

# TIME FOR COFFEE Represses Accumulation of the MYC2 Transcription Factor to Provide Time-of-Day Regulation of Jasmonate Signaling in *Arabidopsis*

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Plants are confronted with predictable daily biotic and abiotic stresses that result from the day–night cycle. The circadian clock provides an anticipation mechanism to respond to these daily stress signals to increase fitness. Jasmonate (JA) is a phytohormone that mediates various growth and stress responses. Here, we found that the circadian-clock component TIME FOR COFFEE (TIC) acts as a negative factor in the JA-signaling pathway. We showed that the *tic* mutant is hypersensitive to growth-repressive effects of JA and displays altered JA-regulated gene expression. TIC was found to interact with MYC2, a key transcription factor of JA signaling. From this, we discovered that the circadian clock rhythmically regulates JA signaling. TIC is a key determinant in this circadian-gated process, and as a result, the *tic* mutant is defective in rhythmic JA responses to pathogen infection. TIC acts here by inhibiting MYC2 protein accumulation and by controlling the transcriptional repression of *CORONATINE INSENSITIVE1* in an evening-phase-specific manner. Taken together, we propose that TIC acts as an output component of the circadian oscillator to influence JA signaling directly.


## INTRODUCTION


Plants face predictable daily environmental changes that generate rhythmic biotic and abiotic stresses within a 24-h period. The circadian clock controls the expression of ~30% of the *Arabidopsis thaliana* transcriptome, which leads to regulation of diverse metabolic processes and developmental programs in response to daily environmental signals (Davis and Millar, 2001; Covington et al., 2008; Harmer, 2009; Sanchez et al., 2011). The plant circadian clock consists of an input pathway, a core oscillator, and output responses. The components of the input pathway recognize environmental signals, such as light intensity, wavelength and duration, and temperature (Salome and McClung, 2005). Perceived environmental signals are then relayed to the core oscillator for resetting or fine-tuning of the clock. The core oscillator produces a ~24-h rhythmic periodicity to direct clock output factors. These output factors regulate diverse plant developmental and physiological responses, such as rhythmic hypocotyl growth, photosynthesis, stomata opening, and seasonal flowering-time regulation (Suárez-López et al., 2001; Nozue et al., 2007; Michael et al., 2008; Harmer, 2009). Clock-mediated synchronization of internal growth to external environmental changes is crucial for plant fitness (Dodd et al., 2005).

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One important pathway for circadian outputs is rhythmic hormone signaling. A major part of phytohormone production, biosynthesis, and response gene expression oscillates under the control of diurnal signals and/or the circadian clock (Thain et al., 2004; Nováková et al., 2005; Salomé et al., 2006; Covington and Harmer, 2007; Yin et al., 2007; Covington et al., 2008; Michael et al., 2008; Mizuno and Yamashino, 2008; Robertson et al., 2009; Goodspeed et al., 2012). Thereby, an array of hormone-signaling systems function as key circadian output pathways. For example, the clock influences auxin signaling in a time-of-day-specific manner, and this leads to rhythmic hypocotyl growth under diurnal conditions (Covington and Harmer, 2007; Rawat et al., 2009). Another example can be seen with the circadian evening factor, TIMING OF CAB EXPRESSION1 (TOC1). It regulates rhythmic abscisic acid (ABA) responses by repressing transcription of the proposed ABA receptor *ABAR*; thus, *toc1* mutants show altered ABA responses (Legnaioli et al., 2009). Recent studies also revealed that gibberellic acid (GA) signaling is gated by the circadian clock, leading to maximal GA sensitivity during the night phase (Arana et al., 2011). Together, hormone-regulated developmental processes and stress signaling are clear targets of clock outputs.

Hormone signaling can act as a clock input factor. Several hormone-signaling systems modulate the core oscillator. For example, auxin, ABA, and cytokinin alter circadian amplitude, period, and phase, respectively (Hanano et al., 2006). Furthermore, ABA induces the expression of the clock evening element *TOC1* in a daytime-specific manner (Legnaioli et al., 2009). Although studies of cross-regulation between the circadian clock and hormone pathways have recently started to emerge, the mechanistic basis for such relationships awaits elucidation.

Jasmonates (JAs) regulate diverse aspects of plant life, from developmental growth processes to physiological responses

(Katsir et al., 2008a; Kazan and Manners, 2008; Browse, 2009). Jasmonic acid, its conjugates, and its precursors are oxylipin-signaling molecules. These are collectively termed the JAs. The most biologically active form of JA was reported to be jasmonyl-isoleucine (JA-Ile) (Thines et al., 2007). The bacterial pathogen *Pseudomonas syringae* produces a functional and structural homolog of JA-Ile called coronatine (COR). This bacterial phytotoxin mimics active JA-Ile in plant tissues and thus alters certain defense responses (Bender et al., 1999; Katsir et al., 2008b). A key player in JA signaling is CORONATINE INSENSITIVE1 (COI1), an F-box protein that forms the E3 ubiquitin ligase SCF<sup>COI1</sup> complex (Xie et al., 1998; Devoto et al., 2002). COI1 binds JA-Ile in cooperation with JAZMONATE ZIM-DOMAIN (JAZ) proteins (Thines et al., 2007; Katsir et al., 2008b). Recent structural studies found that a protein complex of COI1 and JAZ with inositol pentakisphosphate is the complete JA receptor (Sheard et al., 2010; Mosblech et al., 2011). JAZ proteins are JA-signaling repressors, and 12 family members are found in *Arabidopsis*. In the absence of JA, the JAZs interact with MYC2, a positive transcription factor in JA signaling, and repress MYC2 activity by recruiting the transcription repressors NOVEL INTERACTOR OF JAZ (NINJA) and TOPLESS (TPL)/TPL-RELATED PROTEINS (TPRs) (Pauwels et al., 2010). In the presence of JA, JAZ proteins are ubiquitinated by SCF<sup>COI1</sup> and subsequently degraded by the 26S proteasome (Chini et al., 2007; Thines et al., 2007). Consequently, active MYC2 is released from repressors to induce JA-response gene expression (Lorenzo et al., 2004; Dombrecht et al., 2007).

MYC2 encodes a bHLH motif-containing transcription factor (Abe et al., 2003; Lorenzo et al., 2004). *myc2* mutants are less sensitive to JA, in line with MYC2 being a positive regulator in the JA pathway (Lorenzo et al., 2004). MYC2 expression is induced by JA and promotes wound-response gene expression and represses pathogen-response gene expression in a JA-dependent manner (Lorenzo et al., 2004). In *Arabidopsis*, two genes annotated as MYC3 and MYC4 have sequence and functional similarity to MYC2. Activities of MYC2, MYC3, and MYC4 in JA signaling are partially overlapping (Abe et al., 2003; Fernández-Calvo et al., 2011; Niu et al., 2011). The different spatial expression pattern of these three genes directs distinct regulatory functions. For example, MYC2 is highly expressed in root tissue, whereas the expression of MYC3 and MYC4 is limited in this tissue. Therefore, MYC2 has a dominant role in the JA response in roots (Fernández-Calvo et al., 2011; Niu et al., 2011).

TIME FOR COFFEE (TIC) was identified as a circadian-clock regulator necessary for maintaining circadian period and amplitude (Hall et al., 2003; Ding et al., 2007). *tic* mutants display a short period with reduced amplitude under free-running conditions (Ding et al., 2007), and TIC was found to reset the core oscillator around the mid-late-night phase (Hall et al., 2003). TIC protein has been shown to localize to the nucleus. The biochemical activity of TIC is currently unknown, because TIC does not have known functional domains in its primary structure to predict its action. To elucidate a functional role and molecular mechanism for TIC, we identified TIC-interacting proteins and explored their functional relationship. Here, we have found MYC2 as a TIC-interacting protein and discovered a previously

unknown role of TIC in JA signaling. We established that TIC acts as a negative regulator in JA signaling by repressing MYC2 protein accumulation. In addition, we defined a signaling connection between the circadian clock and the JA pathway and found that TIC plays a key role in clock-gated JA responses.

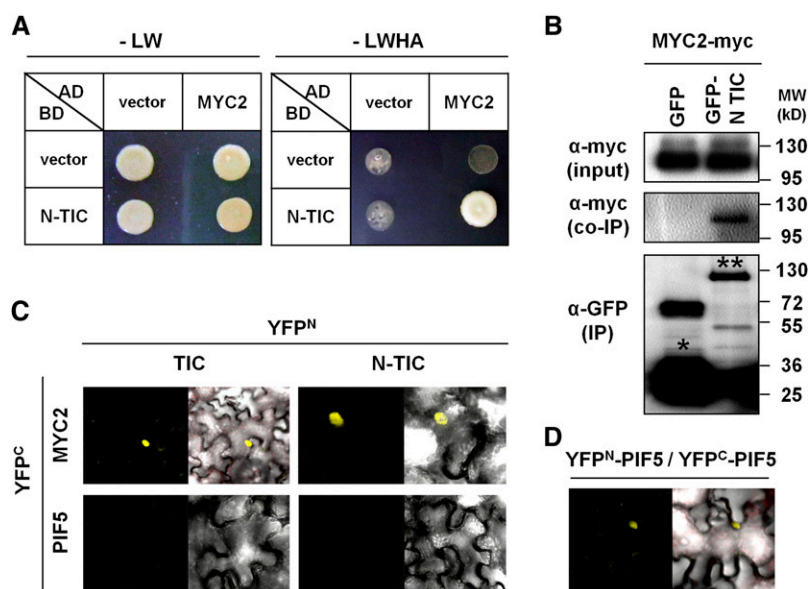
## RESULTS

### MYC2 Protein Interacts with TIC in the Nucleus

As a first step to investigate the molecular function of TIC, we identified TIC interaction partners in a yeast two-hybrid screen. Because of the protein size of the full-length TIC (1555 amino acids, 165 kDa) and lack of known motifs in its primary structure, we first used its 564-amino acid N terminus as bait. Among several TIC-interacting protein candidates, MYC2 was most frequently identified. To confirm this, we generated constructs with full-length MYC2 and N terminus TIC and repeated the yeast two-hybrid assay. N-TIC and MYC2 constructs enabled yeast growth, suggesting a direct protein-protein interaction (Figure 1A). We next tested the interaction between N-TIC and MYC2 in planta by performing a coimmunoprecipitation assay. Green fluorescent protein (GFP)-fused N terminus TIC (amino acids 1 to 564) and myc-epitope-tagged MYC2 were transiently expressed in *Nicotiana benthamiana*, and N-TIC was immunoprecipitated with GFP-TRAP beads. MYC2 specifically precipitated with the N terminus of TIC but did not coprecipitate with GFP alone (Figure 1B). For further analysis of protein interaction, we performed a biomolecular fluorescence complementation (BiFC) assay. The N terminus of yellow fluorescent protein (YFP) was fused to full-length TIC (YFP<sup>N</sup>-TIC) or the N-terminal portion of TIC (YFP<sup>N</sup>-N TIC), and the YFP C terminus was fused to MYC2 (YFP<sup>C</sup>-MYC2) or PIF5 (YFP<sup>C</sup>-PIF5) as a control. Fusion proteins were expressed in *N. benthamiana*, and interaction was determined by measuring YFP fluorescence under a confocal microscope. Both full-length TIC and N-terminal TIC were found to interact with MYC2, and this was exclusively in nuclei (Figure 1C). A TIC-MYC2 interaction was also found with the reciprocal YFP<sup>N</sup>-MYC2 and YFP<sup>C</sup>-TIC combination (see Supplemental Figure 1 online). Association between TIC and MYC2 was specific, because TIC did not interact with another bHLH protein, PIF5 (Figure 1C; see Supplemental Figure 1 online). As a positive experimental control, we confirmed by BiFC assay that PIF5 can successfully form a homodimer under the same experimental conditions (Figure 1D). Taken together, these results demonstrate that TIC physically interacts with MYC2.

### TIC Represses JA-Mediated Inhibition of Root Growth in a MYC2-Dependent Manner

For genetic analysis of TIC and MYC2, we obtained two independent T-DNA insertion mutants of MYC2 (Salk\_061267, Salk\_130877). The Salk\_061267 mutant was already characterized and previously named *myc2-3/jin1-8* (Lorenzo et al., 2004; Sehr et al., 2010), so we refer to this mutant as *myc2-3*. We named the Salk\_130877 line *myc2-4*. In both homozygous



**Figure 1.** TIC Specifically Interacts with MYC2 in the Nucleus.

(A) Yeast two-hybrid assay with N-TIC and MYC2.

(B) Coimmunoprecipitation assay of N-TIC and MYC2. GFP-fused N-TIC and the myc-epitope-fused MYC2 were transiently expressed in *N. benthamiana*. GFP-N TIC was immunoprecipitated and detected using an anti-GFP antibody. Coimmunoprecipitated MYC2 was detected using an anti-myc antibody. \* indicates GFP protein, and \*\* indicates GFP-N TIC protein. co-IP, coimmunoprecipitation; IP, immunoprecipitation; MW, molecular weight.

(C) BiFC assay using N-YFP fused with full-length TIC or N terminus TIC, and C-YFP fused with MYC2 or PIF5.

(D) BiFC assay using N-YFP fused with PIF5 and C-YFP fused with PIF5. Proteins were transiently coexpressed in *N. benthamiana*, and YFP fluorescence was detected under a confocal microscope. BiFC experiments were performed three times, and similar results were observed from each experiment.

T-DNA lines, *MYC2* mRNA was detected only at background levels by quantitative RT-PCR (qRT-PCR), and *MYC2* full-length transcripts were not detected by RT-PCR (see Supplemental Figure 2 online). We also generated *MYC2ox* plants that express *MYC2-myc* under control of the CaMV 35S promoter. *MYC2ox* expressed >10 times more *MYC2* transcript compared with the wild type (see Supplemental Figure 3 online). For our analysis, we used the *tic-2* (SAIL\_753\_E03) allele (Ding et al., 2007) and generated the *tic-2 myc2-3* double mutant from a cross between *tic-2* and *myc2-3*.

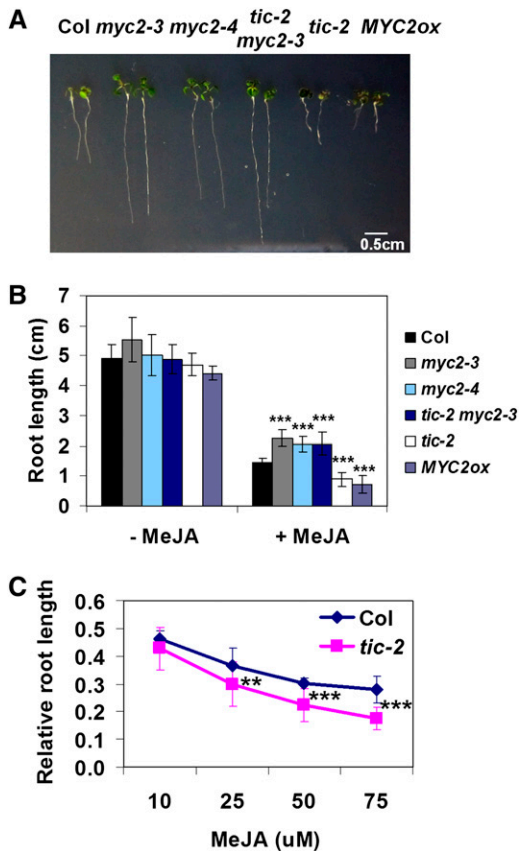
Because MYC2 is an established positive regulator of the JA pathway (Lorenzo et al., 2004; Dombrecht et al., 2007), we asked whether TIC could also be involved in the JA response by testing the inhibition of root growth in response to exogenous methyl jasmonate (MeJA) in Columbia (Col), *tic-2*, *myc2-3*, *myc2-4*, *tic-2 myc2-3*, and *MYC2ox* seedlings. All genotypes had similar root length in the absence of exogenous MeJA (Figure 2B). Consistent with previous reports, both *myc2-3* and *myc2-4* mutants displayed reduced sensitivity to MeJA compared with the wild type; MeJA-treated *myc2* mutants displayed 59% inhibition of root growth relative to untreated plants, whereas wild-type plants showed 71% inhibition. Conversely, *MYC2ox* plants exhibited severely reduced root growth (83% inhibition) on MeJA-containing media. Interestingly, *tic-2* displayed significantly shortened primary roots on MeJA-

containing media (81% inhibition), similar to the response seen in *MYC2ox* plants. The response of the *tic-2 myc2-3* double mutant (56% inhibition) was comparable with that of the *myc2-3* single mutant (Figures 2A and 2B). Therefore, *myc2-3* is genetically epistatic to *tic-2* in JA-dependent inhibition of root elongation.

We additionally examined the responsiveness of *tic-2* to various concentrations of MeJA. The wild type and *tic-2* showed similar responses on 10  $\mu$ M MeJA-containing medium. *tic-2* began to show enhanced sensitivity relative to the wild type on 25  $\mu$ M MeJA medium, and this hypersensitive phenotype was more pronounced at higher concentrations (Figure 2C).

#### TIC Promotes Resistance to *P. syringae* pv *tomato* DC3000 Infection

To investigate the JA-response phenotype of *tic* further, we examined JA-dependent susceptibility to bacterial pathogen infection. Col, *tic-2*, *myc2-3*, and *tic-2 myc2-3* plants were inoculated with the virulent strain *P. syringae* pv *tomato* (*Pst*) DC3000, and in planta bacterial titers were determined on the day of inoculation (day 0) and after 3 d (day 3). Titers of inoculated bacteria at day 0 were similar in all examined genotypes, indicating that *Pst* DC3000 bacteria enter into *tic-2* leaves with the same efficiency as in the wild type. At 3 d after



**Figure 2.** TIC Represses JA-Mediated Inhibition of Root Growth.

(A) Inhibition of root growth of 10-d-old Col (wild type), *myc2-3*, *myc2-4*, *tic-2*, *tic-2 myc2-3*, and *MYC2ox* seedlings on medium containing 50  $\mu$ M MeJA.

(B) Root length of seedlings. The results are the mean of 20 seedlings, and error bars indicate  $\pm$ sd.

(C) Relative root length of each genotype grown on MeJA-containing media to root length without MeJA treatment. The results are the mean of 20 seedlings, and error bars indicate  $\pm$ sd. Asterisks indicate statistically significant differences compared with Col (Student's *t* test, \*\**P* < 0.01, and \*\*\**P* < 0.001).

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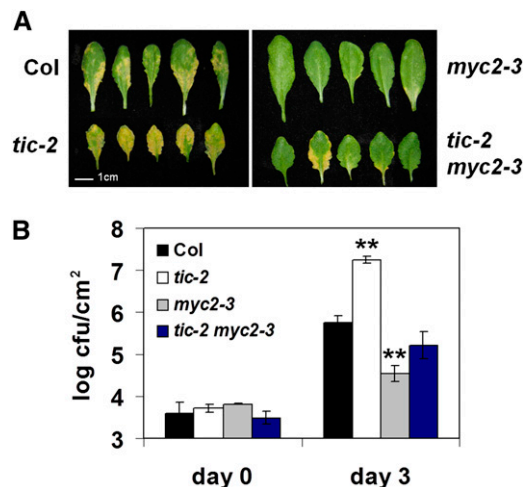
inoculation, *tic-2* allowed ~30 times more bacterial proliferation as compared with the wild type (Figure 3B). The increased susceptibility of *tic-2* to *Pst* DC3000 was also seen at the level of enhanced macroscopic disease symptom development (Figure 3A). Consistent with previous reports (Fernández-Calvo et al., 2011), *myc2-3* mutants were more resistant to pathogen infection. *tic-2 myc2-3* plants exhibited higher resistance than the *tic-2* single mutant; however, this was not as extreme as in *myc2-3* (Figure 3B). Because *Pst* DC3000 produces the JA-Ile structural mimic COR to suppress host defenses by activating JA signaling in a COI1-dependent manner (Bender et al., 1999; Katsir et al., 2008b), these results are consistent with the enhanced sensitivity of *tic-2* to JA. We tested this hypothesis by inoculating plants with *Pst* COR<sup>-</sup>, a mutant strain deficient in

production of COR (Melotto et al., 2006). All examined plant genotypes limited growth of *Pst* COR<sup>-</sup> to a similar extent (see Supplemental Figure 4 online). We concluded that TIC serves to restrict *Pst* DC3000 infection through negative regulation of JA signaling.

### TIC Regulates JA-Responsive Gene Expression in a MYC2-Dependent Manner

We investigated the JA responses of *tic-2* at a molecular level by determining the fold induction of JA-marker gene expression in the presence of exogenous JA relative to untreated tissues. Seedlings were grown under continuous white light (WLC) and treated with MeJA for 1, 3, 6, or 24. For this analysis, we selected three groups of JA-responsive genes: immediate-early-induced genes (*JAZ5* and *LOX3*) (Mandaokar et al., 2006; Chini et al., 2007; Chung et al., 2008), wound-response genes (*VSP2* and *TAT*) (Titarenko et al., 1997; Reymond et al., 2000; Cheong et al., 2002; Farmer et al., 2003; Howe, 2004; Lorenzo et al., 2004), and pathogen-response genes (*PDF1.2* and *PR4*) (Penninckx et al., 1996; Manners et al., 1998; Penninckx et al., 1998; Lorenzo et al., 2003; Lorenzo et al., 2004).

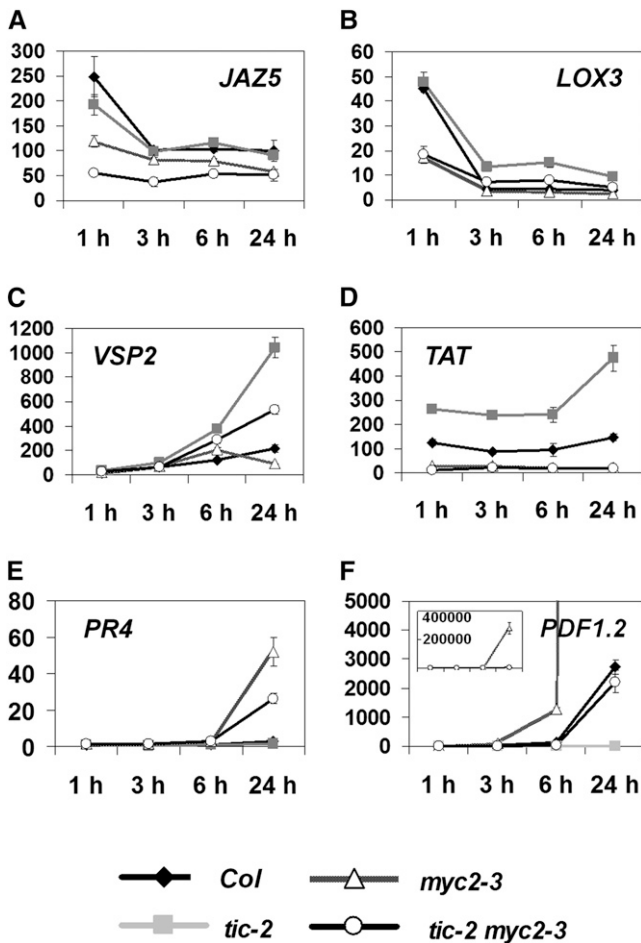
Consistent with a previous report (Lorenzo et al., 2004), we confirmed that *myc2-3* displayed lower induction of immediate-early-response genes and wound-responsive genes and enhanced induction of pathogen-response genes (Figures 4A to 4F). The induction of *JAZ5* and *LOX3* after MeJA treatment was similar in *tic-2* and the wild type (Figures 4A and 4B), but levels of *VSP2* and *TAT* were significantly induced in *tic-2* relative to the wild type (Figures 4C and 4D). In measuring the expression



**Figure 3.** *tic-2* Is More Susceptible to *Pst* DC3000 Infection.

(A) Disease symptoms of Col, *tic-2*, *myc2-3*, and *tic-2 myc2-3* plants after *Pst* DC3000 infection. Plants were grown for 5 weeks and sprayed with *Pst* DC3000. Pictures were taken 4 d after inoculation.

(B) In vivo titers of *Pst* DC3000 in Col, *tic-2*, *myc2-3*, and *tic-2 myc2-3* plants on the day of spraying (day 0) or 3 d after inoculation (day 3). Bacterial counts were expressed as log (cfu/cm<sup>2</sup>). Error bars indicate  $\pm$ se. Asterisks indicate statistically significant differences compared with Col (Student's *t* test, \*\**P* < 0.01).



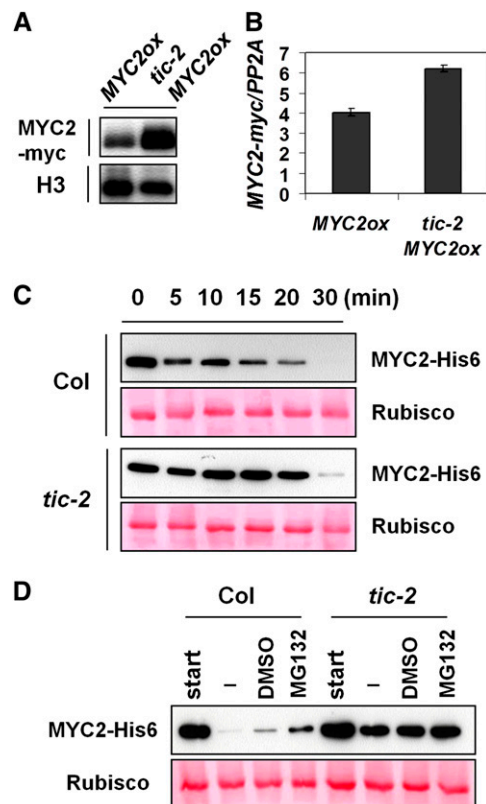
**Figure 4.** TIC Regulates JA-Responsive Gene Expression in a MYC2-Dependent Manner.

Fold change of *JAZ5* (A), *LOX3* (B), *VSP2* (C), *TAT* (D), *PR4* (E), and *PDF1.2* (F) expression by exogenous MeJA treatment relative to mock-treated levels. Plants were grown under WLc for 7 d, and transferred to 50  $\mu$ M MeJA-containing media for 1, 3, 6, and 24 h, respectively. Gene expression relative to *PP2A* at each time point was measured by qRT-PCR, and fold induction relative to basal expression level was determined. Fold change indicates a mean of three technical replicates, and error bars indicate sd. qRT-PCR experiments were repeated three times, each with an independent biological replicate, and similar results were obtained. An inset graph in (F) indicates the full scale of the result.

of pathogen-response marker genes, we found that *PR4* and *PDF1.2* were only marginally induced by exogenous MeJA treatment in *tic-2* compared with the wild type (Figures 4E and 4F). Therefore, TIC represses the wound-response gene expression and promotes the pathogen-response gene expression in a JA-dependent manner. The *myc2-3* mutation was either fully (*JAZ5*, *LOX3*, *TAT*) or partially (*VSP2*, *PR4*, *PDF1.2*) epistatic to *tic-2* (Figures 4A to 4F). These patterns of gene expression are consistent with our physiological results in Figures 2 and 3 showing a MYC2-dependent repressive function of TIC in JA signaling. Collectively, our data suggest that TIC negatively affects JA signaling and that functional MYC2 is required for this.

We determined the expression level of several JA biosynthetic genes in the wild type and *tic-2* under WLc to test whether the increased JA sensitivity of *tic-2* was caused by elevated JA biosynthesis. Transcript levels of various JA biosynthesis genes were not significantly different between *tic-2* and wild-type plants (see Supplemental Figure 5 online). Therefore, enhanced responsiveness of *tic-2* to JA is unlikely to be caused by elevated JA biosynthesis.

We examined JA responses in the arrhythmic clock mutant *elf3-1* (Hicks et al., 1996; McWatters et al., 2000; Reed et al., 2000; Thines and Harmon, 2010; Kolmos et al., 2011) to investigate whether the altered JA sensitivity in *tic-2* is caused by



**Figure 5.** TIC Participates in the MYC2 Protein Depletion.

(A) Immunoblot analysis of MYC2-myc and histone H3 protein in *MYC2ox* and *tic-2 MYC2ox* plants.

(B) qRT-PCR of *MYC2-myc* in *MYC2ox* and *tic-2 MYC2ox* plants. Gene expression indicates a mean of three technical replicates, and error bars indicate sd.

(C) Immunoblot analysis of MYC2-His<sub>6</sub> protein in a cell-free degradation assay. Recombinant MYC2-His<sub>6</sub> protein was incubated in total protein extract of Col or *tic-2* for indicated times. MYC2 protein was detected using anti-his<sub>6</sub> antibody, and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) was visualized after Ponceau S staining. This cell-free assay was repeated three times, always with consistent results.

(D) Immunoblot analysis of MYC2-His<sub>6</sub> protein in a cell-free degradation assay. Recombinant MYC2-His<sub>6</sub> protein was incubated for 20 min with or without DMSO and MG132.

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general clock defects. Root growth of *elf3-1* on MeJA-containing media was similarly inhibited as in the wild type, and MeJA-mediated gene induction of *JAZ5* and *TAT* was comparable between *elf3-1* and the wild type (see Supplemental Figure 6 online). Therefore, we concluded that the altered JA response in *tic-2* is not the consequence of general clock defects.

### MYC2 Protein Accumulation Is Elevated in the *tic* Mutant

We next investigated whether TIC and MYC2 regulate the expression of each other. We examined transcript accumulation of these genes in the reciprocal mutants. *MYC2* transcript levels in *tic-2* grown under WLC were similar to those in wild-type plants (see Supplemental Figure 7A online). Also, *TIC* transcript accumulation was not significantly changed in *myc2* mutants or *MYC2ox* plants (see Supplemental Figure 7B online). Therefore, TIC and MYC2 do not cross-regulate the transcript expression of each other.

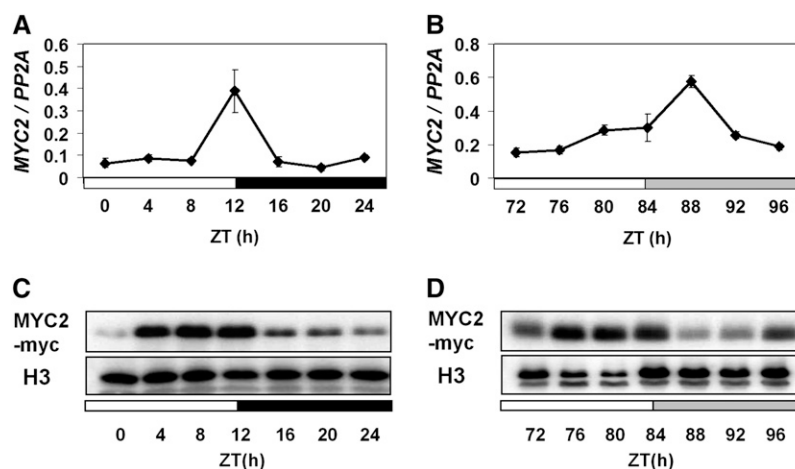
We next tested whether TIC regulates MYC2 protein accumulation. *MYC2ox* plants were crossed with *tic-2* to generate a *tic-2 MYC2ox* line. Although *MYC2-myc* transcript levels were slightly higher in *tic-2 MYC2ox* compared with *MYC2ox*, MYC2 protein accumulation was dramatically increased in *tic-2 MYC2ox* compared with *MYC2ox* (Figures 5A and 5B). We additionally examined MYC2 protein stability in *tic-2* by a cell-free protein degradation assay. Recombinant MYC2-His<sub>6</sub> protein was purified from *Escherichia coli* and incubated with a soluble protein extract from Col or *tic-2*, respectively. The MYC2-His<sub>6</sub> protein was rapidly degraded within 15 min in Col extract, whereas it remained more stable for 20 min in *tic-2* extract (Figure 5C). We further found that the proteasome is involved in this MYC2 depletion processes, because MYC2 protein degradation was restricted by application of the proteasome inhibitor MG132 in Col extract (Figure 5D). Together, these results

indicate that TIC participates in a process leading to MYC2 protein depletion.

### MYC2 Expression Oscillates under Control of the Circadian Clock

We next examined whether the circadian clock controls the expression of MYC2. *MYC2* transcript profiles were investigated under diurnal and circadian conditions. Plants were entrained under 12-h light/12-h dark diurnal conditions for 7 d and then transferred to WLC free-running conditions for a further 3 d. Total RNA was extracted from replicate samples of plants at defined time points, and *MYC2* mRNA levels were measured by qRT-PCR. *MYC2* was rhythmically expressed during a 24-h period, with expression highest at dusk (zeitgeber time [ZT] 12) under diurnal conditions (Figure 6A). This rhythmic expression pattern persisted under free-running conditions, with transcripts becoming most abundant at subjective dusk (Figure 6B).

We additionally tested the protein accumulation pattern of MYC2 under diurnal and free-running conditions. *MYC2ox* plants were used for this analysis to remove circadian control of *MYC2* transcript accumulation (shown above; Figures 6A and 6B). Whereas *MYC2* transcripts were constantly present in *MYC2ox*, MYC2 protein accumulation varied rhythmically throughout the day (Figure 6C; see Supplemental Figure 8 online). Under diurnal conditions, MYC2 protein was abundant at midday (ZT 4 to ZT 12) and decreased in darkness. This protein destabilization required the proteasome, because application of the MG132 led to increased MYC2 steady state accumulation at night (see Supplemental Figure 9A online). We wanted to establish whether MYC2 protein accumulation was directly regulated by light signaling or by diurnal signaling. For this, MYC2 protein levels were measured in *MYC2ox* plants grown under continuous darkness, continuous red light, or continuous far-



**Figure 6.** MYC2 Transcript and Protein Accumulation Are Both Regulated by the Circadian Clock.

qRT-PCR of *MYC2* relative to *PP2A* either under diurnal conditions (A) or under free-running conditions (B). Col plants were grown under 12-h light/12-h dark conditions for 7 d then transferred to continuous light for 3 d. The measurements of gene expression indicate a mean of three technical replicates, and error bars indicate SD. qRT-PCR experiments were repeated three times, each with an independent biological replicate, and similar results were obtained. Immunoblot analysis of MYC2-myc and histone H3 protein in *MYC2ox* plants, either under diurnal conditions (C) or free-running conditions (D). Immunoblot experiments were repeated three times, each with an independent biological replicate, and similar results were obtained.

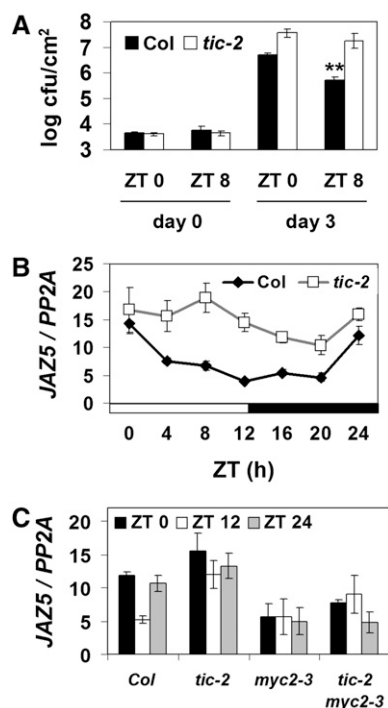
red-light conditions. MYC2 accumulation was not reduced in darkness; rather, its level was comparable with or even higher than those observed under continuous red light or continuous far-red-light conditions (see Supplemental Figure 9B online). Therefore, MYC2 protein does not seem to be stabilized by light signaling. We further determined MYC2 protein levels under free-running conditions and found rhythmic accumulation. MYC2 protein abundance peaked at subjective day and decreased at subjective night (Figure 6D). Taken together, the circadian-clock regulation of MYC2 accumulation uses both transcriptional and posttranslational mechanisms.

### JA Response Is Gated by the Circadian Clock

Several studies have reported connections between the circadian clock and various phytohormone-signaling pathways (Hanano et al., 2006; Salomé et al., 2006; Legnaioli et al., 2009; Rawat et al., 2009). This led us to test whether the JA response is gated by the circadian clock and whether TIC is involved in a clock-gating process. We first compared the susceptibility of Col plants with *Pst* DC3000 at different times of day. Plants grown under short-day conditions (8-h light/16-h dark) were inoculated with *Pst* DC3000 either at dawn (ZT 0) or at dusk (ZT 8), and bacterial growth was determined 72 h later. Although initial titers of *Pst* DC3000 bacteria were similar at both examined time points, ~10 times more *Pst* DC3000 bacteria were measured in Col at day 3 when plants were inoculated at dawn compared with plants infected at dusk (Figure 7A). Notably, this timing effect was significantly attenuated in *tic-2*, because *Pst* DC3000 growth remained high in *tic-2* regardless of the inoculation time of day (Figure 7A). We performed the same experiment with the *Pst* COR<sup>-</sup> strain and found that Col and *tic-2* plants displayed similarly low bacterial titers at day 3 regardless of the inoculation time of day (see Supplemental Figure 10 online). These results suggest that the JA response triggered by COR is higher in the morning than in the evening.

We next examined JA-responsive gene expression at different times of day. Col plants were grown under 12-h light/12-h dark conditions, and MeJA was applied for 1 h at 4-h intervals over 1 d. Then, immediate-early-induced JA-responsive gene expression was determined. Induction of *JAZ5* expression by MeJA treatment was significantly higher at dawn (ZT 0) relative to other time points over a 24-h period (Figure 7B). *MYC2* was also rhythmically induced by exogenous MeJA in a similar pattern with *JAZ5* (see Supplemental Figure 11 online). Therefore, *MYC2* peak-expression phase was shifted from dusk to dawn, in addition to the transcript accumulation being significantly induced by exogenous JA treatment (Figure 6A; see Supplemental Figure 11 online). However, in *tic-2*, *JAZ5* expression on MeJA treatment was not rhythmically regulated, but it was maintained at a high level (Figure 7B). Therefore, the JA response seems to be elevated in *tic-2* compared with the wild type, especially at dusk. These data collectively indicate that the circadian clock gates JA responses in a TIC-dependent manner.

We determined *JAZ5* induction by MeJA in Col, *tic-2*, *myc2-3*, and *tic-2 myc2-3* mutants both at dawn (ZT 0, ZT 24) and at dusk (ZT 12). Consistent with the results in Figure 4A, *JAZ5* transcript levels were low at all examined time points in *myc2-3*



**Figure 7.** JA Response Is Gated by the Circadian Clock.

**(A)** Growth of *Pst* DC3000 in Col and *tic-2* plants 72 h after infection. Plants were grown under short-day (8-h light/16-h dark) conditions for 5 weeks and were inoculated with *Pst* DC3000 either at ZT 0 or ZT 8. Bacterial counts are expressed as a log scale (cfu/cm<sup>2</sup>). Error bars indicate SE. Asterisks indicate statistically significant differences of ZT 8 compared with ZT 0 of each genotype (Student's *t* test, \*\**P* < 0.01).

**(B)** qRT-PCR of *JAZ5* expression in Col and *tic-2* plants. Plants were grown under 12-h light/12-h dark conditions for 7 d and treated with 50 μM MeJA for 1 h at different times as indicated.

**(C)** qRT-PCR of *JAZ5* expression in Col, *tic-2*, *myc2-3*, and *tic-2 myc2-3* plants, treated with 50 μM MeJA for 1 h at ZT 0, ZT 12, and ZT 24. The measurements of gene expression indicate a mean of three technical replicates, and error bars indicate SD. qRT-PCR experiments were repeated three times, each with an independent biological replicate, and similar results were obtained.

as well as in *tic-2 myc2-3* (Figure 7C). Interestingly, the *JAZ5* level in *myc2-3* was similar to that of wild-type plants at dusk (ZT 12). Therefore, *JAZ5* is only marginally induced by MeJA at dusk in a wild-type background.

### *COI1* Transcription Is Controlled by the Circadian Clock in a TIC-Dependent Manner

Our results show that JA-responsive gene induction is differentially regulated depending on the time within diurnal periods (Figure 7B; see Supplemental Figure 11 online). We reasoned that JA signaling, rather than JA biosynthesis, is controlled by the clock for this gating effect. To test this hypothesis, we measured transcript accumulation of the JA receptor *COI1* under 12-h light/12-h dark diurnal conditions. *COI1* transcript levels oscillated, with expression highest at dawn (ZT 0; Figure 8A). This corresponds to the time when plants most strongly

respond to JA (Figure 7). We then measured *COI1* transcript accumulation after MeJA application. Although MeJA application did not change the rhythmic expression pattern of *COI1*, it slightly lowered *COI1* expression at all examined time points (Figure 8A). Strikingly, *COI1* transcripts did not oscillate in *tic-2* and were not reduced by MeJA treatment (Figure 8B). Thus, *COI1* expression maintained an elevated peak during 24 h in *tic-2* (Figure 8C). *COI1* transcript levels in the *tic-2 myc2-3* double mutant were similar to those of *tic-2* (Figure 8D). Therefore, *MYC2* is not required for evening-specific depression of *COI1* in *tic-2*.

### JA Promotes *MYC2* Protein Accumulation in a Circadian Clock-Dependent Manner

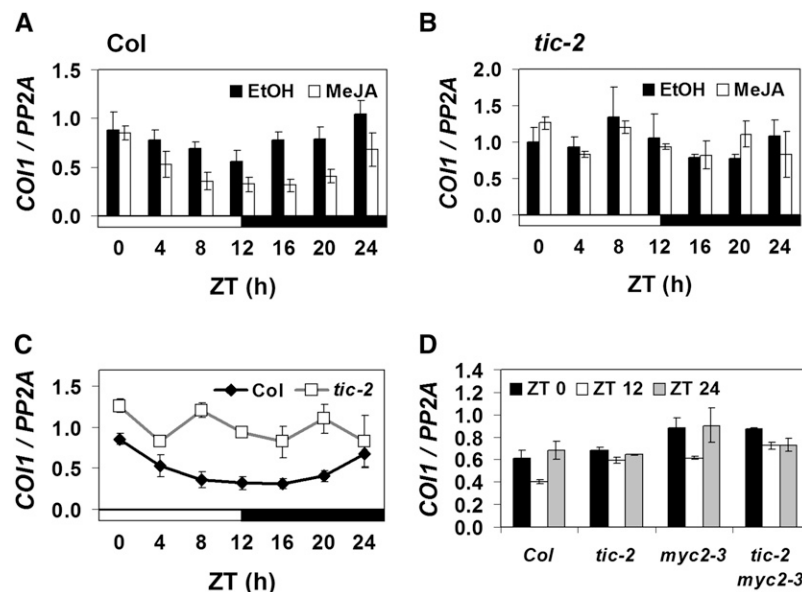
We measured *MYC2* protein accumulation in the *tic-2* background under diurnal conditions. Consistent with the results in Figure 6C, *MYC2* accumulated during the day in *MYC2ox* plants (Figure 9A). In *tic-2 MYC2ox* plants, a similar rhythmic *MYC2* protein accumulation pattern was observed; however, *MYC2* protein levels were dramatically elevated during the entire day. This was particularly pronounced at night phases and at dawn (Figure 9B). We then investigated whether *MYC2* protein accumulation is regulated by JA in a time-of-day-dependent manner. Plants were grown under 12-h light/12-h dark conditions and treated for 1 h with MeJA at dawn (ZT 0) or at dusk (ZT 12),

respectively. *MYC2* protein accumulation was increased by MeJA treatment at ZT 0, but not at ZT 12 (Figure 9C). Therefore, *MYC2* protein accumulation was more susceptible to the effects of JA in the morning. These data collectively indicate that both TIC and JA-mediated temporal *MYC2* stabilization contribute to clock-gated JA responses.

### DISCUSSION

Both the circadian clock and hormone signaling are involved in the regulation of plant growth and development by synchronizing the internal growth state to external environmental changes. Therefore, those signaling systems are tightly interconnected and share regulating networks. In this study, we identified signaling crosstalk between the circadian clock and the JA pathway, mediated by TIC. We found that TIC physically interacts with *MYC2* to deplete *MYC2* protein accumulation. Consequently, TIC acts as a negative regulator of the JA pathway in a *MYC2*-dependent manner. We further showed that the circadian clock gates JA responses and that TIC is required for evening-specific inhibition of JA signaling.

*tic-2* displayed an enhanced responsiveness to JA in the regulation of root growth, susceptibility to *Pst* DC3000 infection, and JA-dependent wound-response gene expression (Figures 2, 3, 4C, and 4D). By contrast, TIC promotes JA-dependent pathogen-response gene expression (Figures 4E and 4F), and



**Figure 8.** TIC Is Required for Rhythmic *COI1* Expression under Diurnal Conditions.

qRT-PCR of *COI1* expression relative to *PP2A*. Plants were grown under 12-h light/12-h dark conditions for 7 d and treated either with 0.01% ethanol (EtOH) as a mock control ([A] and [B]) or with 50  $\mu$ M MeJA for 1 h at different times as indicated ([A] to [D]).

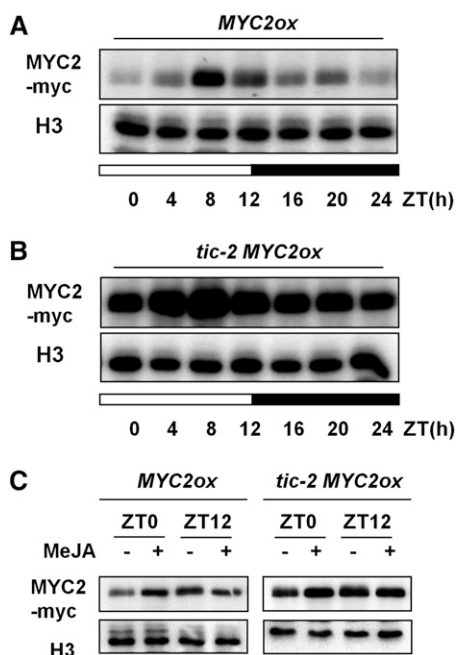
(A) *COI1* expression in Col plants in response to either ethanol (gray bar) or MeJA (white bar) at different times of day.

(B) *COI1* expression in *tic-2* plants in response to either ethanol (gray bar) or MeJA (white bar) at different times of day.

(C) Comparison of *COI1* expression in Col and *tic-2* on MeJA treatment for 1 h under diurnal conditions.

(D) *COI1* expression by 50  $\mu$ M MeJA treatment for 1 h at ZT 0, ZT 12, and ZT 24 in Col, *tic-2*, *myc2-3*, and *tic-2 myc2-3* plants. The measurements of gene expression indicate a mean of three technical replicates, and error bars indicate sd. qRT-PCR experiments were repeated three times, each with an independent biological replicate, and similar results were obtained.





**Figure 9.** MYC2 Protein Accumulation Is Promoted by JA Treatment Predominantly at Dawn.

Immunoblot analysis of MYC2-myc and histone H3 protein levels in *MYC2ox* (A) and *tic-2 MYC2ox* plants (B). Plants were grown under 12-h light/12-h dark conditions for 7 d.

(C) Immunoblot analysis of MYC2-myc and histone H3 protein levels in *MYC2ox* and *tic-2 MYC2ox* plants, with or without 50  $\mu$ M MeJA treatment for 1 h either at ZT 0 (dawn) or ZT 12 (dusk). Immunoblot experiments were repeated three times, each with an independent biological replicate, and similar results were obtained.

TIC acts as a negative regulator of MYC2 by promoting MYC2 protein degradation (Figure 5). For JA-driven repression of root growth, *myc2* was completely genetically epistatic to *tic* (Figures 2A and 2B), whereas MYC2 was only partially necessary for JA-responsive gene expression and resistance to *Pst* DC3000 infection (Figures 3 and 4). These data suggest the presence of additional downstream regulators of TIC in JA signaling. MYC2, MYC3, and MYC4 are known to be functionally redundant mainly in aerial tissues in a wide range of JA responses, such as JA-induced gene expression and the pathogen and herbivore infection responses (Fernández-Calvo et al., 2011). MYC family genes also have functional specificity. MYC2 predominantly acts in the root tissue, which supports our root growth results of *tic-2 myc2-3* on JA-containing media. It is possible that MYC3 and MYC4 function downstream of TIC in the aerial parts of the plant, and this awaits to be tested.

We showed here cross-regulation of JA signaling and the circadian clock. JA-signaling activity varied depending on the time of day, which is caused by a circadian gating effect. This allows higher JA response in the morning phase. Plants were more susceptible to infection by *Pst* DC3000 at dawn than at dusk, and JA-responsive gene (*JAZ5* and *MYC2*) induction on JA treatment was highest at dawn (Figure 7; see Supplemental Figure 11 online). TIC was required for the clock gating of JA responses.

Susceptibility to *Pst* DC3000 infection and JA-responsive gene induction were increased in *tic-2*, regardless of the specific time of a day. Although *tic-2* shows only marginally increased sensitivity to JA compared with the wild type at dawn, the difference between the wild type and *tic-2* became pronounced at dusk (Figure 7). Therefore, we found that TIC is required at dusk to inhibit JA responses. Because TIC was shown to reset the circadian core clock at the late-night phase (Hall et al., 2003), the function of TIC on the circadian core oscillator and clock output pathways seems to be temporally divided.

In microarray studies, it was reported that clock-regulated JA-responsive genes were mostly expressed in the morning phase (Covington et al., 2008). Some JA-biosynthesis genes were also identified to be rhythmically expressed in a 24-h frame (Mockler et al., 2007). In a recent in planta study, the JA level was found to be rhythmic, with maximum abundance in the middle of the day and lower levels during the night (Goodspeed et al., 2012). Notably, the JA synthesis and signaling pathway is acutely induced by stress signals, such as wound or pathogen infection, whereas it is maintained at only basal levels under nonstressed conditions. Therefore, rhythmic biosynthesis of JA and its signaling gene expression under nonstressed conditions would not likely correlate with the stress-triggered rhythmic JA responses that we have shown in this study.

Daily plant growth is promoted during the night phase and is known to be regulated by rhythmic auxin and GA signaling (Covington and Harmer, 2007; Nozue et al., 2007; Rawat et al., 2009; Arana et al., 2011). Our results support that clock-gated JA responses also contribute to a temporal balance between daily growth and stress responsiveness, because JA response activates stress responses at the expense of energy needed for plant growth (Walters and Heil, 2007). Such a mechanism may allow plants to promote growth in the evening with less-active JA signaling and restrict growth in the morning when JA response is more active. Oxidative stress caused by the dark-to-light transition is one example of a daily event in which JA signaling is vital to plants (Sasaki-Sekimoto et al., 2005; Wolucka et al., 2005; Dombrecht et al., 2007). In previous genomic analysis, clock-regulated genes responsive to the reactive oxygen species or to oxidative damage were shown to be expressed early in the morning phase (Covington et al., 2008). This is consistent with our results showing the clock-gated active JA response in the morning.

The JA pathway is important for defense against necrotrophic pathogen infection and wounding by feeding insects (Howe, 2004; Browse, 2009; Goodspeed et al., 2012). Based on our data, we speculate that plants can predict both the timing of pathogen infection and insect attack by time-specific defense-pathway activation to maximize the defense response against a particular pathogen or pest. Previous studies have shown that plant immune responses against obligate biotrophic pathogens are regulated by the circadian clock (Wang et al., 2011). It is possible that this response allows plants to anticipate pathogen infection in the morning. In particular, the circadian morning element CCA1 was found to direct *R* gene expression. TIC has been shown to shorten the circadian period and reduce the expression amplitude of the core oscillator, such as CCA1 (Ding et al., 2007). It will be useful to test the functional relationship

between TIC and obligate biotrophic pathogen infection in the future.

Here we suggest two components contributing to the JA-gating mechanism: regulation of the expression of the JA receptor *COI1* and JA-mediated temporal regulation of MYC2 protein accumulation. We showed here that *COI1* transcript accumulation oscillates, with the highest expression at dawn, which corresponds to the time when JA response is high (Figure 8). Previously, the activity of COI1 was shown to correlate with JA responses (Li et al., 2006; Mosblech et al., 2011). This supports our hypothesis that rhythmic *COI1* expression would correlate with rhythmic JA responses. We additionally found that the expression of *COI1* was modestly downregulated by exogenous JA treatment. This might be caused through transcriptional feedback regulation, which could create homeostasis of JA signaling. In *tic-2*, *COI1* transcript accumulation did not oscillate and was not reduced by JA. The transcriptional regulation of *COI1* in *tic-2* was not a MYC2-dependent event, because *myc2-3 tic-2* double mutants displayed a similar *COI1* expression pattern as *tic-2* (Figure 8D). We additionally found that JA predominantly promotes MYC2 protein accumulation in the morning. TIC was shown to repress the basal MYC2 protein level during the entire diurnal period; however, its role in the regulation of JA-mediated MYC2 protein accumulation was limited. The underlying molecular mechanism for temporal regulation of JA-mediated MYC2 accumulation should be investigated in future studies.

## METHODS

### Plant Material and Growth Conditions

*Arabidopsis thaliana* Col accession is the genetic background of the wild type and transgenic lines used in this study. Plants were grown on Murashige and Skoog (MS) media (one-half-strength MS [Sigma-Aldrich], 0.8% phytoagar, and 0.05% MES [Duchefa], pH 5.7, 1% Suc) at 22°C under various light conditions. Mutant lines *myc2-3* (salk\_061267) and *myc2-4* (salk\_130877) were obtained from the Nottingham Arabidopsis Stock Centre. *tic-2* (Ding et al., 2007) and *elf3-1* (Hicks et al., 1996; Kolmos et al., 2011) have been previously reported. The *tic-2 myc2-3* double mutant was generated by crossing the corresponding parental homozygous lines and genotyping F2 segregating progenies to select homozygous mutations. Primers for genotyping mutants are listed in Supplemental Table 1 online. To generate *MYC2ox* transgenic plants, full-length *MYC2* cDNA was amplified with gene-specific primers (see Supplemental Table 1 online), cloned into the *Sma*I and *Avr*II sites of the *pBI-HTM* vector (kindly provide by Giltsu Choi), and transformed into Col by *Agrobacterium tumefaciens*-mediated transformation (Davis et al., 2009).

### Yeast Two-Hybrid Assay

Full-length *MYC2* and N terminus *TIC* (encoded amino acids 1 to 564) were amplified with Gateway-compatible primers (see Supplemental Table 1 online) and PCR products inserted into pDONR201 with a Gateway BP kit (Invitrogen). A *MYC2* construct was used in Gateway LR reactions in combination with the destination yeast-expression vector, pACT-attR (GAL4 AD). A *TIC* construct was used in Gateway LR reactions in combination with the destination yeast-expression vector, pAS-attR (GAL4 BD). To examine protein-protein interactions in yeast, corresponding plasmids were cotransformed into *Saccharomyces cerevisiae* AH109 according to the Clontech manual, and transformed cells were spread on selective yeast synthetic dropout media.

### Root Measurements

For the JA-mediated root-growth-inhibition assay, seeds were plated on MS agar media with or without 50  $\mu$ M MeJA (Duchefa), placed for 3 d at 4°C in the dark, and subsequently placed vertically under WLC for 10 d. Three independent biological replicates were measured for each sample, and similar results were obtained from each independent experiment. Comparison between Col and mutants (*tic-2*, *myc2-3*, *myc2-4*, *MYC2ox*, and *tic-2 myc2-3*) were performed by Student's *t* test.

### Gene Expression Analysis

Seedlings were grown for 7 d before sampling under various photoperiod conditions, as indicated in the results. For the analysis of JA-induced gene expression, plants were treated with 50  $\mu$ M MeJA (Duchefa) or 0.01% ethanol (mock control) for the time indicated for each experiment. Total RNA was extracted from seedlings using Spectrum Plant Total RNA Kit (Sigma-Aldrich) according to the manufacturer's instructions. cDNA was synthesized from 4  $\mu$ g of total RNA with Maxima First Strand cDNA Synthesis Kit (Fermentas). To amplify genes, 5  $\mu$ L of 1/25 diluted cDNA was used as the template. qRT-PCR analysis was performed using SYBR and iQ5 (Bio-Rad). Primer sequences for qRT-PCR are listed in Supplemental Table 1 online. The resulting gene expression levels were normalized with the level of *PP2A* as described previously (Czechowski et al., 2005). Data analysis was performed using three technical replicates from each biological sample, and similar results were obtained in three biological replicates.

### Protein Extraction and Immunoblotting

For protein extraction, 100 seedlings were grown on MS-agar plates for 7 d. For the analysis of JA-dependent protein accumulation, plants were treated with 50  $\mu$ M MeJA (Duchefa) or 0.01% ethanol (mock control) for 1 h at the indicated time of each experiment. Seedlings were ground in liquid nitrogen and homogenized in a denaturing buffer (100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-Cl, 8 M urea) with vigorous vortexing. Cell debris was removed by centrifugation at 20,817 relative centrifugal force for 15 min at 4°C. For immunoblot analysis, clarified supernatants were separated in a SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore). For detection of GFP-N TIC and MYC2-myc, the membrane was incubated with anti-GFP antibodies (Yau et al. 2008) and anti-myc antibodies (Cell Signaling), respectively, in PBS buffer containing 0.05% Tween-20. For detection of histone H3, the membrane was incubated in the same buffer with antihistone H3 antibodies (Upstate Biotechnology). Bands were visualized with an enhanced chemiluminescence kit (GE Healthcare), according to manufacturer's instructions.

### Coimmunoprecipitation

Four-week-old *Nicotiana benthamiana* leaves were infiltrated with *A. tumefaciens* GV3101 strain harboring a vector that generates MYC2 protein fused with myc tag, or N terminus TIC (amino acids 1 to 564) fused with a GFP tag. Briefly, transformed *A. tumefaciens* were grown at 28°C in yeast-enriched broth media for 2 d, centrifuged, and resuspended with MS media containing 10% Suc, 2.6 mM MES, pH 5.7, and 150  $\mu$ M acetosyringone. Cells were incubated at room temperature in the dark for 3 h, then transformed *Agrobacterium* were mixed with OD<sub>600</sub> ratios as MYC2:N-TIC:p19 = 0.7:0.7:1.0 and infiltrated into the *N. benthamiana* leaves. At 3 d after infiltration, 100 mg of leaves were collected, ground in liquid nitrogen, and homogenized in a immunoprecipitation buffer (50 mM Tris-Cl, pH, 7.5, 150 mM NaCl, 10% glycerol, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride) in addition to 50  $\mu$ M MG132 (Sigma-Aldrich) and 1 $\times$  complete mini protease inhibitor cocktail (Roche).

Resuspended cell lysates were centrifuged at 20,817 relative centrifugal force for 15 min at 4°C. The clarified supernatant was incubated with 20  $\mu$ L of preequilibrated GFP-Trap-A bead (Chromotek) for 2.5 h at 4°C and washed three times with 1 mL of immunoprecipitation buffer. The immunoprecipitated sample was released by incubation with 2 $\times$  SDS sample buffer followed by a 95°C treatment for 5 min. These samples were then analyzed by immunoblotting, as described above.

#### BIFC Assay

Full-length *TIC*, N terminus *TIC*, *MYC2*, and *PIF5* were cloned into gateway compatible N-YFP and C-YFP vectors (Kwaaitaal et al., 2010) and transformed into *A. tumefaciens* GV3101. Different combinations of the *A. tumefaciens* were infiltrated into *N. benthamiana* as described above. At 3 d after infiltration, YFP fluorescence was visualized using a Leica confocal microscope. BIFC experiments were performed three times, and the same results were observed from each experiment.

#### Cell-Free Protein Degradation Assay

For MYC2-His<sub>6</sub> protein purification, *MYC2* cDNA in pDONR201 was moved into the Gateway-compatible pET-28a vector by Gateway LR reactions. The clone was transformed into the *Escherichia coli* BL21 strain, and MYC2-His<sub>6</sub> protein was isolated using Ni-NTA column (Qiagen).

Cell-free protein degradation assays were performed as described (Chini et al., 2007), with minor modifications. Briefly, total plant protein was extracted with the extraction buffer (20 mM Tris-HCl, pH 7.4, 25 mM NaCl, 0.01% Nonidet P-40, and 1 $\times$  complete mini protease inhibitor cocktail [Roche]). Protein concentration was determined by the Bradford assay, and 200  $\mu$ g total protein was added to the reaction mix (20 mM Tris-HCl, pH 7.4, 2 mM MgCl<sub>2</sub>, 2 mM ATP, 10  $\mu$ g/ $\mu$ L ubiquitin [Sigma-Aldrich]), and 24  $\mu$ g of purified recombinant MYC2-His<sub>6</sub> protein was added. For proteasome inhibitor assays, either 200  $\mu$ M MG132 or 2% DMSO was added. The protein mixture was incubated at 30°C for different times as indicated, and the reaction was stopped by adding 5 $\times$  SDS sample buffer.

#### Bacterial Infection Assays

Plants were grown under short-day (8-h light/16-h dark) conditions for 5 weeks. For spray-infection of *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000 or *Pst* COR<sup>-</sup> strains (Melotto et al., 2006), bacteria were adjusted to 10<sup>8</sup> colony-forming units (cfu)/mL in 10 mM MgCl<sub>2</sub> containing 0.04% (v/v) Silwet L-77 (Lehle seeds). In planta bacterial titers were determined 3 to 4 h after spray infection (day 0) and 3 d after infection. For this, the surface of leaves was sterilized with 70% ethanol followed by two washing steps with water, and samples were taken using a cork borer. Bacterial titers were determined by shaking leaf discs in 10 mM MgCl<sub>2</sub> with 0.01% Silwet L-77 at 28°C for 1 h, as described previously (Tornero and Dangl, 2001; Garcia et al., 2010). Means and SE were calculated from at least three biological replicates per experiment.

#### Accession Numbers

Sequence data from this article can be found in The Arabidopsis Information Resource or GenBank/EMBL databases under the following accession numbers: *TIC* (locus AT3G22380, GenBank NM\_113136), *MYC2* (locus AT1G32640, GenBank NM\_102998), *PIF5* (locus AT3G59060, GenBank NM\_180690), *JAZ5* (locus AT1G1738, GenBank 0 NM\_101599), *LOX3* (locus AT1G17420, GenBank NM\_101603), *VSP2* (locus AT5G24770, GenBank NM\_122386), *TAT* (locus AT2G24850, GenBank NM\_128044), *PR4* (locus AT3G04720, GenBank NM\_111344), *PDF1.2* (locus AT5G44420, GenBank NM\_123809), *PP2A* (locus AT1G13320, GenBank NM\_001198052).

#### Supplemental Data

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

**Supplemental Figure 1.** TIC Interacts with MYC2 in the Nucleus.

**Supplemental Figure 2.** Identification of *myc2* Mutants.

**Supplemental Figure 3.** MYC2 Transcript Levels in MYC2ox Plants.

**Supplemental Figure 4.** Examined Genotypes Are Not Susceptible to *Pst* COR<sup>-</sup>.

**Supplemental Figure 5.** JA Biosynthetic Gene Expression Is Not Altered in *tic-2* Relative to the Wild Type.

**Supplemental Figure 6.** *elf3-1* Does Not Show JA-Defective Phenotypes.

**Supplemental Figure 7.** TIC and MYC2 Do Not Participate in Cross-Regulation of Respective Transcript Accumulation.

**Supplemental Figure 8.** MYC2 Is Overexpressed without Oscillation in MYC2ox Plants.

**Supplemental Figure 9.** MYC2 Protein Is Degraded by the Proteasome.

**Supplemental Figure 10.** Susceptibility to *Pst* COR<sup>-</sup> Is Similar Regardless of Genotypes and Infection Time of Day.

**Supplemental Figure 11.** Circadian Clock Gates the JA-Induced MYC2 Expression.

**Supplemental Table 1.** Primer Lists.

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#### AUTHOR CONTRIBUTIONS

J.S. and S.J.D. designed the research, J.S., K.H., and A.S.V. performed the research, J.S., K.H., J.E.P., and S.J.D. analyzed data, and J.S., K.H., J.E.P., and S.J.D. wrote the article.

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**TIME FOR COFFEE Represses Accumulation of the MYC2 Transcription Factor to Provide Time-of-Day Regulation of Jasmonate Signaling in *Arabidopsis***

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