermediate 12-oxophytodienoic acid (Stintzi and Browse, 2000), were evaluated for root growth in the presence of 0.1, 0.25, and 0.5 μM IAA. Figure 7A shows that at all three concentrations the *fad* mutant grew less than its wild-type counterpart, which is Columbia-0 (Col-0). This effect was not dependent on the jasmonate signaling pathway because *coi1-17* did not show

diminished growth on IAA. The genetic background for *opr3* is the Wassilewskija ecotype, and it also displayed decreased growth compared to the corresponding wild type. Although the effect seen here is modest, these results suggest that endogenous root JA-Trp is involved in regulating response to exogenous auxin.

The ability of increased endogenous Trp to affect auxin resistance was tested in the *trp5* mutant, which accumulates soluble Trp about 3-fold above wild-type levels due to altered feedback regulation of anthranilate synthase (Li and Last, 1996). Figure 7B shows that over a range of 0.05 to 0.2 μM , *trp5* was more resistant to the root inhibiting effects of IAA than was the wild type, while at higher concentrations, no difference was seen. This result supports the idea that the additional endogenous Trp in the mutant is available for conjugation to IAA, thereby providing increased resistance to auxin.

Trp Conjugate Activity Does Not Require Auxin Transport

The inability of the Trp conjugates to suppress NAA-inhibited root growth (Fig. 5B) might indicate that these conjugates act at the level of auxin transport, since NAA cellular import is independent of an influx carrier (Delbarre et al., 1996). The conjugates were tested for their effect on lateral root development, which is strongly suppressed when the transport inhibitor NPA is applied to the shoot-root junction (Rashotte et al., 2000). Four-day-old seedlings were transferred to new media, and 100 μM JA-Trp, IAA-Trp, or NPA were applied in agar. Table II shows that after 6 d, lateral root production was greatly impaired

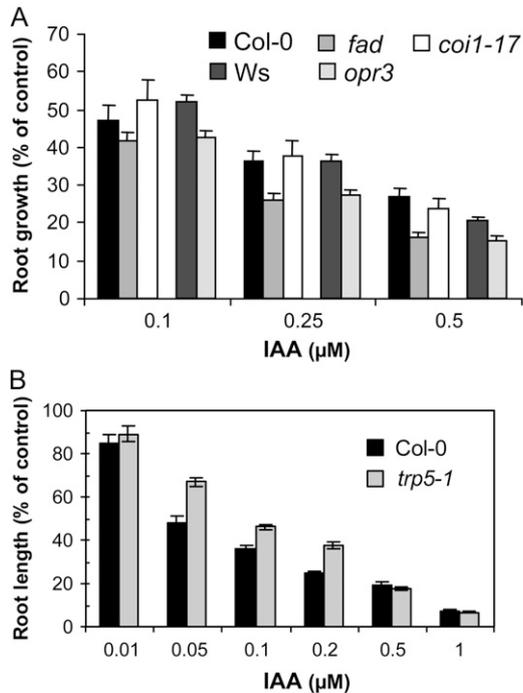


Figure 7. Sensitivity of mutants to IAA. A, Seedling root length of jasmonate biosynthesis mutants (*fad3-2 fad7-2 fad8* and *opr3*) and signaling mutant (*coi1-17*) after 7 d of growth on IAA. The genetic background for *opr3* is Wassilewskija (Ws) and for the other two mutants is Col-0. Values are expressed as percentage of the length when each genotype was grown on control medium ($n = 13\text{--}15$ seedlings). Error bars represent 95% confidence interval for each ratio of the means. B, Growth of the Trp-overaccumulating mutant *trp5-1* on IAA. Values are expressed as in A and determined after 6 d of growth ($n = 25$ seedlings).

by NPA, as expected. In contrast, treatment with the Trp conjugates actually enhanced the number of roots modestly. Also consistent with earlier findings (Rashotte et al., 2000), NPA modestly reduced root growth by about 36%, whereas the conjugates had no effect on growth. This evidence indicates that JA-Trp and IAA-Trp do not act by the same mechanism as the auxin transport inhibitor NPA.

AUX1 is required for auxin transport, and *aux1* mutants are resistant to exogenous auxin and strongly agravitropic (Marchant et al., 1999). To further examine whether the Trp conjugates act at the level of auxin transport, the sensitivity of *aux1-7* to IAA-Trp was examined. JA-Trp was not evaluated here because it inhibits growth to a greater extent than IAA-Trp (Fig. 5B), thereby masking the suppression of IAA inhibition. Figure 8A shows that even though the mutant root growth is strongly resistant to IAA, IAA-Trp at 10 and 25 μM still reduces inhibition caused by up to 3 μM IAA. Although the effect of IAA-Trp was not as striking as for the wild type, 25 μM IAA-Trp completely abolished IAA inhibition for *aux1-7* roots grown on 1 and 2 μM IAA. This suggests that IAA-Trp does not act at the level of AUX1-mediated auxin transport.

JA-Trp Requires TIR1 for IAA Inhibition

TIR1 is a critical auxin receptor, so its role in the activity of JA-Trp was evaluated in *tir1-1*. Figure 8B shows that in the absence of IAA root length of *tir1-1* did not differ from Col-0 when grown on 0, 10, or 25 μM JA-Trp. This mutant is modestly resistant to IAA, and yet *tir1-1* roots were only 56% to 72% of the wild-type length when JA-Trp at 10 or 25 μM was included along with 0.2 to 1 μM IAA. The decreased efficacy of the conjugate in *tir1-1* indicates that TIR1 signaling is required for maximal JA-Trp activity.

One way that JA-Trp and IAA-Trp might act is to compete directly with IAA for binding of TIR1. This was tested in an in vitro pull-down assay that uses a glutathione *S*-transferase (GST)-Aux/IAA7 fusion protein to select SCF^{TIR1} from cell extracts of transgenic plants that carry a myc-tagged TIR1 (Dharmasiri et al., 2003). As seen in Figure 8C, 0.2 and 1 μM IAA effectively stimulated the interaction. However, adding either conjugate to the reaction at 40 μM had no effect on the ability of IAA to promote interaction between TIR1 and Aux/IAA7.

DISCUSSION

With the exception of the well-established role of JA-Ile as a hormonal signal, amino acid conjugates of both JA and IAA have been regarded as inactive metabolites of the hormones. This study identifies an unexpected function for JA-Trp and IAA-Trp as endogenous inhibitors of several physiological responses to auxin. The activity of IAA-Trp is particularly interesting because synthesis of this conjugate not only removes free IAA from the active auxin pool, as for IAA conjugation to other amino acids, but also converts it to an antagonist of any remaining IAA. Thus, IAA-Trp is a kind of "super inactivator" of IAA.

JA-Trp was first identified in *Vicia faba*, particularly in flowers and fruits, and it was reportedly elevated in asparagus (*Asparagus officinalis*) shoots following harvest and during senescence (Brückner et al., 1988; Gapper et al., 2002). JA-Trp had little or no ability to induce genes that were activated by JA or JA-Ile in

Table II. Effect of Trp conjugates on root growth and lateral root number

Root length is the amount of new growth 4 d after treatment began, and lateral roots were counted 6 d after treatment. Values are the means of 12 plants with SE in parentheses.

Treatment ^a	Root Length	Lateral Roots
	mm	no.
Control	21.8 (1.5)	4.2 (0.7)
NPA	13.8 (0.7)	0.3 (0.2)
JA-Trp	21.3 (1.2)	6.4 (0.5)
IAA-Trp	22.5 (1.1)	6.4 (0.7)

^aTreatments were 100 μM of indicated compound applied in agar to the shoot-root junction of 4-d-old seedlings.

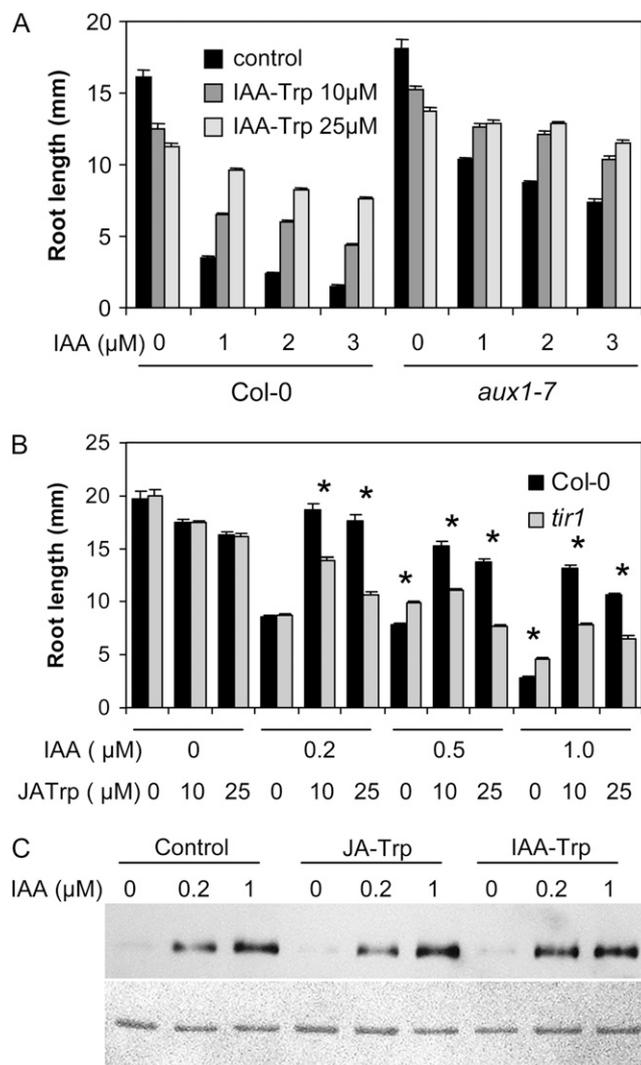


Figure 8. Role of AUX1 and the TIR1 in mediating Trp conjugate activity. A and B, Root lengths for *aux1* and *tir1* were determined 5 and 7 d, respectively, after growth in medium containing the indicated amounts of IAA and Trp conjugates. Values are the mean of 17 and 19 seedlings for experiments with *aux1* and *tir1*, respectively, with se indicated. Asterisks in B indicate means that differed significantly between the two genotypes for a given treatment (*t* test, $P < 0.01$). C, Pull down of TIR1-myc with GST-Aux/IAA7. IAA was added to the interaction mix at the concentrations indicated, and JA-Trp or IAA-Trp was added at 40 mM. The bottom panel is a loading control for Aux/IAA7 stained with Ponceau Red.

barley (*Hordeum vulgare*), suggesting it was not a jasmonate signal (Kramell et al., 1997; Miersch et al., 1999). That JA-Trp is not a typical jasmonate signal is supported here because it only minimally inhibits root growth compared with JA-Ile. Conversely, JA and JA-Ile do not produce an agravitropic root response like the Trp conjugate, and agravitropism was COI1 independent, contrary to most characterized jasmonate responses. These results also agree with recent biochemical studies showing that unlike JA-Ile, JA-Trp does not promote SCF^{COI1} interaction with JAZ proteins

(Thines et al., 2007). On the other hand, some JA-responsive Arabidopsis genes are regulated independently of COI1 (Devoto et al., 2005). The mechanism is unknown, but it seems plausible that conjugation of JA to Trp might be involved, which could then alter gene expression through an auxin signaled pathway.

Endogenous JA-Trp and IAA-Trp

Although JA-Trp and IAA-Trp were detected at low levels or not at all in some Arabidopsis tissues, they may still have a significant role. Auxin activity is highly regulated by a complex network of interacting mechanisms, and endogenous auxin antagonists might be expected to remain low in most cells under conditions of normal growth. Higher concentrations might occur in response to specific environmental stimuli or in localized tissues. For this study, conjugates were extracted from whole roots, seedlings, and leaves, which would dilute higher concentrations that might occur at more restricted sites of accumulation.

The increase in auxin sensitivity in JA synthesis mutants (Fig. 7A) supports, although does not prove, that endogenous JA-Trp is functional. The defect in these mutants was modest, but the loss might be partially compensated for by IAA-Trp or by other endogenous auxin inhibitors. Interestingly, a role for JA in gravitropic response was previously found in rice (*Oryza sativa*) coleoptiles, which accumulated a gradient of JA that was opposite the IAA gradient that formed under gravistimulation (Gutjahr et al., 2005). Flooding coleoptiles with JA delayed the onset of gravitropic bending, and the jasmonate-deficient *hebiba* mutant responded more slowly to gravity than did wild-type rice coleoptiles. It would be interesting to know whether JA-Trp also accumulates asymmetrically in these rice coleoptiles, as this seems a plausible hypothesis to explain how JA functions in the observed gravity response.

Substrate Availability May Limit Conjugate Synthesis

Arabidopsis roots synthesized excess IAA-Trp and JA-Trp when the appropriate substrates were provided to roots, indicating that substrate availability limits conjugate production under normal conditions. Trp appears limiting because addition of this amino acid to media containing IAA decreased root sensitivity to auxin essentially as effectively as did IAA-Trp itself. Furthermore, roots incubated in only JA and Trp produced IAA-Trp in addition to JA-Trp, indicating that endogenous IAA was available for conjugation. The Trp-overaccumulating mutant *trp5* was also more resistant to IAA than the wild type, possibly due to increased IAA-Trp production. On the other hand, wounding leaves to markedly increase endogenous JA only minimally increased JA-Trp, consistent with a limitation in Trp availability. Previous evidence that amino acid availability influences the IAA conjugate spectrum comes from the Arabidopsis Gln-overaccu-

mutating mutant *gluS*, which had elevated levels of IAA-Gln in place of IAA-Asp (Barratt et al., 1999). Synthesizing IAA-Trp under cellular conditions where Trp is abnormally high might be a particularly effective strategy since as a precursor for IAA synthesis, Trp overabundance might also lead to excess auxin.

Presently, we do not know which enzymes are involved in Trp conjugate synthesis. Members of the GH3 family seem likely candidates because in vitro assays indicate they conjugate JA and IAA to several amino acids, including Trp (Staswick and Tiryaki, 2004; Staswick et al., 2005). Preliminary phenotypic analysis of several GH3 mutants did not identify obvious candidates for the Trp conjugating enzymes that function in vivo (data not shown), but redundancy among the IAA-conjugating family members may require the evaluation of multigene mutants. Functional redundancy between IAA-Trp and JA-Trp could also complicate the genetic identification of the conjugating enzymes that are involved.

Trp Conjugate Activity

Trp appears to be a structural requirement for an effective conjugate because nine other amino acid conjugates of JA were ineffective promoters of agravitropism and no amino acid other than Trp suppressed the root inhibiting activity of IAA. On the other hand, JA-Trp and IAA-Trp produced similar physiological responses, even though JA and IAA are structurally quite diverse. BA-Trp and CA-Trp were essentially inactive, suggesting that there is structural specificity for this component, although it is possible that uptake of these conjugates was ineffective or that they were inactivated in vivo by a mechanism that does not affect the active Trp conjugates. Because two diverse Trp conjugates were active, it would not be surprising if other related compounds are also auxin antagonists. Initial results indicate that dihydro JA-Trp is at least as active as JA-Trp in the agravitropic response (data not shown). Certain hydroxylated jasmonates are abundant in plants, and they also might be conjugated to form active Trp conjugates (Miersch et al., 2008). Trp conjugates of other compounds related to IAA could also exist and may contribute to regulation of auxin activity.

The amount of exogenous Trp conjugates necessary for activity is considerably higher than the activity range for exogenous IAA. For example, IAA is strongly inhibitory to root growth at 1 μM , while 10- to 50-fold more of the Trp conjugates was required to markedly counter this auxin activity. However, the observed activity is generally consistent with other auxin inhibitors that have been investigated (Oono et al., 2003; Armstrong et al., 2004; Yamazoe et al., 2005; Rojas-Pierce et al., 2007; Hayashi et al., 2008). Low activity could also result if JA-Trp and IAA-Trp were metabolized after uptake by plants. Enzymatic hydrolysis of IAA amide conjugates is known to release free IAA (Rampey et al., 2004). However, the characterized

Arabidopsis auxin conjugate hydrolases have relatively low activity on IAA-Trp (LeClere et al., 2002), and the fact that IAA-Trp only weakly inhibited root growth (Fig. 5B) suggests that little free IAA was derived from this conjugate. It would not be surprising if plants had other mechanisms to limit excess accumulation of the Trp conjugates, such as the oxidative catabolism of IAA-Glu (Ljung et al., 2002).

This study suggests that JA is released from JA-Trp because root inhibition by this conjugate was dependent on both JAR1 and COI1. The higher agravitropic activity of (+)-JA-Trp and its lower root inhibiting activity relative to (-)-JA-Trp could arise if the naturally occurring conjugate was a better substrate for enzymatic cleavage. Specific hydrolases active on JA amide conjugates have not been reported, although the auxin conjugate hydrolase IAR3 also apparently acts on JA conjugates (LeClere et al., 2002). As for auxin conjugates, other inactivating modifications of JA-Trp are also possible.

Mechanism of Action

The mechanism for Trp conjugate activity is still unclear. The greater IAA sensitivity of *tir1-1* roots when grown with JA-Trp (even though this mutant is resistant to IAA; Fig. 8B), indicates that the TIR1 signaling pathway is required. However, the conjugates did not interfere with IAA in the pull-down assays even at a 200-fold molar excess over IAA. Inactivity in a similar assay was also found for terfestatin A, an auxin inhibitor from *Streptomyces* sp. F40 (Yamazoe et al., 2005). On the other hand, alkyl-substituted IAAs antagonized the activity of IAA in the interaction assay (Hayashi et al., 2008). It is possible that the Trp conjugates interfere with TIR1 activity in plant cells. Only one of 29 Arabidopsis Aux/ IAA proteins was examined here, and the conjugates might affect TIR1 interaction with other Aux/IAAs differently. Conjugates may also require interaction with other cellular components for activity, and these may be too dilute in the extracts used for pull-down experiments. Alternatively, the antagonism might occur elsewhere in the TIR1 signaling pathway. The conjugates do not appear to affect the auxin transport mechanisms involving AUX1 or NPA-sensitive transport. We are currently analyzing mutants with diminished response to JA-Trp to elucidate the mechanism for Trp conjugate activity.

Auxin antagonists vary markedly in how they affect plant growth. For example, a synthetic auxin inhibitor having an alkyl substitution at the α -position of IAA stimulated root growth markedly, which was attributed to the inhibition of endogenous auxin (Hayashi et al., 2008). In contrast, PCIB suppressed root growth, and this was dependent on TIR1 and Aux/IAA7, suggesting it was not a xenobiotic effect (Oono et al., 2003). PCIB action was hypothesized to be the result of either a direct interference with IAA binding of TIR or a disruption of auxin homeostasis leading to excess IAA production (Biswas et al., 2007). In this study, JA-

Trp and IAA-Trp did not promote root growth at any concentration tested, and the weak inhibitory activity observed was likely due largely to JA and IAA activity, as described earlier.

Coordination among hormone signaling paths is a common theme in plants, and shared functions among jasmonate and auxin signaling components are well known (Schwechheimer et al., 2002; Tiryaki and Staswick, 2002; Gray et al., 2003). Jasmonates are integral to normal flower development, and both JA production in flowers and flower development require the ARF6 and ARF8 transcription factors. JA methylester also alters flux through the Trp pathway and increases the level of IAA in Arabidopsis (Nagpal et al., 2005; Dombrecht et al., 2007). The antagonistic activity of JA-Trp on IAA adds to this complex list of mechanisms by which the JA and IAA pathways intersect to coordinate growth, development, and stress response.

MATERIALS AND METHODS

Plant Materials, Growth, and Biochemical Assays

Arabidopsis (*Arabidopsis thaliana*), ecotype Col-0, was used in all experiments except as noted. The mutants *trp5* (Li and Last, 1996) and *tir1-1* (Ruegger et al., 1997) were obtained from the Arabidopsis Biological Resource Center (Columbus, OH), *coi1-17* and *jar1-1* were previously described (Staswick et al., 1992; Suza and Staswick, 2008), and the triple mutant *fad3-2 fad7-2 fad8* (McConn and Browse, 1996) and *opr3* (Stintzi and Browse, 2000) were from J. Browse. The DR5-GUS reporter line was provided by T. Guilfoyle (Ulmasov et al., 1997). Seedlings were sown on agar media containing the compounds indicated using surface-sterilized seeds essentially as previously described (Staswick and Tiryaki, 2004). Growing roots within the agar by germinating seeds on slots cut into the Murashige and Skoog (MS) media (half-strength MS, pH 6.0, 0.5% Suc, and 1% agar [w/v]) was found to produce roots that grew straightest in the absence of added hormones. Hormones or conjugates were added from 1,000-fold stock solutions in ethanol immediately before pouring plates. Plates containing seeds were incubated at 4°C for 2 to 4 d and then placed on edge in a Percival plant culture chamber and grown at 22°C under 16-h-fluorescent-light/8-h-dark cycles. Root lengths were measured from the point of germination to the root tip. When root length of genotypes differed on control plates, the treatment values were expressed as a percentage of the control length for each genotype, and confidence intervals (95%) were calculated using the delta method. Reorientation of roots to the new gravity vector (Fig. 3) was tested as described by Oono et al. (2003) except that MS medium was used. DR5-GUS experiments were carried out as described by Oono et al. (2003), and fluorimetric determination of enzyme activity followed the methods of Jefferson (1987). Transport inhibitor studies were done according to Rashotte et al. (2000) as described in Table II. Each experiment was done at least three times.

Tissue for hormone extraction was grown as indicated in Table I. Mature plants were grown in Redi Earth (W.R. Grace) in plastic pots in a Conviron growth chamber under the light (approximately 100 $\mu\text{E m}^{-2} \text{s}^{-2}$) and temperature conditions described above. Harvested tissue was quickly frozen in liquid N and then ground to a powder and stored at -80°C.

Pull-down assays were performed essentially as described by Dharmasiri et al. (2003). Reactions with 800 μg total protein extracted from TIR1-myc seedlings were reacted with glutathione agarose-bound GST-Aux/IAA7 protein in 500 μL of homogenization buffer (50 mM Tris-HCl, pH 7.2, 100 mM NaCl, 0.1% Tween 20 [v/v], 10 μM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μM MG132, and miniprotease inhibitor cocktail [Roche]). After rotating 30 min at 4°C, agarose beads were washed three times for 4 min in 1 mL of homogenization buffer minus protease inhibitors. Agarose-bound proteins were dissolved in SDS-PAGE sample buffer, electrophoresed, and transferred to nitrocellulose as described earlier (Staswick, 1989). Immunodetection of TIR1-myc was with an anti-c-myc antibody (Roche) using a Supersignal West Pico chemiluminescent detection kit as described by the manufacturer (Thermo Scientific).

Synthesis of Amino Acid Conjugates

Hormones and other chemicals were from Sigma-Aldrich. Amino acid conjugates were prepared by mixed anhydride condensation reactions essentially as outlined earlier, except that acetonitrile replaced tetrahydrofuran in the reaction (Kramell et al., 1988, 1999; Staswick and Tiryaki, 2004). Conjugates were separated from precursors and side products by silica gel chromatography (1.5 \times 50 cm) in chloroform:ethyl acetate:acetic acid (14:6:1 or 3:5:1 [v/v]). Organic solvents were evaporated under a stream of nitrogen from the appropriate column fractions identified by thin-layer chromatography. Conjugates were further purified on reverse phase C18 solid phase extraction columns (Burdick and Jackson; 500 mg, 8 mL) as described earlier (Staswick and Tiryaki, 2004). The two enantiomers of JA-Trp were separated on a semipreparative HPLC column (Luna 5 μ C18, 250 mm \times 15 mm; Phenomenex) using isocratic 75% methanol (v/v) at 3 mL min⁻¹. Purity and structure of each conjugate was verified by gas GC/MS following derivatization with diazomethane. The spectra for JA-Trp was consistent with that reported previously (Kramell et al., 1988), and that of IAA-Trp agreed with a standard provided by B. Bartel (LeClere et al., 2002). BA-Trp and CA-Trp produced molecular masses on GC/MS that agreed with the expected values for the derivatized compounds. JA conjugates with other amino acids were either described previously (Staswick and Tiryaki, 2004) or synthesized here by similar methods. Internal stable isotope standards for conjugate quantitation by GC/MS were synthesized by scaling down the above protocol and using [¹³C₆]IAA for IAA-Trp or [D₆]Trp for JA-Trp (Cambridge Isotope Laboratories). Products gave the expected modified mass spectra compared to the nonisotopic compounds. The other internal standards used were previously described (Staswick and Tiryaki, 2004; Staswick et al., 2005).

Extraction and Quantitation of Jasmonates from Tissue

Solvents were HPLC grade, and chloroform contained 1% ethanol (v/v) as stabilizer. The general methods of Kramell et al. (2000) were followed with modifications as described. Weighed frozen tissue (0.2–0.4 g) was added to 7 mL of 85% (v/v) methanol with butylated hydroxytoluene (100 ng mL⁻¹) and appropriate quantities of internal standards. Tissue was ground 1 min on high speed with an Omni tissue homogenizer (Omni International) and then centrifuged 3 min at 6,000 rpm. Diethylaminoethyl Sephadex A-25 (Sigma-Aldrich) was equilibrated in 0.5 M sodium acetate in methanol and then washed thoroughly with methanol. Ion exchange was performed with a 2-mL bed volume of DEAE resin in a 10-mL disposable syringe with a filter paper frit to retain the resin. After sample loading, columns were washed with 12 mL of methanol and then eluted with 14 mL of 12% formic acid (v/v) in methanol. The eluate was dried in a stream of nitrogen at 48°C. Residue was dissolved in 800 μL of methanol, dried, dissolved in 300 μL of 5% HCl (v/v), and extracted with an equal volume of chloroform. The extract was dried and derivatized in 50 μL of acetone with 1 μL of 1-ethylpiperidine and 5 μL of 2,3,4,5,6-pentafluorobenzyl bromide for 30 min at 55°C as described (Epstein and Cohen, 1981). After drying, the residue was dissolved in 150 μL of chloroform and applied to a mini column with 2 mL of bed volume of silica equilibrated in chloroform. The column was washed with 2 mL of hexane and then eluted with 2 mL of ethyl acetate. After drying, the sample was resuspended in 100 μL of ethylacetate for analysis by GC/MS using negative chemical ionization. A Finnigan Trace GC with an Rtx 5MS column (15 m \times 0.25 mm, 0.1 μm) from Restec coupled to a DSQ mass spectrometer was used for the analysis. The injector port was at 280°C, and the gradient for column temperature changes was 25°C min⁻¹. The start temperature of 150°C was held for 1 min, 195°C for 4 min, 245°C for 5 min, and 320°C for 8 min. R_t for pentafluorobenzyl esters of JA-Trp and IAA-Trp were 18.36 and 19.55 min, respectively. The reagent gas was methane with a source temperature of 200°C, and the instrument was operated in selected ion monitoring mode. Frequent cleaning of the ion volume, lenses, and injector liner was necessary for maximal sensitivity of Trp conjugates. Quantitative data were obtained by comparing the integrated peak areas for the respective molecular ion of each compound and the internal standards.

Supplemental Data

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

Supplemental Figure S1. Effect of JA-Trp on root growth of *coi1-17* and *jar1-1*.

Supplemental Figure S2. Growth of Arabidopsis roots on IAA in presence of amino acids.

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