Xenobiotic- and Jasmonic Acid-Inducible Signal Transduction Pathways Have Become Interdependent at the Arabidopsis *CYP81D11* Promoter[1][C][W]

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Plants modify harmful substances through an inducible detoxification system. In Arabidopsis (*Arabidopsis thaliana*), chemical induction of the cytochrome P450 gene *CYP81D11* and other genes linked to the detoxification program depends on class II TGA transcription factors. *CYP81D11* expression is also induced by the phytohormone jasmonic acid (JA) through the established pathway requiring the JA receptor CORONATINE INSENSITIVE1 (COI1) and the JA-regulated transcription factor MYC2. Here, we report that the xenobiotic- and the JA-dependent signal cascades have become interdependent at the *CYP81D11* promoter. On the one hand, MYC2 can only activate the expression of *CYP81D11* when both the MYC2- and the TGA-binding sites are present in the promoter. On the other hand, the xenobiotic-regulated class II TGA transcription factors can only mediate maximal promoter activity if TGA and MYC2 binding motifs, MYC2, and the JA-isoleucine biosynthesis enzymes DDE2/AOS and JAR1 are functional. Since JA levels and degradation of JAZ1, a repressor of the JA response, are not affected by reactive chemicals, we hypothesize that basal JA signaling amplifies the response to chemical stress. Remarkably, stress-induced expression levels were 3-fold lower in *coi1* than in the JA biosynthesis mutant *dede2-2*, revealing that COI1 can contribute to the activation of the promoter in the absence of JA. Moreover, we show that deletion of the MYC2 binding motifs abolishes the JA responsiveness of the promoter but not the responsiveness to COI1. These findings suggest that yet unknown cis-element(s) can mediate COI1-dependent transcriptional activation in the absence of JA.

In plants, reactive chemicals released by humans, neighboring plants, or pathogenic microbes are inactivated by an inducible set of detoxifying enzymes that modify and eliminate these compounds in three steps. First, hydroxylases, cytochrome P450 monooxygenases, or peroxidases introduce reactive side groups. In the second step, these are conjugated to sugar moieties or glutathione by either glycosyl transferases or glutathione *S*-transferases. Finally, the conjugates are transported to the vacuole or to the apoplast (Sandermann, 1992).

The molecular mechanisms leading to the activation of the detoxification program are only poorly explored. Many studies have pointed at members of the bZIP family of TGA transcription factors to mediate xenobiotic-induced transcriptional activation (Pascuzzi et al., 1998; Klinedinst et al., 2000; Baerson et al., 2005; Fode et al., 2008; Mueller et al., 2008). Moreover, the TGA-interacting GRAS protein SCARECROW-LIKE14 (SCL14) is required for the induction of a subset of detoxification genes in Arabidopsis (*Arabidopsis thaliana*; Fode et al., 2008). Still, the primary events leading to the activation of TGA/SCL14-regulated promoters after xenobiotic stress have remained unknown.

One of the direct target genes of the TGA/SCL14 complex is *CYP81D11* (At3g28740; Fode et al., 2008), a putative cytochrome P450 monooxygenase gene that is induced by reactive chemicals, including the allelochemical benzoxazolin-2(3H)-one (BOA; Baerson et al., 2005), the herbicide 2,4-dichlorophenoxyacetic acid (Fode et al., 2008), the herbicide safener benoxacor (Baerson et al., 2005), and the auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA; Fode et al., 2008). Furthermore, *CYP81D11* transcription is activated by a variety of nonhormone lipid peroxidation products like phytoprostanes, oxophytodienoic acid (Mueller et al., 2008), and cis-jasmone (Bruce et al., 2008; Matthes et al., 2010). Although the substrate of CYP81D11 is not known, its presumed function as a monooxygenase and its expression pattern suggest a role in plant detoxification processes (Fode et al., 2008; Mueller et al., 2008). Another function was unraveled by behavioral choice experiments with the aphid parasitoid *Aphidius ervi*, which was preferentially attracted to transgenic plants overexpressing CYP81D11. It was hypothesized that CYP81D11 might

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be involved in the biosynthesis of volatiles serving indirect defense responses against insect pests (Bruce et al., 2008; Matthes et al., 2010).

In addition to the chemicals listed above, *CYP81D11* is activated by jasmonic acid (JA; Mueller et al., 2008), a phytohormone that accumulates upon wounding or pathogen attack. The biosynthesis pathway starts from the polyunsaturated fatty acid α-linolenic acid, which is processed by enzymes of the octadecanoid pathway (Feussner and Wasternack, 2002). Target genes of the JA pathway are under the negative control of JAZ proteins, which interact with transcriptional activators like MYC2, MYC3, or MYC4 (Chini et al., 2007; Cheng et al., 2011; Fernández-Calvo et al., 2011; Niu et al., 2011). After binding of the active JA derivative JA-Ile to the F-box protein CORONATINE INSENSITIVE1 (COI1; Yan et al., 2009), degradation of JAZ repressor proteins is initiated. According to the current model of JA signaling (Pauwels and Goossens, 2011), this allows the transcriptional activation of JA-responsive genes by the above-mentioned MYC transcription factors. Examples of direct target genes of MYC2 are JAZ repressor and lipoxygenase genes (Chini et al., 2007; Hou et al., 2010).

Here, we report that the xenobiotic- and the JA-induced signaling pathways have become interdependent at the *CYP81D11* promoter. MYC2 as well as the MYC2 binding sites are essential for JA induction. MYC2 stringently requires TGA factors to confer JA-regulated gene expression. In the context of xenobiotic-induced gene expression, TGA factors, which are considered to respond to chemical stress, are supported by MYC2 and basal JA levels. Moreover, a JA-Ile-independent COI1 function was identified that influences promoter activity even in the absence of the MYC2 binding motifs.

RESULTS

JA- and Xenobiotic-Induced Stress Pathways Have Become Interdependent at the *CYP81D11* Promoter

Previous expression studies of the *CYP81D11* gene had indicated that at least one of the class II TGA transcription factors TGA2, TGA5, and TGA6 and the JA-Ile receptor COI1 are required for efficient induction by either phytoprostanes or JA (Mueller et al., 2008). Since phytoprostanes and TGA transcription factors are associated with xenobiotic-induced stress while COI1 is associated with JA-induced responses, these results suggested that the two pathways have become interdependent at this promoter.

To investigate whether COI1 would also be involved in responses to harmful chemicals not derived from oxidized fatty acids, we treated *coi1* plants with the electrophilic halogenated phenol TIBA, which is effective with respect to activating *CYP81D11* expression (Fode et al., 2008). Indeed, induction of *CYP81D11* expression after spraying of plants with TIBA was compromised in the absence of COI1, irrespective of whether we used the well-characterized *coi1-1* allele (Xie et al., 1998; Supplemental Fig. S1) or the recently described T-DNA mutant line *coi1-t* (Mosblech et al., 2011; Fig. 1A). The insertion line exhibits phenotypic features previously reported for the *coi1-1* mutant, such as male sterility and methyl jasmonate-insensitive root growth, and was used for most of our experiments. Induction by the allelochemical BOA (Baerson et al., 2005), which is structurally different from TIBA, also depended on COI1 (Fig. 1B). TIBA induction of other TGA-requiring genes encoding glutathione *S*-transferases (*GSTU1* and *GSTU7*) and transcription factor *NAC032* (Fode et al., 2008) was not affected by the *coi1-1* allele (Supplemental Fig. S2A), indicating that the *coi1* mutant is not in all aspects TIBA insensitive. Unlike *CYP81D11*, these genes are not subject to the COI1-promoted JA pathway. Their moderate induction by JA was even enhanced in the *coi1-1* background (Supplemental Fig. S2B).

While COI1 plays an unexpected role in xenobiotic-induced signal transduction, class II TGA factors play an unexpected role in the JA pathway: This was first described when treating submersed seedlings grown in 2% Suc with JA (Mueller et al., 2008) and was reproduced in 6-week-old soil-grown plants (Fig. 1C). JA induction depends on MYC2, as revealed by real-time reverse transcription (RT)-PCR analysis of *CYP81D11* transcript levels in the TIBA-treated *jin1-1/myc2* mutant, which contains a premature stop codon in the MYC2 coding region (Fig. 1D). The importance of TGA factors for the JA pathway was not observed when other MYC2-dependent JA-induced marker genes were analyzed. For instance, MYC2-dependent genes like *VSP2* (Supplemental Fig. S3A) and *LOX2* (Zander et al., 2010) can be induced in the absence of TGA factors. Even the *GRX480* promoter, which contains MYC2 and TGA binding sites and which is not expressed in the *tga2-1 tga5-1 tga6-1* mutant after salicylic acid treatment (Ndamukong et al., 2007; Godoy et al., 2011), requires MYC2 but not TGA factors to be induced by JA (Supplemental Fig. S3).

TGA and MYC2 Binding Motifs Are Essential for JA-Induced *CYP81D11* Promoter Activity

In order to investigate whether the integration of TGA factors into the JA signal transduction network occurs directly at the *CYP81D11* promoter, we tested the functional relevance of putative TGA binding sites for JA induction. A perfect TGA target site is defined as a TGACGTCA sequence with the TGACG pentamer being sufficient for binding (Qin et al., 1994; Spoel et al., 2003). In the *CYP81D11* promoter, the sequence TGACGaCA is located between base pair positions -232 and -223 with respect to the transcriptional start site (Fig. 2A). A combination of two TGA factor recognition motifs spaced by 4 bp is found in other stress-induced promoters and is referred to as an *activation*

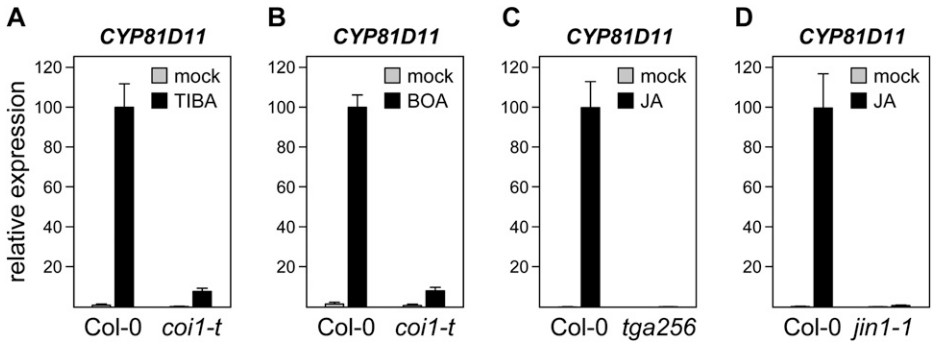

Figure 1. COI1 and class II TGA factors are required for chemical- and JA-induced *CYP81D11* expression. Quantitative RT-PCR analysis of relative *CYP81D11* transcript levels was performed after chemical stress and JA treatment. Six-week-old soil-grown plants of the indicated genotypes were either sprayed with 0.1 mM TIBA or 2 mM BOA or incubated in the presence of methyl jasmonate (see "Materials and Methods"). Whole rosettes were harvested for RNA isolation after 8 h of chemical and 24 h of JA treatment. Relative transcript levels (fold over the reference gene *UBQ5*) in treated wild-type plants were set to 100%. Mean values ± SE obtained from the following numbers of individual plants are shown: four plants per treatment, three plants for mock-treated *coi1-t* plants (A); six mock- and BOA-treated wild-type plants, three mock-treated *coi1-t* plants, eight BOA-treated *coi1-t* plants, all values originating from two independent experiments (B); three plants per treatment; the mutant *tga2-1 tga5-1 tga6-1* lacking all three class II TGA factors is labeled *tga256* (C); five plants per genotype and treatment (D).

sequence-1-like (*as-1*-like) element (Ellis et al., 1993). Often, the second TGA binding motif is less conserved. The *CYP81D11* promoter contains a TGACaTat motif lying 4 bp upstream of the TGACGaCA motif. The sequence TGACaTatgacaaTGACGaCA thus resembles an *as-1*-like element (Fode et al., 2008). To analyze the importance of the two potential TGA binding sites, we generated transgenic lines carrying either the wild-type *CYP81D11* promoter (from −893 to the start codon ATG) or an *as-1*-deficient promoter fused to the *GUS* gene (*CYP81D11*$_{Proas\text{-}1m}$:*GUS*). Analysis of 17 independent transgenic lines showed that the wild-type *CYP81D11*$_{Prowt}$:*GUS* construct was induced by a factor of 9 after JA treatment (Fig. 2B). Mutation of the *as-1*-like element strongly compromised basal and JA-induced promoter activities.

In addition to the *as-1*-like element, the *CYP81D11* promoter contains a G-box (CACGTG) and a G-box-related sequence (CACATG) between base pair positions −206 and −192. Either of these sequences might serve as a binding site for MYC2 (Godoy et al., 2011; Fig. 2A). To assess their importance, both elements were mutated, resulting in the promoter *CYP81D11*$_{Pro\text{-}Gm}$. Analysis of 19 independent transgenic plant lines revealed that basal activity of the promoter was reduced by a factor of 2 and that responsiveness to JA was abolished (Fig. 2C). In summary, at least one of the TGA binding motifs of the *as1*-like element and at least one of the G-boxes are important for the JA responsiveness of the promoter.

MYC2 Activates Transcription from the *CYP81D11* Promoter Only in the Presence of TGA Factors

Next, we analyzed the expression from a chimeric promoter containing the *as-1*-like element and the two G-boxes (base pair positions –249 to –187) upstream of the cauliflower mosaic virus (*CaMV*) *35S* core promoter (–47 to –1; Fig. 3A) as a function of coexpressed MYC2. The promoter was fused to the firefly *luciferase* gene as a reporter, and its expression was monitored in transiently transformed protoplasts along with an effector plasmid encoding a *CaMV 35S*:*MYC2* construct. Ectopic expression of MYC2 led to a strong activation of this promoter (Fig. 3B). MYC2 did not activate the promoter when the two G-boxes were mutated to nonfunctional sequences. Moreover, mutation of the *as-1*-like element compromised MYC2-mediated activation. In this assay, the *CYP81D11 as-1*-like element could be functionally replaced by the *as-1* element (TGACGTaAgggaTGACGcac) of the *CaMV 35S* promoter. Consistently, MYC2-mediated activation of the chimeric promoter was abolished in *tga2-1 tga5-1 tga6-1* protoplasts (Fig. 3B).

Chemical Induction of the *CYP81D11* Promoter Is Enhanced by MYC2 and MYC2 Binding Motifs

In order to investigate the importance of MYC2 and TGA binding motifs for promoter activation after chemical induction, we treated the transgenic *CYP81D11*$_{Pro}$:*GUS* lines with TIBA (Fig. 4, A and B). As already observed after JA induction, mutation of the *as-1*-like element strongly reduced basal activities and induced promoter activities. This finding is consistent with the finding that TIBA induction is compromised in the *tga2-1 tga5-1 tga6-1* mutant (Fode et al., 2008). In contrast, mutation of the G-boxes, which had abolished the JA responsiveness of the promoter (Fig. 2C), did not alter the responsiveness to TIBA. (For side-by-side analysis of individual transgenic lines treated with either TIBA or JA, see

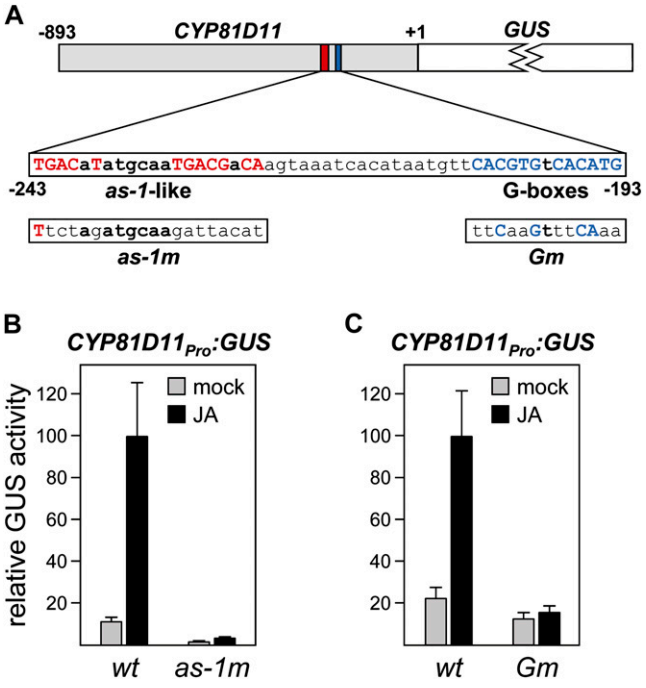

Figure 2. TGA and MYC2 binding motifs are essential for *CYP81D11* promoter activity after treatment with JA. A, Relative position of the *as-1*-like element and the two G-boxes within the *CYP81D11* promoter. Conserved positions of the perfect TGACGTCA binding motif and the G-box are shown in uppercase boldface letters, and nonconserved bases are shown in lowercase boldface letters. The indicated promoter sequence was fused to a reporter gene containing a fusion of the *eGFP* and the *GUS* genes. The sequence of the respective mutated cis-elements is shown below the wild-type sequence. Base pair position −893 refers to the annotated transcriptional start site +1. B and C, GUS activities obtained from JA-treated transgenic seedlings containing chimeric $CYP81D11_{Pro}$:*GUS* genes. For each construct, approximately 50 F2 seedlings of each independent plant line were grown for 16 d on Murashige and Skoog (MS) agar before exposure to JA (see "Materials and Methods") for 24 h. Control seedlings remained untreated. Bars represent average relative GUS activities ± SE of the following number of transgenic lines: 16 lines transformed with the wild-type (wt) promoter, 17 lines transformed with the mutated promoter (B); 18 lines transformed with the wild-type promoter, 19 lines transformed with the mutated promoter (C). GUS activities obtained from JA-treated plants encoding the wild-type promoter were set to 100%. [See online article for color version of this figure.]

Supplemental Figure S4) Still, expression from the *CYP81D11* promoter lacking the G-boxes was reduced between 2- and 3-fold both in mock- and TIBA-treated samples, indicating a general positive influence of the G-boxes. Like mutation of the G-boxes, mutation of *MYC2* reduced expression by a factor of 3 (Fig. 4C), which contrasts with the stringent requirement for MYC2 after JA induction (Fig. 1D). The same reduction was observed in TIBA-induced leaves from transgenic *JAZ1Δ3A-GUS* plants, which accumulate a dominant negative JAZ1 derivative that blocks JA signaling (Thines et al., 2007; Fig. 4D).

Chemically Induced *CYP81D11* Promoter Activity Occurs in the Absence of Increased JA-Ile Levels and Increased JAZ Degradation

Since COI1 and MYC2 were important for maximal *CYP81D11* promoter activity, we determined jasmonate levels after TIBA treatment. JA or JA-Ile levels did not change at 8 h after application of TIBA (Fig. 5, A and B), suggesting that JA-Ile-mediated accelerated JAZ degradation was not required for the observed promoter-enhancing activity of MYC2. In order to substantiate this conclusion, we monitored GUS activities in transgenic plants expressing a JAZ1-GUS fusion protein under the control of the *CaMV 35S* promoter (Thines et al., 2007). Steady-state levels of the JAZ1-GUS protein were unaffected in TIBA-treated seedlings, whereas exposure to JA led to the expected decrease (Fig. 5C). Consistent with the undetectable increase of JA-Ile and the undetectable degradation of JAZ proteins, expression of *VSP2* is not induced by TIBA (Supplemental Fig. S5).

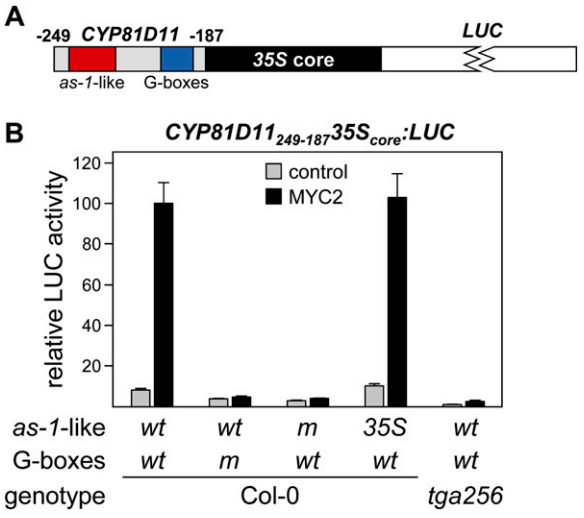

Figure 3. TGA factors and MYC2 functionally interact at the *CYP81D11* promoter. A, Scheme of a chimeric $CYP81D11_{249\text{-}187}35S_{core}$ promoter. The region spanning base pair positions −249 to −187 was fused to the *CaMV 35S* −47 core promoter upstream of the firefly *luciferase* gene (*LUC*). B, Relative LUC activities of promoter mutants derived from the chimeric $CYP81D11_{249\text{-}187}35S_{core}$ promoter as a function of coexpressed MYC2. Mutations (m) of the *as-1*-like element and the G-boxes are as in Figure 2A. A fourth construct encodes the *CaMV 35S as-1* element instead of the *as-1*-like element of the *CYP81D11* promoter. Reporter plasmids were transformed into Arabidopsis mesophyll protoplasts with either an empty effector plasmid or an effector plasmid encoding the *MYC2* open reading frame under the control of the *CaMV 35S* promoter. Firefly LUC activities were normalized to *Renilla* LUC activities. LUC activity obtained from the MYC2-activated $CYP81D11_{249\text{-}187}35S_{core}$ promoter in wild-type (wt) protoplasts was set to 100%. Values are means of four replicates ± SE. Mutant *tga2-1 tga5-1 tga6-1* lacking all three class II TGA factors is labeled *tga256*. [See online article for color version of this figure.]

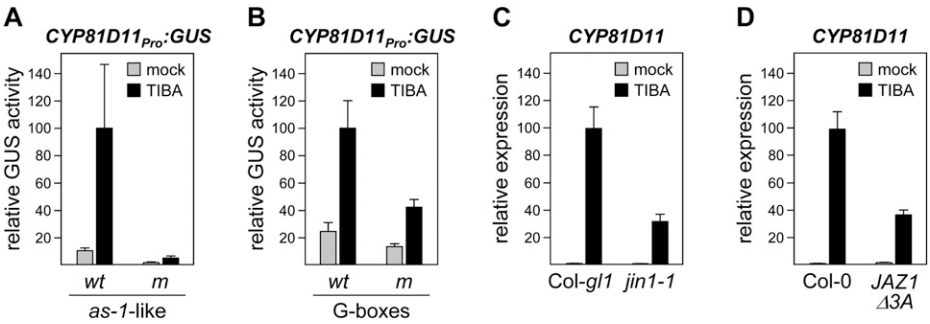

Figure 4. TGA and MYC2 binding sites are required for *CYP81D11* promoter activity after treatment with TIBA. A and B, Relative GUS activities obtained from TIBA-treated transgenic seedlings containing chimeric *CYP81D11*$_{Pro}$*:GUS* genes (for constructs, see Fig. 2A). For each construct, approximately 50 F2 seedlings of each independent plant line were grown for 16 d on MS agar before subjecting them to either mock or TIBA treatment for 8 h. Bars represent average relative GUS activities ± SE of the following number of transgenic lines: 16 lines transformed with the wild-type (wt) promoter, 17 lines transformed with the mutated (m) promoter (A); 18 lines transformed with the wild-type promoter, 19 lines transformed with the mutated promoter (B). GUS activities obtained from TIBA-treated plants encoding the wild-type promoter were set to 100%. C and D, Quantitative RT-PCR analysis of relative *CYP81D11* transcript levels after TIBA treatment. Six-week-old soil-grown plants of the indicated genotypes were either sprayed with 0.1 mM TIBA or 0.1% DMSO (mock). Whole rosettes were harvested for RNA isolation after 8 h of treatment. Relative transcript levels (fold over the reference gene *UBQ5*) in TIBA-treated wild-type plants were set to 100%. Mean values ± SE obtained from the following number of individually harvested plants are shown: four plants per treatment (C); five mock-treated wild-type plants, six TIBA-treated wild-type and *JAZ1Δ3A-GUS* plants, and seven mock-treated *JAZ1Δ3A-GUS* plants (D).

Chemical Induction of *CYP81D11* Involves a JA-Ile-Independent COI1 Function

The *coi1-t* allele reduced TIBA-induced *CYP81D11* promoter activity by a factor of 10 (Fig. 1A), whereas deletion of the G-boxes, the *jin1-1/myc2* allele, and the dominant negative JAZ1Δ3A-GUS protein reduced *CYP81D11* promoter activity only by a factor of 3 (Fig. 4). Likewise, expression of *CYP81D11* in the hormone biosynthesis mutant *dde2-2*, which cannot synthesize the JA precursor oxophytodienoic acid (Park et al., 2002; von Malek et al., 2002), was reduced by a factor of 3 (Fig. 6A). Side-by-side analysis of the *coi1-t* and the *dde2-2* mutants documented that reduction of steady-state mRNA levels in the *dde2-2* mutant was not as stringent as in the *coi1-t* mutant (Fig. 6A), pointing at a JA-independent COI1 function. This finding was confirmed by comparison of *CYP81D11* expression levels in *dde2-2* and the *dde2-2 coi1-t* double mutant: TIBA induction in the *dde2-2* background was further compromised by the *coi1-t* allele, indicating that the COI1 protein is directly or indirectly involved in chemical induction of the *CYP81D11* promoter even in the absence of its ligand JA-Ile. The more pronounced reduction of *CYP81D11* transcript levels in *coi1-t* as compared with *dde2-2* was

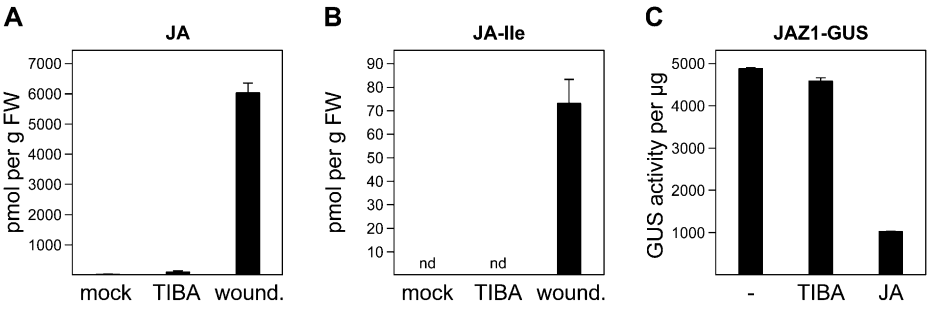

Figure 5. JA, JA-Ile, and JAZ1-GUS levels remain unaffected after TIBA treatment. A and B, HPLC-MS/MS analysis for the detection of JA and JA-Ile levels. Six-week-old soil-grown plants were either sprayed with 0.1 mM TIBA or 0.1% DMSO or wounded with forceps. Whole rosettes or wounded leaves were harvested for phytohormone extraction after 8 h of TIBA treatment or 2 h after wounding. Mean values ± SE obtained from four (mock and TIBA treatment) and two (wounded) individual plants are shown. FW, Fresh weight; nd, not detected. C, GUS activities obtained from seedlings expressing *JAZ1-GUS* after TIBA and JA treatment. Approximately 50 seedlings were grown for 16 d on MS agar before spraying with TIBA or incubation in the presence of JA for 8 h. Bars represent average GUS activities ± SE of seedlings harvested from two agar plates.

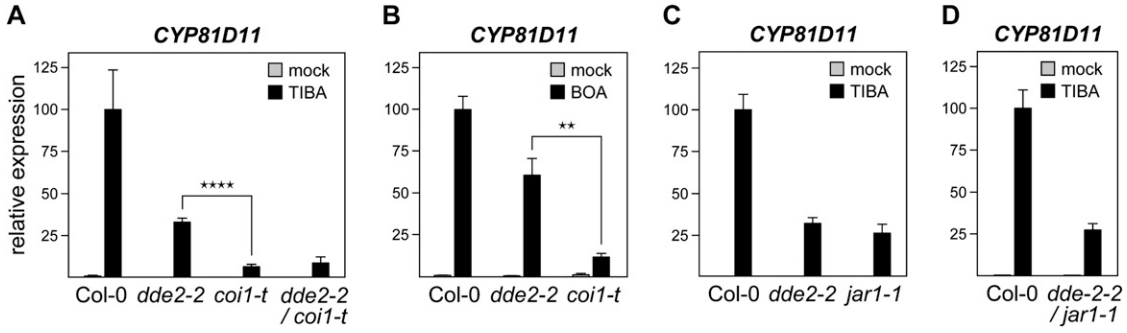

Figure 6. *CYP81D11* expression is more stringently reduced in the JA receptor mutant *coi1-t* as compared with the JA biosynthesis mutants *dde2-2* and *jar1-1*. Quantitative RT-PCR analysis of relative *CYP81D11* transcript levels after TIBA and BOA treatment is shown. Six-week-old soil-grown plants of the indicated genotypes were either sprayed with 0.1 mM TIBA or 2 mM BOA or subjected to the corresponding mock treatments (see "Materials and Methods"). Relative transcript levels (fold over the reference gene *UBQ5*) in treated wild-type plants were set to 100%. Mean values ± SE obtained from the following number of individual plants are shown: five mock-treated plants from each genotype, four TIBA-treated wild-type plants, four TIBA-treated *dde2-2* plants, five TIBA-treated *coi1-t* plants, and six TIBA-treated *dde2-2 coi1-t* plants (A); four plants were analyzed with the exception of mock-treated *coi1-t* plants, where three plants were analyzed (B–D). Asterisks indicate significant differences between induced *dde2-2* and *coi1-t* mutant plants (unpaired *t* test: **** $P < 0.0001$, ** $P = 0.0028$).

also observed after treatment with the allelochemical BOA (Fig. 6B), indicating that this unusual result was not unique to TIBA.

The *dde2-2* mutant is reported to be a complete knockout with respect to JA synthesis after wounding and pathogen attack (Chehab et al., 2011). Likewise, JA-Ile levels were below the levels of detection in this mutant after TIBA treatment (Supplemental Fig. S6). Still, we aimed to rule out the possibility that residual JA-Ile levels might activate COI1 even in the *dde2-2* mutant. To this end, we generated a homozygous *dde2-2 jar1-1* (for *jasmonate resistant1-1*; Staswick et al., 2002) double mutant, envisioning that biochemically undetectable JA levels would be converted with lowered efficiency into the active JA-Ile conjugate due to the absence of a functional JAR1 enzyme. Like the corresponding single mutants (Fig. 6C), the *dde2-2 jar1-1* double mutant showed higher *CYP81D11* transcript levels than the *coi1-t* mutant, supporting the idea that COI1 influences TIBA-induced *CYP81D11* promoter activity independently from JA-Ile (Fig. 6D).

DARK INDUCED11 (*DIN11*) is another gene that is induced in a COI1- and DDE2-dependent manner even though JA-Ile levels are not increased (Fig. 7, B and C). In contrast to *CYP81D11*, no difference in dark-induced *DIN11* expression was detected when comparing the biosynthesis mutant *dde2-2* and the receptor mutant *coi1-t* (Fig. 7A).

Other Elements Than the MYC2 Binding Motifs Integrate COI1-Dependent Processes at the *CYP81D11* Promoter

Our data so far indicate that the *as-1*-like element and the MYC2 binding motifs are essential for the activation of the promoter by exogenous JA. In the absence of either of the two cis-acting modules, JA cannot activate the promoter, indicating that no functionally redundant JA/COI1-responsive sites are present in the promoter. In order to investigate whether other sites might be important to integrate a potentially JA-Ile-independent COI1 function under conditions that activate the stress pathway, we aimed to analyze the *CYP81D11* promoter lacking these cis-elements in the *coi1-t* genetic background. For this, we chose a transient expression system. In Arabidopsis mesophyll protoplasts, the endogenous *CYP81D11* gene is highly expressed (Fig. 8A). Similar to TIBA-induced expression in leaves, constitutive expression in protoplasts is more strongly compromised in the *coi1-t* mutant than in the *dde2-2* mutant, indicating that protoplasts experience stress conditions that require the JA-Ile-independent COI1 function for maximal promoter activation. Mutation of the two G-boxes reduced promoter activity by a factor of 3 (Fig. 8B), which is comparable to the reduction observed in TIBA-induced plants (Fig. 4B). Importantly, the *coi1-t* allele still affected the promoter even when the G-boxes, or the *as-1*-like element, or both elements were mutated, indicating that the promoter contains other sequences that facilitate COI1 to contribute to activation (Fig. 8B). In the absence of these yet unknown COI1 integration sites, COI1 stringently operates through the G-boxes, as proven by analysis of a chimeric promoter containing the *as-1*-like element together with the G-box upstream of the *CaMV 35S* core promoter. This construct was not affected by *coi1-t* when the G-boxes were mutated (Fig. 8B).

DISCUSSION

Many environmental stimuli and developmental cues activate transcription through hormone-dependent signaling pathways. In addition, cellular damage caused by reactive xenobiotic or endogenous chemicals might induce gene expression in a more direct manner

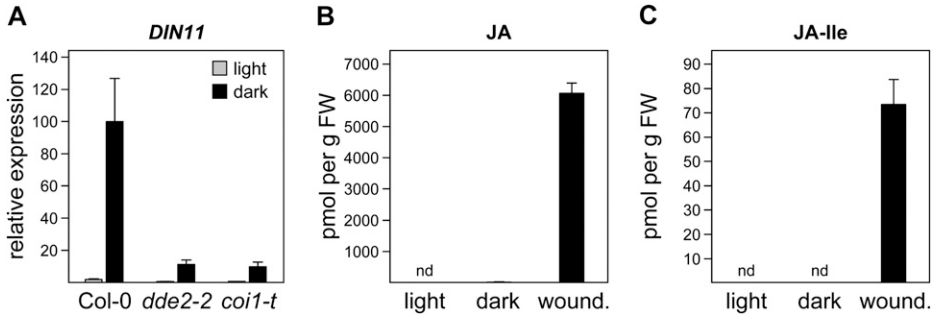

Figure 7. Dark-induced *DIN11* expression requires COI1 and but no increase in JA-Ile. A, Quantitative RT-PCR analysis of *DIN11* transcript levels in wild-type *dde2-2* and *coi1-t*. Six-week-old plants grown under a 12-h-light/12-h-dark cycle on soil were kept in the dark for 36 h (dark), while control plants (light) were kept under the normal light regime for this period. Whole rosettes were harvested for RNA isolation. Values (fold over the reference gene *UBQ5*) from dark-grown wild-type plants were set to 100%. Mean values ± SE obtained from four individual plants per genotype and treatment are shown with the exception of light-treated *coi1-t* plants, where three plants were analyzed. B and C, HPLC-MS/MS analysis for the detection of JA and JA-Ile levels. Material for light- and dark-treated samples was obtained as described in A. As positive controls, leaves were harvested at 2 h after wounding with forceps. Mean values ± SE obtained from four (light regime) and two (wounding) individual plants are shown. FW, Fresh weight; nd, not detected.

(Walley and Dehesh, 2010). The *CYP81D11* gene appeared as a suitable marker gene for exploring the mechanisms underlying transcriptional activation upon chemical stress, as it had emerged as one of the most highly induced genes in a number of transcriptome studies after treatment of Arabidopsis plants with different structurally unrelated chemicals (Baerson et al., 2005; Fode et al., 2008; Mueller et al., 2008; Matthes et al., 2010). Moreover, the identification of the TGA/SCL14 complex at the promoter had already yielded the first regulatory proteins required for induction. Starting from the observation that induction of *CYP81D11* transcription after application of reactive phytoprostanes requires the JA-Ile receptor COI1 (Mueller et al., 2008) and that induction by JA requires TGA factors, we asked how COI1 and other components of the JA signal transduction chain are integrated into the chemical stress pathway and how TGA factors are integrated into the JA pathway.

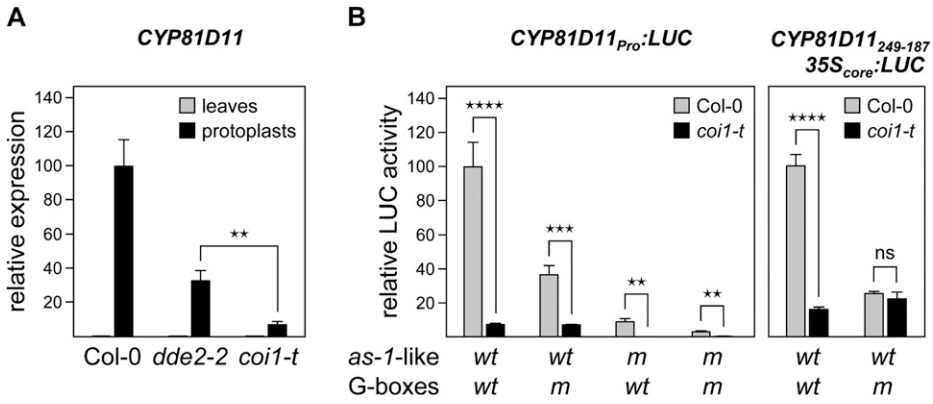

Figure 8. COI1 influences the *CYP81D11* promoter in the absence of JA-responsive cis-elements. A, Quantitative RT-PCR analysis of relative *CYP81D11* transcript levels in intact leaves and mesophyll protoplasts from wild-type, *dde2-2*, and *coi1-t* plants. Relative transcript levels (fold over the reference gene *UBQ5*) in protoplasts of wild-type plants were set to 100%. Mean values ± SE obtained from five samples from two independent experiments are shown. Asterisks indicate significant differences between *dde2-2* and *coi1-t* protoplasts (unpaired *t* test: ** $P = 0.0035$). B, *Luciferase* (LUC) activities of *CYP81D11* promoter constructs as a function of COI1. Reporter plasmids were transformed into Arabidopsis wild-type and *coi1-t* protoplasts. wt (wild-type) and m (mutant) refer to the intactness of the *as-1*-like element and the G-boxes, respectively. Firefly LUC activities were normalized to *Renilla* LUC activities. LUC activity obtained from the wild-type *CYP81D11* promoter in wild-type protoplasts was set to 100%. Values shown are means ± SE of four to six replicates from two independent experiments. The promoter constructs are depicted in Figure 2A (*CYP81D11* promoter) and Figure 3A (chimeric promoter containing the region spanning the *as-1*-like element and the two G-boxes upstream of the *CaMV 35S* core promoter). Asterisks indicate significant differences between wild-type and *coi1-t* protoplasts (unpaired *t* test: **** $P < 0.0001$, *** $P < 0.0005$, ** $P < 0.005$; ns, not significant).

TGA Factors Are Essential for MYC2 Function at the JA-Induced *CYP81D11* Promoter

TGA factors belong to the first plant transcription factors to be identified when searching for trans-factors binding to the *as-1* element of the viral *CaMV 35S* promoter (Katagiri et al., 1989). After analysis of the *tga2-1 tga5-1 tga6-1* triple knockout mutant, it had become evident that the three redundant class II TGA factors TGA2, TGA5, and TGA6 play a role in the induction of gene expression after application of the plant defense hormone salicylic acid. Later, it was discovered that TGA2 and/or TGA5 are essential for the activation of ethylene (ET)-induced defense genes (Zander et al., 2010). Moreover, microarray studies showed that a number of detoxification genes are not induced in the *tga2-1 tga5-1 tga6-1* mutant (Mueller et al., 2008), which correlates well with impaired survival of this mutant on toxic chemicals (Fode et al., 2008). In addition, TGA factors seemed to be important for JA-induced *CYP81D11* expression in seedlings grown on Suc (Mueller et al., 2008), placing these factors into another defense pathway. This finding was confirmed in this study using soil-grown plants (Fig. 1C).

The *CYP81D11* promoter contains at least two binding motifs that are essential for JA induction, an *as-1*-like element and two adjacent G-boxes. Since MYC2 is connected to the JA signaling cascade through interaction with JAZ repressors (Chini et al., 2007) and since it is important for JA induction of the *CYP81D11* promoter (Fig. 1D), we assume that the JA signal is integrated through MYC2. In an unbiased approach to select for MYC2 binding sites, the sequences CACGTG and CACATG were identified (Godoy et al., 2011). The *CYP81D11* promoter contains the sequence CACGTGxCACATG, which is stringently required for JA induction (Fig. 2). Therefore, it is very likely that JA-activated MYC2 binds to the CACGTGxCACATG sequence within the *CYP81D11* promoter.

The *as-1*-like element of the *CYP81D11* promoter fulfills the binding site requirement for TGA factors and can be replaced by the *as-1* element of the *CaMV 35S* promoter (Katagiri et al., 1989) to assist MYC2 function in transient assays (Fig. 3). Since TGA factors and G-boxes are required for JA induction, we have to consider whether TGA factors might bind to the G-boxes. TGA factors can recognize G-boxes if these overlap with a TGACG motif (Schindler et al., 1992), which is not the case in the CACGTGtCACATG sequence of the *CYP81D11* promoter. Therefore, we propose that TGA factors target the *as-1*-like element while MYC2 binds to the G-boxes and that the combination of both binding sites works as a nonseparable functional unit after JA induction (Fig. 9A). Whether TGA factors assist MYC2 binding or whether they are required for MYC2 to activate the promoter is not known.

Evidence for TGA factors being necessary for the activity of regulated transcriptional processes has been reported before (Lam and Chua, 1990): the *as-1* element is required to mediate light-responsive gene expression from a chimeric promoter consisting of four light-responsive cis-elements fused to the *CaMV 35S* core promoter. Likewise, such a functional interaction between TGA factors and ET-activated transcriptional activators might explain why marker genes of the JA/ET-dependent defense pathway are not expressed in ET-treated *tga2-1 tga5-1 tga6-1* plants (Zander et al., 2010). It can be envisioned that TGA factors are necessary components of the protein complexes (also called enhanceosomes) that have to assemble to initiate transcription at the corresponding promoters.

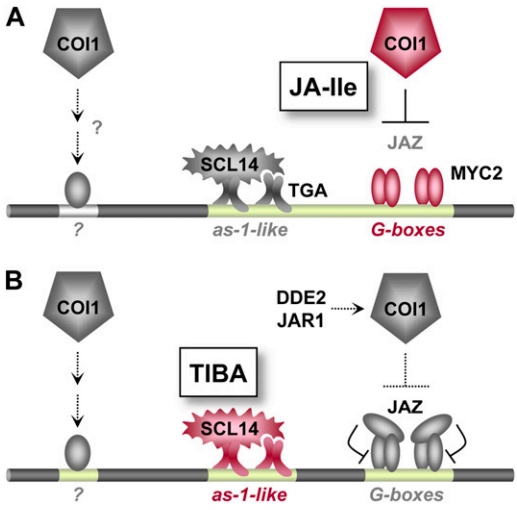

Figure 9. Working model describing the regulation of the *CYP81D11* promoter in response to JA-Ile and TIBA. The model aims to illustrate that JA and TIBA use different but interdependent signaling pathways to influence promoter activity and that COI1 can influence the promoter through a yet unknown mechanism that is distinct from the well-established JA-Ile-regulated mechanism merging on the G-boxes. It does not represent stoichiometric ratios between the interacting regulatory proteins. A, Under conditions of increased JA-Ile levels, COI1 accelerates the degradation of JAZ proteins, thus allowing MYC2 to activate the promoter. MYC2 stringently requires the presence of TGA transcription factors at the *as-1*-like element, as documented by the representation of the *as-1*-like sequence and the G-boxes as one functional unit on the DNA (shown in yellow). Activation of the JA pathway is indicated in red. B, Under conditions of increased TIBA levels, the TGA/SCL14 complex is activated. Deletion of the G-boxes, mutation in MYC2, expression of a dominant negative JAZ protein, and impaired JA-Ile biosynthesis reduce TIBA-induced promoter activity by a factor of 3. Since the TIBA-induced pathway is still functional in the absence of the G-boxes, the *as-1*-like sequence and the G-boxes are drawn as two separate entities (shown in yellow). In addition, COI1 influences the promoter even in the absence of its ligand. It remains to be elucidated which proteins are targeted by this COI1 activity, whether this mechanism involves protein degradation, and which trans-factors are involved. Activation of the chemically induced pathway is shown in red.

MYC2, DDE2, and JAR1 Enhance Activation of the Stress-Induced *CYP81D11* Promoter

TGA transcription factors are not only stringently required for JA-induced expression of the *CYP81D11*

promoter (Mueller et al., 2008) but they are also needed for *CYP81D11* induction by different chemicals, including TIBA (Fode et al., 2008), phytoprostanes (Mueller et al., 2008), and cis-jasmone (Matthes et al., 2010). As *as-1*-like elements are responsive to multiple stress stimuli (Xiang et al., 1996; Redman et al., 2002), it seems probable that regulatory processes merge directly at the complex between TGA factors and their interacting transcriptional coactivator SCL14 (Fig. 9B).

Both basal and TIBA-induced promoter activities were 3-fold reduced upon mutation of the G-boxes (Fig. 4B), suggesting that these elements function as amplifiers of promoter activity. TIBA-induced *CYP81D11* mRNA levels were diminished by the same factor of 3 in the *jin1-1/myc2* mutant, in the *dde2-2* and *jar1-1* mutants, and in transgenic plants expressing the dominant negative JAZ protein JAZ1Δ3A-GUS (Figs. 4 and 6). If we assume that it is not a coincidence that disturbing the JA pathway by independent means reduces promoter activity by a factor of 3, we propose the following model (Fig. 9B): MYC2 bound to the G-boxes can contribute to promoter activation in the presence of steady-state levels of JA-Ile, which activate basal COI1-mediated degradation of JAZ proteins. If this process is either disturbed in the *dde2-2* and *jar1-1* mutants or if MYC2 is inhibited by JAZ1Δ3A-GUS, or if the binding sites or MYC2 are mutated, promoter activity is reduced by a factor of 3. However, HPLC-tandem mass spectrometry (MS/MS) analysis of JA-Ile levels in TIBA-treated wild-type plants did not provide biochemical evidence for these postulated resting JA-Ile levels influencing COI1 activity (Supplemental Fig. S6). Therefore, we cannot exclude that other ligands that require DDE2 and JAR1 to be synthesized are responsible for this activity.

Simultaneous activation of the JA/MYC2/G-box-dependent pathway together with the TIBA/TGA/*as-1*-dependent pathway leads to the hyperinduction of the *CYP81D11* promoter (Supplemental Fig. S7), confirming the notion that two separate activation mechanisms exist. These have become interdependent with the JA pathway depending completely on TGA factors and with the chemical pathway depending only partially on the JA pathway (Fig. 9).

COI1 Enhances Stress-Induced *CYP81D11* Promoter Activity Even in the Absence of Its Ligand

Whereas *CYP81D11* transcript levels were 3-fold reduced in the JA biosynthesis mutants *dde2-2* and *jar1-1*, TIBA-induced *CYP81D11* transcript levels were 10-fold reduced in the JA receptor mutant *coi1* (Fig. 6). This difference might be explained by assuming that residual COI1 action might affect the same processes that are controlled in the presence of JA-Ile. To challenge this hypothesis, we chose to analyze the dark-induced expression of *DIN11* because it resembles *CYP81D11* in being activated in a COI1-dependent manner, although JA-Ile levels do not increase (Fig. 7). Since *DIN11* expression was affected to the same degree in the biosynthesis and the receptor mutants, we conclude that the JA-Ile-independent residual COI1 activity is not a general phenomenon.

Moreover, we provide preliminary evidence that the JA-Ile-independent COI1 activity does not target the same process as the JA-Ile-dependent COI1 activity (i.e. degradation of JAZ proteins that control MYC2 activity at the G-boxes). First, deletion of the G-boxes, which are essential for JA induction, had a less stringent effect (3-fold) than the *coi1* alleles (10-fold). Second, we observed that even a *CYP81D11* promoter derivative that does not contain the G-boxes was less active in *coi1-t* than in wild-type cells (Fig. 8B). Therefore, other sequences have to be postulated that allow COI1 to enhance activation of the promoter in the absence of the MYC2 binding sites. This sequence is not the *as-1*-like element, as revealed by the observation that a promoter that lacks the G-boxes and the *as-1*-like element is affected by the *coi1-t* allele (Fig. 8B). If the postulated COI1 integration site is removed, as in the $CYP81D11_{249\text{-}187}35S_{core}$ promoter, COI1 activity depends exclusively on the G-boxes. In contrast to the G-boxes, the hypothetical JA-Ile-independent COI1 integration site cannot respond to the JA signal, as proven by the inability of a promoter without the MYC2 binding motifs to be activated by JA (Fig. 2). As a consequence of these considerations, it can be envisioned that the MYC2 boxes integrate the COI1 function that depends on JA-Ile, whereas the other site integrates the residual COI1 activity that is still observed in the absence of JA-Ile (Fig. 9B). Since there is in vitro evidence of JA-Ile-independent binding of JAZ repressors to COI1 at least at high concentrations (Chini et al., 2007; Sheard et al., 2010) and since JAZ repressors interact with other transcription factors such as EIN3 (Zhu et al., 2011) and DELLA proteins (Hou et al., 2010), it might be that a transcription factor that does not bind to the G-boxes is activated by JA-Ile-independent COI1-mediated degradation of JAZ proteins. Another consideration is that COI1 might interact with other proteins (such as histone deacetylases; Devoto et al., 2002) to support TIBA-induced activation of the *CYP81D11* promoter in the absence of JA-Ile (Fig. 9B).

It was recently reported that root growth inhibition by ET was more pronounced in the two JA-Ile biosynthesis mutants *dde2-2* and *opr3* as compared with *coi1-16* (Adams and Turner, 2010), establishing another example for a JA-Ile-independent COI1 activity.

In conclusion, the *CYP81D11* promoter is activated by two different signal transduction networks that have become interdependent. It may be speculated that the xenobiotic pathway, probably acting through the TGA/SCL14 complex, activates *CYP81D11* as part of phase I of the detoxification machinery. Assuming that the monooxygenase function of CYP81D11 was coopted for the synthesis of a volatile that attracts parasitoids after insect attack (Bruce et al., 2008), its activation through wound-induced JA-Ile might have

evolved through the acquisition of MYC2 binding sites. The two signaling pathways became interdependent at this specific promoter, with TGA factors being crucially important for MYC2 function and with MYC2 activity contributing to the strong activation in response to xenobiotic stress. The postulated dual function of the CYP81D11 protein explains why the interdependence of TGA factors and JA-dependent processes is not found in other promoters of either the JA or the detoxification pathway. Whether the JA-Ile-independent COI1 function is related to the integration of the two pathways or whether it has evolved as a consequence of other processes that influence the complex *CYP81D11* promoter awaits further investigations.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) plants (ecotype Columbia [Col-0]) were grown in growth cabinets with light intensity at 35 to 45 μmol photons m^{-2} s^{-1}, 60% humidity, under a 12-h-light/12-h-dark regime at 22°C in square pots (8.5 × 8.5 cm) filled with steamed soil (Archut, Fruhstorfer Erde, T25, Str1 fein). If different growth conditions were used, these are indicated in the figure legends. Sown seeds were incubated for at least 1 d at 4°C to promote germination. Transgenic *JAZ1-GUS* and *JAZ1Δ3A-GUS* plants encoding chimeric *JAZ1* genes under the control of the *CaMV 35S* promoter (Thines et al., 2007) were obtained from J. Browse (Washington State University). Mutants *coi1-t* (Mosblech et al., 2011), *tga6-1 tga2-1 tga5-1* (Zhang et al., 2003), *dde2-2* (Park et al., 2002), *jar1-1* (Staswick and Tiryaki, 2004), and *jin1-1/myc2* (Berger et al., 1996) were described before and either obtained from the respective laboratories or from the Nottingham Arabidopsis Stock Centre (*jar1-1*). The *coi1-t* mutant was used for most of the experiments because it is in the same genetic background as the JA biosynthesis mutants, whereas *coi1-1* is in the *gl1* background. Double mutants *dde2-2 coi1-t* and *dde2-2 jar1-1* were made by genetic crosses and confirmed with the relevant diagnostic PCR markers.

Chemical Treatments

For TIBA treatments, plants were sprayed with 0.1 mM TIBA (prepared by diluting a 0.1 M stock solution in dimethyl sulfoxide [DMSO]). The corresponding mock treatment was performed by spraying with 0.1% DMSO. For BOA treatments, plants were sprayed with 2 mM BOA (prepared by a 1:500 dilution of a 1 M stock solution in ethanol). The corresponding mock treatments were done by spraying with 0.2% ethanol; the incubation time was 8 h. For JA treatments, plants were placed for 24 h within a closed glass container containing 1 μL of methyl jasmonate per 1 L of head space deposited on filter paper. Control plants were incubated under the same conditions without JA. Chemicals were obtained from Sigma-Aldrich.

Quantitative RT-PCR Analysis

RNA extraction and quantitative RT-PCR analysis were performed as described (Fode et al., 2008). Calculations were done according to the $2^{-\Delta CT}$ method (Livak and Schmittgen, 2001) using the *UBQ5* (At3g62250) gene as a reference (Kesarwani et al., 2007). Primers serving to amplify and quantify *CYP81D11* (At3g28740), *VSP2* (At5g24770), and *UBQ5* (At3g62250) transcript levels are indicated in Supplemental Table S1. All other primers used for quantitative RT-PCR experiments were obtained from Qiagen.

Construction of Recombinant Plasmids

The promoter region from position −893 to the ATG of the annotated coding region of the *CYP81D11* gene (At3g28740) was amplified from the Arabidopsis Col-0 genome (extracted with the DNeasy Plant Mini Kit [Qiagen]) using primers P1 and P2 (for primer sequences, see Supplemental Table S1), which add Gateway recombination sites to the amplified fragment. The Gateway technology (Invitrogen) allowed insertion of the fragment into pDONR201, yielding pDONR201/CYP81D11$_{Pro}$. Mutation of the *as-1*-like element between positions −244 and −223 was achieved by PCR using primer pairs P1/P4 and P2/P3 and pDONR201/CYP81D11$_{Pro}$ as a template, resulting in two fragments that subsequently served as templates for overlapping PCR with primers P1 and P2. Mutations in the G-boxes between base pair positions −206 to −192 were introduced by cloning annealed oligonucleotides (O1/O2 and O3/O4) with *Nde*I and *Eco*RV or *Xba*I and *Eco*RV, respectively, into the correspondingly cut pDONR201/CYP81D11$_{Pro}$ and pDONR201/CYP81D11$_{Proas\text{-}1m}$. The wild-type promoter fragment and the fragments with the mutated TGA and/or MYC2 binding sites were recombined into the binary vector pBGWFS7 (http://www.psb.ugent.be/gateway/; Karimi et al., 2002), resulting in a translational fusion starting with the ATG of the *CYP81D11* coding region, followed by the sequence of the Gateway cassette, followed by the in-frame ATG of the *eGFP-GUS* fusion gene. For transient assays in protoplasts, the wild-type and the mutated promoter fragments were recombined upstream of the firefly *luciferase* gene in the binary vector pBGWL7 (http://www.psb.ugent.be/gateway/). Plasmids containing the −249 to −187 promoter fragment upstream of the *CaMV 35S* core (−47) promoter were generated using classical cloning steps. The promoter fragments with the same mutations in the relevant cis-elements as introduced into the full-length promoter were synthesized as oligonucleotides (for oligonucleotide sequences O5–O12, see Supplemental Table S1), which were annealed and cloned into an appropriate intermediate vector. The final vector used for transient assays is a pUC-based vector that contains the chimeric promoters upstream of the firefly *luciferase* gene (Supplemental Fig. S8). The *MYC2* (At1g32640) coding region was amplified from cDNA with primers P5 and P6, which add Gateway recombination sites, and recombined into pDONR207 (Invitrogen). Subsequently, the fragment was recombined into the binary vector pB2GW7-HA to obtain an appropriate effector plasmid for transient assays. pB2GW7-HA originates from the binary vector pB2GW7.0 (http://www.psb.ugent.be/gateway/) containing the expression cassette of pE-35S-HA-GW (Weiste et al., 2007), which allows the expression of hemagglutinin-tagged proteins.

Stable and Transient Transformations of Plant Cells

For the generation of transgenic plants, binary plasmids were electroporated into *Agrobacterium tumefaciens* strain GV3101 (pMP90). The resulting agrobacteria were used to transform Col-0 plants using the floral dipping method (Clough and Bent, 1998). For the analysis of promoter activities in the absence of effectors, 10 μg of pBGWL7-derived reporter plasmids were transformed into either wild-type or *coi1-t* protoplasts. For the analysis of promoter activities as a function of MYC2, 1 μg of pUC-derived reporter plasmids and 13 μg of the effector plasmid (pB2GW7-HA-MYC2) were transformed in Arabidopsis wild-type and *tga2-1 tga5-1 tga6-1* protoplasts (Yoo et al., 2007). To normalize for the experimental variability, 1 μg of the plasmid pUBQ10Ruc containing the *Renilla luciferase* gene under the control of the Arabidopsis *UBQ10* promoter was added to each sample. Protoplasts were incubated in washing and incubation solution for 16 h before harvest.

Luciferase and GUS Assays

Quantitative GUS assays were performed using 4-methyl-umbelliferyl-β-D-glucuronide (Sigma-Aldrich) as a substrate (Jefferson, 1989). The released fluorescence was measured with a Cyto Fluor Series 4000 plate reader (Perspective Biosystems). The total amount of protein was determined using the Bradford assay solution. Luciferase activities of transformed protoplasts were determined with the Dual Luciferase Reporter assay system from Promega using the CentroXS^3 LB 960 luminometer from Berthold Technologies.

Determination of JA and JA-Ile Levels

Extraction was performed as described previously for lipids, with some modifications (Matyash et al., 2008). Plant material (200 mg) was extracted with 0.75 mL of methanol containing 10 ng of D_6-JA and 10 ng of D_4-JA-Leu (kindly provided by Dr. Otto Miersch, Institute for Plant Biochemistry, Halle, Germany), each as an internal standard. After vortexing, 2.5 mL of methyl-*tert*-butyl ether was added, and the extract was shaken for 1 h at 4°C. For phase separation, 0.6 mL of water was added. The mixture was incubated for 10 min at room temperature and centrifuged at 450*g* for 15 min. The upper phase was collected, and the lower phase was reextracted with 0.7 mL of methanol:water (3:2.5, v/v) and 1.3 mL of methyl-*tert*-butyl ether as described above. The combined upper phases were dried under streaming nitrogen and resuspended in 100 μL of acetonitrile:water:acetic acid (20:80:0.1, v/v/v).

The analysis of constituents was performed using an Agilent 1100 HPLC system (Agilent) coupled to an Applied Biosystems 3200 hybrid triple quadrupole/linear ion trap mass spectrometer (ABSciex). Nanoelectrospray (nanoESI) analysis was achieved using a chip ion source (TriVersa NanoMate; Advion BioSciences). For the analysis, 10 μL of extract was injected. Reverse-phase HPLC separation was performed on an EC 50/2 Nucleodure C18 gravity 1.8-μm column (50 $\times$ 2.1 mm, 1.8 μm particle size; Macherey-Nagel). The binary gradient system consisted of solvent A (water:acetic acid, 100:0.1, v/v) and solvent B (acetonitrile:acetic acid, 100:0.1, v/v) with the following gradient program: 28% solvent B for 2 min, followed by a linear increase of solvent B up to 92% within 6 min, and an isocratic run at 92% solvent B for 4 min. To reestablish starting conditions, a linear decrease to 28% solvent B in 2 min was performed, followed by an isocratic equilibration at 28% solvent B for 3 min. The flow rate was 0.3 mL min^{-1}. For stable nanoESI, 130 μL min^{-1} 2-propanol:acetonitrile:water:acetic acid (70:20:10:0.1, v/v/v/v) delivered by a 2150 HPLC pump (LKB Bromma) was added just after the column via a mixing tee valve. By using another postcolumn splitter, 790 nL min^{-1} eluent was directed to the nanoESI chip. Ionization voltage was set to -1.7 kV. Phytohormones were ionized in a negative mode and determined in multiple reaction monitoring mode. Mass transitions were as follows: 215/59 (declustering potential [DP] -45 V, entrance potential [EP] -9.5 V, collision energy [CE] -22 V) for D_6-JA, 209/59 (DP -45 V, EP -9.5 V, CE -22 V) for JA, 325/133 (DP -80 V, EP -4 V, CE -30 V) for D_4-JA-Leu, and 322/130 (DP -80 V, EP -4 V, CE -30 V) for JA-Ile. The mass analyzers were adjusted to a resolution of 0.7 atomic mass units full width at half-height. The ion source temperature was 40°C, and the curtain gas was set at 10 (given in arbitrary units). Quantification was carried out using a calibration curve of intensity (mass-to-charge ratio) of (unlabeled)/(deuterium labeled) versus molar amounts of unlabeled (0.3–1,000 pmol).

Supplemental Data

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

Supplemental Figure S1. TIBA-induced *CYP81D11* transcription is compromised by independent *coi1* alleles.

Supplemental Figure S2. COI1 is not required for chemical- and JA-induced expression of marker genes of the detoxification response.

Supplemental Figure S3. JA-induced MYC2-dependent expression of *VSP2* and *GRX480* is independent from TGA transcription factors.

Supplemental Figure S4. Side-by-side analysis of independent transgenic lines containing either the $CYP81D11_{Prowt}$:*GUS* or the $CYP81D11_{Pro\text{-}Gm}$:*GUS* construct after TIBA and JA treatment.

Supplemental Figure S5. The JA marker gene *VSP2* does not respond to TIBA.

Supplemental Figure S6. JA-Ile levels are below the limit of detection after TIBA treatment.

Supplemental Figure S7. The combined treatment with TIBA and JA leads to hyperinduction of *CYP81D11* expression.

Supplemental Figure S8. Vector map of plasmids used for transient assays as shown in Figure 3.

Supplemental Table S1. List of oligonucleotide sequences.

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