The Plant Cell, Vol. 32: 242–262, January 2020, www.plantcell.org © 2020 ASPB.

A Jasmonate-Activated MYC2–Dof2.1–MYC2 Transcriptional Loop Promotes Leaf Senescence in Arabidopsis

Mengna Zhuo, Yasuhito Sakuraba, and Shuichi Yanagisawa[1]

Plant Functional Biotechnology, Biotechnology Research Center, The University of Tokyo, Tokyo 113-8657, Japan

ORCID IDs: 0000-0003-1258-7905 (M.Z.); 0000-0002-0432-6767 (Y.S.); 0000-0002-3758-5933 (S.Y.).

DNA binding-with-one-finger (Dof) proteins are plant-specific transcription factors closely associated with a variety of physiological processes. Here, we show that the Dof protein family in Arabidopsis (*Arabidopsis thaliana*) functions in leaf senescence. Disruption of *Dof2.1*, a jasmonate (JA)-inducible gene, led to a marked reduction in promotion of leaf senescence and inhibition of root development as well as dark-induced and age-dependent leaf senescence, while overexpression of *Dof2.1* promoted these processes. Additionally, the *dof2.1* knockout mutant showed almost no change in the transcriptome in the absence of JA; in the presence of JA, expression of many senescence-associated genes, including *MYC2*, which encodes a central regulator of JA responses, was induced to a lesser extent in the *dof2.1* mutant than in the wild type. Furthermore, direct activation of the *MYC2* promoter by Dof2.1, along with the results of epistasis analysis, indicated that Dof2.1 enhances leaf senescence mainly by promoting *MYC2* expression. Interestingly, MYC2 was also identified as a transcriptional activator responsible for JA-inducible expression of *Dof2.1*. Based on these results, we propose that Dof2.1 acts as an enhancer of JA-induced leaf senescence through the MYC2–Dof2.1–MYC2 feedforward transcriptional loop.

INTRODUCTION

DNA binding-with-one-finger (Dof) proteins are transcription factors with a highly conserved DNA binding domain (reviewed in Yanagisawa, 2002). The DNA binding domain of Dof proteins contains a single C2–C2 zinc finger motif that is distinguishable from the zinc finger motifs in animal and yeast proteins (Yanagisawa, 1995, 1996). Therefore, Dof proteins are considered unique to plants (Yanagisawa and Sheen, 1998). Genes encoding Dof proteins are found in a variety of photosynthetic organisms ranging from unicellular algae to flowering plants (Moreno-Risueno et al., 2007; Shigyo et al., 2007); however, the number of *Dof* genes varies considerably among species. For example, the green alga *Chlamydomonas reinhardtii* harbors only one *Dof* gene, whereas flowering plants possess ~30 copies (Yanagisawa, 2015). Although the Dof protein in *C. reinhardtii* is likely involved in regulation of fatty acid metabolism (Ibáñez-Salazar et al., 2014), Dof proteins in flowering plants are involved in a variety of physiological processes (reviewed in Yanagisawa, 2002, 2015), suggesting functional divergence of Dof proteins coupled with multiple rounds of duplication of *Dof* genes during evolution of flowering plants.

The Arabidopsis (*Arabidopsis thaliana*) genome harbors 36 genes encoding Dof proteins (Yanagisawa, 2002). Here we use the names for Dof proteins in accordance with the complete list of *A. thaliana* Dof proteins (Yanagisawa, 2002), although synonyms are also mentioned, if any. Four closely related Dof proteins (CYCLING DOF FACTOR1 [CDF1]/Dof5.5, CDF2/Dof5.2, CDF3/Dof3.3, and CDF5/Dof1.10) control photoperiodic flowering (Imaizumi et al., 2005; Fornara et al., 2009), whereas COGWEEL1/Dof1.5 and CDF4/Dof2.3 are associated with phytochrome signaling and gibberellic acid (GA) biosynthesis (Park et al., 2003; Bueso et al., 2016). Two homologous Dof proteins, DOF AFFECTING GERMINATION1/Dof3.7 and DOF AFFECTING GERMINATION2/Dof2.5, antagonistically regulate GA biosynthesis and seed germination (Gualberti et al., 2002; Gabriele et al., 2010), whereas DOF6/Dof3.2 promotes the expression of abscisic acid (ABA) biosynthesis- and signaling-associated genes, and negatively regulates seed germination (Rueda-Romero et al., 2012). Additionally, DOF6/Dof3.2 physically interacts with REPRESSOR OF GA-LIKE2, a transcriptional repressor of GA signaling, to regulate seed germination (Ravindran et al., 2017). OBF BINDING PROTEIN1 (OBP1)/Dof3.4, OBP2/Dof1.1, and OBP3/Dof3.6 were initially identified as partners of OBF4, a basic leucine zipper transcription factor that interacts with auxin and salicylic acid-responsive *ocs* elements first identified in the promoters of the octopine synthase gene (Chen et al., 1996). Subsequently, OBP1 was shown to play a key role in cell cycle re-entry by regulating cell cycle-related genes including *CYCLIN D3;3* and *Dof2.3* (Skirycz et al., 2008), while OBP2 and OBP3 were found to influence glucosinolate metabolism (Skirycz et al., 2006) and phytochrome signaling (Ward et al., 2005), respectively. Furthermore, Dof4.2 influences phenylpropanoid metabolism (Skirycz et al., 2007), and Dof4.2, together with its related Dof protein Dof4.4, regulates shoot branching and seed coat formation (Zou et al., 2013). Moreover, Dof4.7, Dof5.1, and STOMATAL CARPENTER1/Dof5.7 are involved in floral organ abscission (Wei et al., 2010), adaxial-abaxial polarity (Kim et al., 2010), and guard cell maturation (Negi et al., 2013), respectively. Involvement of HIGH CAMBIAL ACTIVITY2 (HCA2)/Dof5.6 and Dof5.8 in vascular development has also been shown (Guo et al., 2009; Konishi and Yanagisawa, 2015). However, nearly half of the Dof proteins in Arabidopsis have not yet been characterized, implying that potentially several critical roles of Dof proteins remain unidentified.

[1] Address correspondence to asyanagi@mail.ecc.u-tokyo.ac.jp.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Shuichi Yanagisawa (asyanagi@mail.ecc.u-tokyo.ac.jp).
www.plantcell.org/cgi/doi/10.1105/tpc.19.00297

IN A NUTSHELL

Background: Dof (DNA-binding with one finger) proteins are plant-specific transcription factors associated with a variety of physiological processes; however, it has not been shown that Dof proteins are involved in jasmonate (JA) responses. JA and its derivatives are lipid-derived phytohormones involved in a variety of plant processes, including leaf senescence, inhibition of root elongation, and responses to wounding and pathogen. In Arabidopsis, the bHLH transcription factor MYC2 is a central regulator of JA responses. Previous intensive studies revealed that post-translational regulation of MYC2 occupies a central position in the mechanism underlying JA signaling and responses: In the absence of JA, JASMONATE-ZIM-DOMAIN (JAZ) proteins form complexes with MYC2, the TOPLESS transcriptional co-repressor, and NOVEL INTERACTOR OF JAZ (NINJA) adaptor protein to inactivate MYC2. In the presence of JA, however, JAZ proteins interact with CORONATINE INSENSITIVE 1 (COI1), an F-box protein of the SCF E3 ubiquitin ligase complex, and then JAZ proteins are degraded by the 26S proteasome, leading to the release of MYC2 from JAZ containing-complexes.

Question: *MYC2* expression is strongly induced by JA. Thus, the induction of *MYC2* expression might be associated with JA responses. However, the biological significance and underlying molecular mechanism of this induction are not yet understood.

Findings: Disruption of *Dof2.1*, a JA-inducible gene, led to a marked reduction in promotion of leaf senescence and inhibition of root development, while overexpression of *Dof2.1* promoted these processes, indicating that Dof2.1 functions as an enhancer of JA-induced leaf senescence. Furthermore, Dof2.1 enhanced JA-induced leaf senescence by directly activating the *MYC2* promoter. MYC2 was also identified as a transcriptional activator responsible for JA-inducible expression of *Dof2.1*. Thus, Dof2.1 acts as an enhancer of JA-induced leaf senescence through a MYC2-Dof2.1-MYC2 feedforward transcriptional loop. This finding also indicates that a Dof protein is involved in JA responses.

Next steps: Although we revealed that Dof2.1 directly promotes *MYC*2 expression, other genes whose expression levels are directly regulated by Dof2.1 are yet to be identified. Thus, identification of other direct target genes of Dof2.1 may expand our understanding of the function of Dof2.1 in stress responses.

Jasmonate (jasmonic acid, JA) and its derivatives are lipid-derived phytohormones involved in a variety of plant processes such as leaf senescence, root elongation inhibition, seed fertility and development, and wounding and pathogen responses (reviewed in Wasternack, 2007; Wasternack and Hause, 2013; Chini et al., 2016; Zhang et al., 2017). In Arabidopsis, MYC2, a bHLH-type transcription factor, plays a central role in JA responses (reviewed in Kazan and Manners, 2013). MYC2 binds to the G-box motif and its variants in the regulatory region of many JA response-related genes (Yadav et al., 2005; Dombrecht et al., 2007; Godoy et al., 2011) to promote or reduce their expression (Dombrecht et al., 2007). Consistent with this, disruption and overexpression of *MYC2* affect most JA-related biological processes in Arabidopsis (Qi et al., 2015; Vijayan et al., 1998; Lorenzo et al., 2004; Chen et al., 2011). Intensive studies revealed that posttranslational regulation of MYC2 occupies a central position in the mechanism underlying JA signaling and responses (Chini et al., 2007; Thines et al., 2007; Pauwels et al., 2010; Shin et al., 2012; Jung et al., 2015). In the absence of JA, JASMONATE-ZIM-DOMAIN (JAZ) proteins form complexes with MYC2, TOPLESS transcriptional corepressor, and NOVEL INTERACTOR OF JAZ (NINJA) adaptor protein to inactivate MYC2 (Pauwels et al., 2010). In the presence of JA, however, JAZ proteins interact with CORONATINE INSENSITIVE1 (COI1), an F-box protein of the SCF E3 ubiquitin ligase complex (Xie et al., 1998). Because the JAZ–COI1 complex binds to jasmonoyl-L-isoleucine, a bioactive JA derivative (Fonseca et al., 2009), it acts as a receptor in the JA signaling pathway (Sheard et al., 2010). JAZ proteins are subsequently degraded by the 26S proteasome. The JA-dependent degradation of JAZ proteins releases MYC2 from JAZ-containing complexes, thereby triggering JA responses (Thines et al., 2007; Chini et al., 2007). By contrast, although *MYC2* expression is strongly induced by JA (Lorenzo et al., 2004), the molecular mechanism underlying this rapid induction of *MYC2* expression has not been well understood. However, it may be relevant to JA responses because overexpression of *MYC2* enhances JA responses in Arabidopsis (Lorenzo et al., 2004).

Leaf senescence is the final stage of leaf development that includes degradation of intracellular organelles and decomposition of macromolecules for remobilization of nutrients from senescing leaves to developing tissues and/or storage organs (Woo et al., 2019). Leaf senescence is induced by unfavorable environmental stimuli, such as drought, high salinity, light deprivation, and pathogen attack, while the initiation of leaf senescence is tightly regulated by endogenous developmental factors, such as the levels of phytohormones (Woo et al., 2019). Among phytohormones, ABA, JA, ethylene, salicylic acid, and strigolactones are known to promote leaf senescence (Jibran et al., 2013). During the JA-induced leaf senescence, MYC2 has been shown to directly activate genes encoding chlorophyll catabolic enzymes, including *STAY-GREEN1* (*SGR1*) and *NON-YELLOW COLORING1* (Zhu et al., 2015).

In this study, we explore new connections between the Dof plant-specific transcription factor family and regulation of biological processes unique to plants. By checking the expression patterns of Arabidopsis *Dof* genes, we found that JA treatment induced expression of *Dof2.1* (At2g28510). Thus, we investigated the role of Dof2.1 in JA responses. The results revealed that Dof2.1 functions as an enhancer of JA-induced leaf senescence. Interestingly, MYC2 directly regulated the JA-inducible expression of *Dof2.1*, whereas Dof2.1 promoted *MYC2* expression by directly binding to the *MYC2* promoter, indicating the presence of

a feedforward transcriptional loop. The physiological significance of this MYC2- and Dof2.1-containing feedforward transcriptional loop was verified by epistasis analysis between *Dof2.1* and *MYC2* using the *myc*2 knockout (*myc2*-KO) mutant overexpressing *Dof2.1* and the *dof2.1* knockout (*dof2.1*-KO) mutant overexpressing *MYC2*. The results in this study therefore reveal a critical component of the machinery responsible for JA-induced leaf senescence.

RESULTS

JA-inducible Expression of *Dof2.1*

We first checked the expression patterns of 24 Arabidopsis *Dof* genes using the Arabidopsis eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). The results of this analysis suggested that JA might activate *Dof2.1* (At2g28510; Supplemental Figure 1). Three *Dof* genes that were phylogenetically closely related to *Dof2.1* (*TARGET OF MONOPTEROS 6* [*TMO6*]/*Dof5.3*, *HCA2*/*Dof5.6*, and *DOF6*/*Dof3.2*; Yanagisawa, 2015) appeared to play distinct roles for the following reasons: *TMO6*/*Dof5.3* and *HCA2*/*Dof5.6* were not induced by JA (Supplemental Figure 1, data on *Dof3.2* expression was not available in the database); and *HCA2*/*Dof5.6* and *DOF6*/*Dof3.2* have been shown to be involved in vascular development (Guo et al., 2009) and ABA responses (Rueda-Romero et al., 2012), respectively. To evaluate the possibility that *Dof2.1* is associated with JA responses, we first confirmed the JA-inducible expression of *Dof2.1* in rosette leaves of 3-week–old plants treated with, or left without, 100 μM of methyl jasmonate (MeJA), a bioactive JA derivative, by RT-qPCR (Figure 1A). Expression of *Dof2.1* increased during MeJA treatment, similar to expression of VEGETATIVE STORAGE PROTEIN1 (*VSP1*), a typical JA-inducible gene (Berger et al., 1995). However, *TMO6*/*Dof5.3*, *HCA2*/*Dof5.6*, and *DOF6*/*Dof3.2* were not induced by MeJA treatment (Supplemental Figure 2).

To further investigate the JA-inducible expression of *Dof2.1*, we generated transgenic Arabidopsis plants expressing the β-glucuronidase (GUS) reporter enzyme under the control of the *Dof2.1* promoter (*proDof2.1-GUS* lines). MeJA treatment increased GUS activity in *proDof2.1-GUS* seedlings significantly (Figure 1B). These results indicate that, owing to the JA-inducible *Dof2.1* promoter, JA treatment induced *Dof2.1* expression within 4 h (Figure 1A).

Dof2.1 Enhances JA-induced Leaf Senescence

To investigate the role of Dof2.1 in JA responses, we used an Arabidopsis T-DNA insertion line (GK-668G12-022986). Because the T-DNA was inserted in the second exon of *Dof2.1*, transcripts of *Dof2.1* were not detected in this line (Supplemental Figure 3), indicating that it was a *dof2.1*-KO mutant. We also generated two independent transgenic Arabidopsis lines expressing a Dof2.1-GFP fusion protein under the control of the Cauliflower mosaic virus *35S* promoter. The expression level of *Dof2.1-GFP* in both transgenic lines was more than 15-fold higher than that of *Dof2.1* in wild-type plants (Supplemental Figure 4). These lines, hereafter

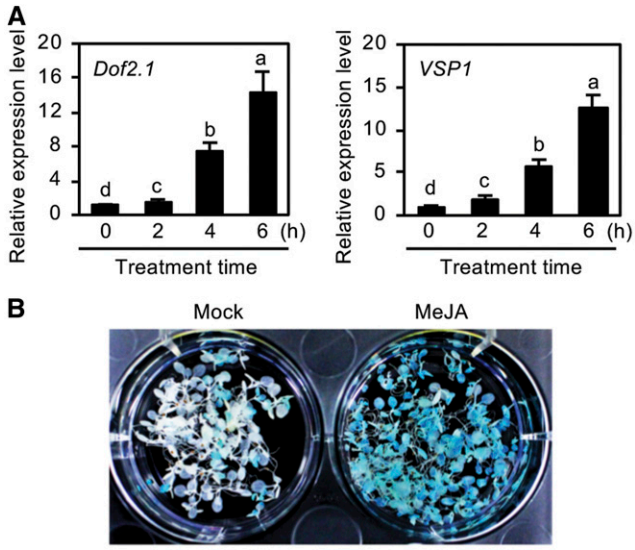

Figure 1. MeJA-Inducible Expression of *Dof2.1*.

(A) MeJA-induced accumulation of *Dof2.1* transcripts. Total RNA was prepared from rosette leaves detached from 3-week–old wild-type plants and then floated on 3 mM of MES buffer containing 100 μM of MeJA for the indicated times. Transcript levels of *Dof2.1* and *VSP1* were normalized first against *ACT2* transcript levels and then against the value obtained from samples at time zero. Data represent the mean ± SD (SD) of five biological replicates (one rosette leaf per replicate). The different letters above each bar indicate that means differ significantly at the 0.05 level in Tukey's multiple comparison test.

(B) *GUS* expression under the control of the *Dof2.1* promoter (−1,847 to +153 bp relative to the transcription start site) in 7-d–old seedlings subjected to MeJA (100 μM) treatment (MeJA) or no treatment (Mock) for 6 h. Each experiment was conducted twice with similar results.

referred to as *Dof2.1* overexpression (*Dof2.1*-OX) lines, were then subjected to phenotypic analysis.

JA promotes leaf senescence (Weidhase et al., 1987), which is used as a marker for JA responses. Thus, we examined JA-induced leaf senescence in wild-type, *dof2.1*-KO, and *Dof2.1*-OX plants. Rosette leaves detached from wild-type, *dof2.1*-KO, and *Dof2.1*-OX plants were similar in color before MeJA treatment; however, *Dof2.1*-OX leaves turned yellow much faster during 3 d of MeJA treatment, while *dof2.1*-KO leaves remained relatively green throughout the treatment (Figure 2A). Additionally, after 3 d of MeJA treatment, chlorophyll and carotenoid contents and maximum quantum yield of photosystem II (F_v/F_m) were significantly higher in *dof2.1*-KO leaves than in wild-type and *Dof2.1*-OX leaves (Figures 2B to 2D), although no differences in yellowing, the chlorophyll content, and the F_v/F_m were observed among wild-type, *dof2.1*-KO, and *Dof2.1*-OX leaves after 3 d of mock treatment (Supplemental Figure 5). These results indicate that JA-induced leaf senescence is delayed by the disruption of *Dof2.1* and promoted by the overexpression of *Dof2.1*.

This conclusion was further verified by phenotypic analysis of three independent *dof2.1*-KO complementation lines, expressing *Dof2.1* under the control of its native promoter (*pro-Dof2.1*:*Dof2.1*/*dof2.1*-KO), and estradiol-inducible *Dof2.1* transgenic

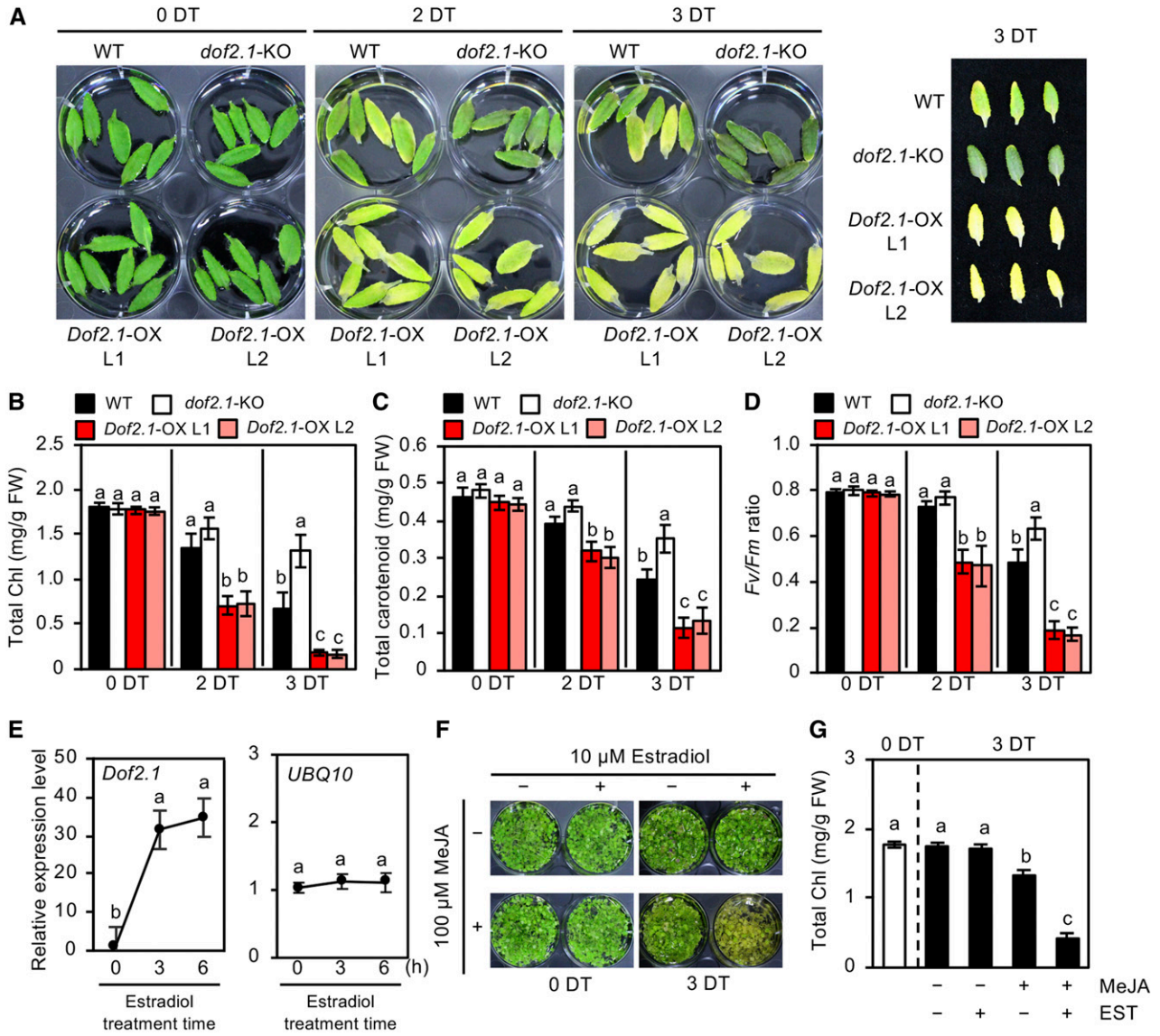

Figure 2. Correlation between *Dof2.1* Expression Level and MeJA-Induced Leaf Senescence.

(A) to **(D)** Effects of MeJA on color **(A)**, total chlorophyll content **(B)**, total carotenoid content **(C)**, and the F_v/F_m **(D)** of fifth-rosette leaves detached from 3-w–old plants of the wild-type, *dof2.1* knockout mutant (*dof2.1*-KO), and two independent transgenic *Dof2.1*-overexpressing lines (*Dof2.1*-OX lines L1 and L2). Detached rosette leaves were floated on 3 mM of MES buffer containing 100 μM of MeJA for the indicated times. In **(B)** to **(D)**, data represent the mean ± SD of sox biological replicates (one rosette leaf per replicate). The same letters above each bar indicate that means did not differ significantly at the 0.05 level in Tukey's multiple comparison test. Chl, chlorophyll; DT, days after the beginning of MeJA treatment; FW, fresh weight; WT, wild type.

(E) Expression levels of *Dof2.1* and *UBQ10* in 7-d–old seedlings of the estradiol-inducible *Dof2.1* transgenic line (*Dof2.1*-EI) treated with 10 μM of estradiol for the indicated times. *Dof2.1* and *UBQ10* transcript levels were normalized first against transcript levels of *ACT2* and then against the value obtained from samples at time zero. The same letters above each bar indicate that means did not differ significantly at the 0.05 level in Tukey's multiple comparison test. Data represent the mean ± SD of six biological replicates (six seedlings per replicate).

(F) and **(G)** Leaf color **(F)** and total chlorophyll content **(G)** of 7-d–old *Dof2.1*-EI seedlings floated on 3 mM of MES buffer containing 10 μM of estradiol and then on 3 mM of MES buffer containing both 10 μM of estradiol and 100 μM of MeJA for the indicated times. In **(G)**, data present the mean ± SD of six biological replicates (six seedlings per replicate), and the same letters above each bar indicate that means did not differ significantly at the 0.05 level in Tukey's multiple comparison test. Each experiment was conducted twice **(A)** to **(E)** or three times **([F]** and **[G])** with similar results. Chl, chlorophyll; EST, estradiol; FW, fresh weight.

line (*Dof2.1*-EI). Because *Dof2.1* is expressed under the control of a β-estradiol–inducible promoter (Zuo et al., 2000) in the *Dof2.1*-EI line, estradiol treatment rapidly induced *Dof2.1* expression (Figure 2E). A delay in JA-induced leaf senescence by the *dof2.1* mutation was mostly diminished in three complementation lines, although the chlorophyll content retained slightly higher in two complementation lines after 3 d of MeJA treatment, compared with wild type (Supplemental Figure 6). On the other hand, JA-induced leaf senescence of the *Dof2.1*-EI line was enhanced by the presence of estradiol, while estradiol alone did not affect leaf senescence (Figures 2F and 2G). Furthermore, we show that JA-induced leaf senescence was facilitated in a T-DNA activation tagging line (*dof2.1-D*) in which *Dof2.1* expression was enhanced by the insertion of a T-DNA carrying constitutive enhancer elements into the promoter region (Supplemental Figure 7). These results conclusively indicate that Dof2.1 acts as an enhancer of JA-induced leaf senescence.

Dof2.1 Promotes Both Dark-induced and Age-dependent Leaf Senescence

JA signaling promotes leaf senescence in response to light deprivation and natural aging (Qi et al., 2015). Similar to expression of *SGR1*, a senescence-associated marker gene (Sakuraba et al., 2014b), expression of *Dof2.1* increased during both dark-induced and age-dependent leaf senescence, although *Dof2.1* expression peaked earlier than *SGR1* expression in both dark-induced and natural senescence (Figures 3A and 3B). Thus, we investigated the effects of *Dof2.1* disruption and overexpression on dark-induced and age-dependent leaf senescence. The *dof2.1*-KO mutant exhibited delayed leaf yellowing, retaining of chlorophyll during dark-induced senescence; however, both phenotypes were enhanced in the *Dof2.1*-OX lines (Figures 3C and 3D). Furthermore, under dark treatment, the *dof2.1*-KO mutant and *Dof2.1*-OX line showed contrasting differences in the levels of photosynthesis-associated proteins (RbcL, PsbC, Lhcb1, and Lhcb4; Figure 3E). Furthermore, although wild-type, *dof2.1*-KO, and *Dof2.1*-OX plants did not show any differences in the number of rosette leaves at bolting and the time to bolting (Supplemental Figure 8), the *dof2.1* KO mutation and overexpression of *Dof2.1* also caused contrasting differences in the chlorophyll content and F_v/F_m of 6-week–old plants but not in those of 4-week–old plants (Figures 3F to 3I). These results suggest that Dof2.1 also promotes both dark-induced and age-dependent leaf senescence.

Dof2.1 Enhances JA-Dependent Inhibition of Root Development and Germination as Well as Wounding Responses

Because JA signaling also inhibits root growth and elongation (Dathe et al., 1981), we investigated JA responses in roots of wild-type, *dof2.1*-KO, and *Dof2.1*-OX seedlings (Figure 4). We measured the primary root length, lateral root number, total lateral root length, and fresh root weight of wild-type, *dof2.1*-KO, and *Dof2.1*-OX seedlings grown in the absence of MeJA for 5 d and then grown in the presence or absence of 5 μM of MeJA for 4 d. Primary root length and fresh root weight of wild-type, *dof2.1*-KO, and *Dof2.1*-OX seedlings were similar when they were transferred onto MeJA-containing plates (Figures 4A to 4C). Furthermore, root development of wild-type, *dof2.1*-KO, and *Dof2.1*-OX seedlings that were additionally grown for 4 d in the absence of MeJA was comparable. However, MeJA-induced inhibition of root development was alleviated in *dof2.1*-KO seedlings but enhanced in *Dof2.1*-OX seedlings (Figures 4A, 4D, and 4G). We note that attenuation and enhancement of JA-induced inhibition of primary root elongation was similarly observed in the *dof2.1*-KO and *Dof2.1*-OX seedlings, respectively, that were continuously grown in the presence of MeJA ever since germination on MeJA-containing plates (Supplemental Figure 9). Moreover, inhibition of germination by JA (Dave et al., 2011) was enhanced by overexpression of *Dof2.1* but attenuated by the *dof2.1* KO mutation (Supplemental Figure 10).

Consistent with the fact that wounding response is a typical JA-regulated process (Yan et al., 2007), wounding was found to elevate the expression level of *Dof2.1* in parallel with increases in the expression of a wounding response marker gene, *LIPOXYGENASE 2* (*LOX2;* Figure 5A; Reymond et al., 2000). Wounding was also shown to activate the *Dof2.1* promoter (Figure 5B). Thus, we further investigated wounding responses in *dof2.1*-KO and *Dof2.1*-OX plants. The results of the analysis suggested that the response to wounding was reduced and enhanced in *dof2.1*-KO and *Dof2.1*-OX plants, respectively (Figure 5C), accompanied with decreased or increased expression of *LOX2* marker gene (Figure 5D). Interestingly, it was found that wounding-induced expression of *MYC2*, a key gene for JA signaling, was also modified in *dof2.1*-KO and *Dof2.1*-OX plants (Figure 5D). These results suggest that Dof2.1 may be involved in not only JA-induced leaf senescence but also other JA responses.

Disruption of *Dof2.1* Modifies Expression Levels of JA Signaling- and Senescence-Related Genes in the Presence of MeJA

Next, to investigate the molecular mechanism underlying Dof2.1-mediated enhancement of leaf senescence, we performed transcriptome analysis using wild-type and *dof2.1*-KO rosette leaves treated with or without MeJA. The results showed minimal differences in gene expression between the *dof2.1*-KO mutant and wild-type leaves in the absence of MeJA but significant differences in expression levels of numerous genes in the presence of MeJA; compared with wild-type leaves, the *dof2.1*-KO mutant leaves exhibited significant upregulation (>2-fold) of 1,745 genes and significant downregulation (<0.5-fold) of 665 genes in the presence of MeJA (Figure 6A; Supplemental Data Sets 1 and 2).

Hierarchical average linkage cluster analysis further clarified the role of Dof2.1 in JA-responsive gene expression (Supplemental Figure 11). Importantly, 263 genes out of 533 genes whose expression levels were strongly elevated by MeJA treatment (>5-fold) in wild-type leaves were significantly downregulated in the *dof2.1*-KO leaves in the presence of MeJA, while 309 genes out of 692 genes whose expression levels were strongly reduced by MeJA treatment (<0.2-fold) in wild-type leaves were significantly upregulated in the *dof2.1*-KO leaves in the presence of MeJA (Figure 6B). Scatterplot analysis further clarified that the *dof2.1* KO mutation significantly alleviated both MeJA-dependent

upregulation and downregulation in the gene expression profile (Figure 6C). These results are concordant with the results of phenotypic analysis and suggest that Dof2.1 globally enhances JA-responsive gene expression during JA-induced leaf senescence.

To verify the results of transcriptome analysis, expression levels of typical JA response- and leaf senescence-associated genes in wild-type, *dof2.1*-KO, and *Dof2.1*-OX plants during JA-induced leaf senescence were examined by RT-qPCR (Figure 7A). The results of RT-qPCR were consistent with those of DNA microarray-based transcriptome analysis and further revealed that *dof2.1* KO mutation and *Dof2.1* overexpression exerted opposite effects on expression of MeJA-responsive genes. Importantly, the results clearly demonstrate that expression levels

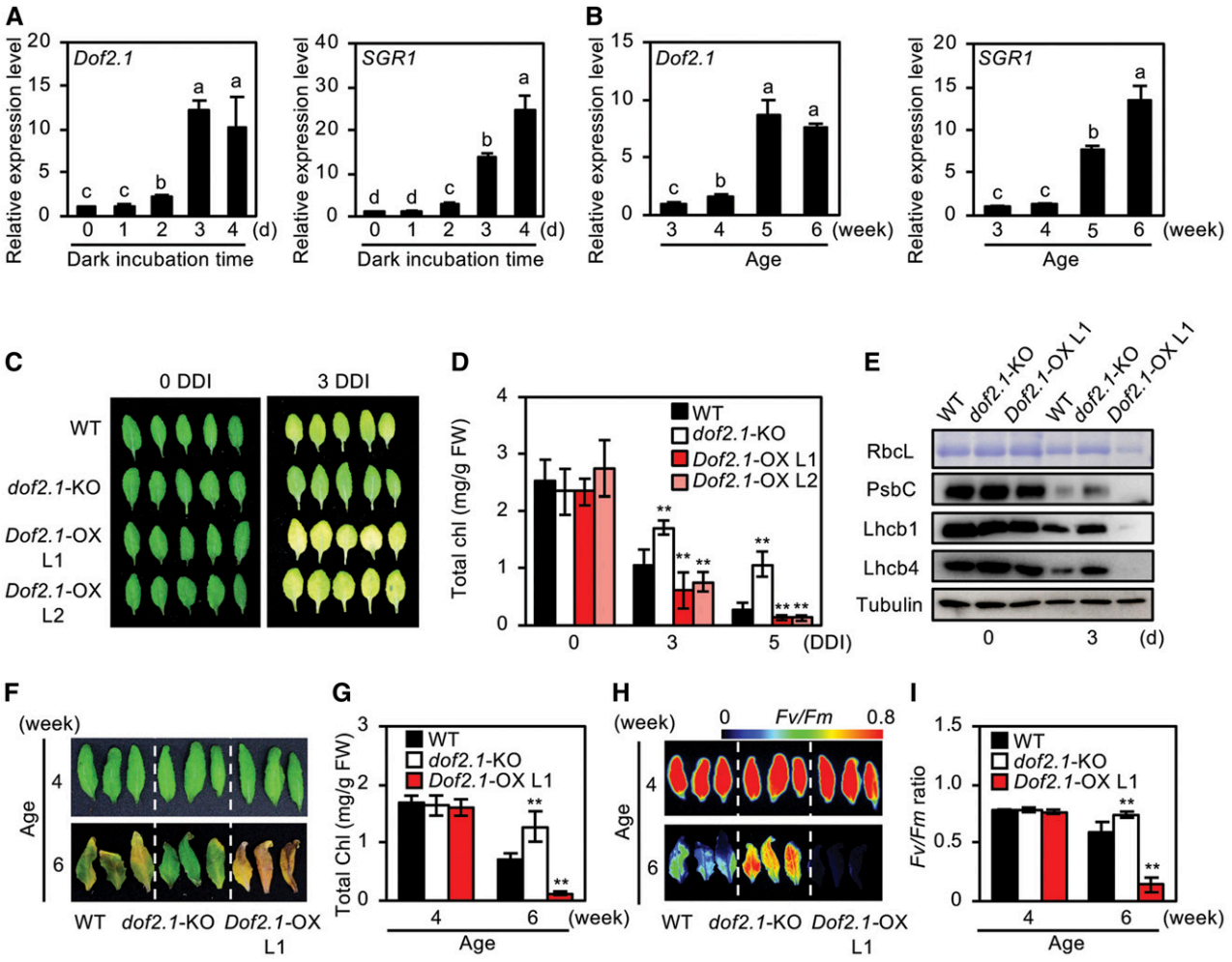

Figure 3. *Dof2.1*-Mediated Acceleration of Dark-Induced and Age-Dependent Leaf Senescence.

(A) and **(B)** Upregulation of *Dof2.1* transcripts during dark-induced **(A)** and age-dependent **(B)** leaf senescence. Total RNA was isolated from the fifth rosette leaf detached from 3-week–old plants and floated on 3 mM of MES buffer in the dark for the indicated times **(A)** and from the fifth rosette leaf of 3- to 6-week–old plants grown under continuous light **(B)**. Expression levels of *Dof2.1* and *SGR1* (control) were normalized first against transcript levels of *ACT2* and then against the value obtained from samples at time zero **(A)** or 3-week–old plants **(B)**. Data represent the mean ± SD of four biological replicates (one rosette leaf per replicate). The same letters above each bar indicate that means did not differ significantly at the 0.05 level in Tukey's multiple comparison test.
(C) to **(E)** Color **(C)**, total chlorophyll content **(D)**, and levels of photosynthesis-associated proteins **(E)** in fifth rosette leaves detached from 3-week–old wild-type, *dof2.1*-KO, and *Dof2.1*-OX (lines L1 and L2) plants. Detached rosette leaves were floated on 3 mM of MES buffer in the dark for the indicated times. In **(D)**, data represent the mean ± SD of six biological replicates (one rosette leaf per replicate). Asterisks ($^{**}P < 0.01$) above each bar indicate significant differences identified between wild type and other samples using Student's *t* test. In **(E)**, PsbC, Lhcb1, and Lhcb4 proteins were detected by immunoblotting, while the large subunit of RbsC was detected by Coomassie Brilliant Blue staining. α-Tubulin was used as a loading control. FW, fresh weight; DDI, days of dark incubation; WT, wild type.
(F) to **(I)** Color **(F)**, total chlorophyll content **(G)**, F_v/F_m image **(H)**, and F_v/F_m values **(I)** of the rosette leaves of 4- and 6-week–old wild-type, *dof2.1*-KO, and *Dof2.1*-OX (line L1) plants grown under continuous light. In **(G)** and **(I)**, data represent the mean ± SD of six biological replicates (one rosette leaf per replicate), and asterisks ($^{**}P < 0.01$) above each bar indicate significant differences identified between wild type and other samples using Student's *t* test. Each experiment was conducted twice **(A)**, **(B)**, **(E)** to **(I)** or three times **(C)** and **(D)** with similar results. FW, fresh weight; WT, wild type.

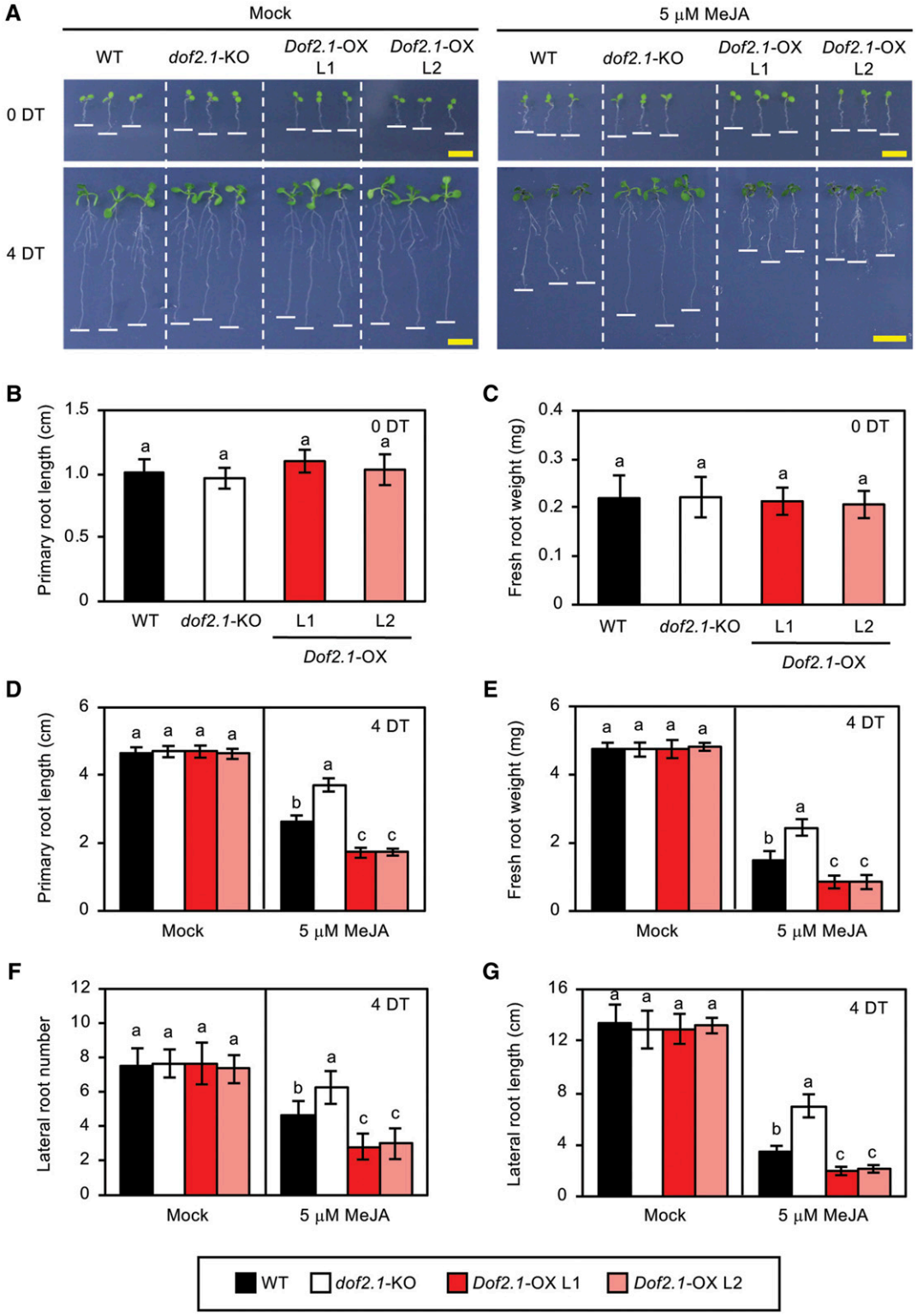

Figure 4. Dof2.1-Dependent Enhancement of MeJA-Mediated Inhibition of Root Elongation.

(A) Root growth of wild-type, *dof2.1*-KO, *Dof2.1*-OX line L1, and *Dof2.1*-OX line L2 seedlings grown on half strength MS agar plates for 5 d (upper representations, 0 d after the beginning of MeJA treatment) and then on half strength MS agar plates containing 5 μM of MeJA or no MeJA (Mock) for an

of *Dof2.1* affect expression of *MYC2* (Supplemental Data Sets 1 and 2; Figure 7A). Unlike most genes under the control of Dof2.1, *MYC2* expression in the *dof2.1*-KO mutant and *Dof2.1*-OX lines was modified significantly in the absence of MeJA (Figures 6A, 7A, and B7B). Thus, we hypothesized that Dof2.1 enhances leaf senescence by elevating *MYC2* expression. This hypothesis was further supported by the observation that expression levels of many MYC2-regulated genes, which were previously identified using a *myc2*-KO mutant (*jin1-9*; Dombrecht et al., 2007), were also modified by the *dof2.1* KO mutation (Figure 6C; Supplemental Figure 12). Furthermore, time-course analysis revealed that changes in *MYC2* expression observed in the *dof2.1*-KO mutant and *Dof2.1*-OX lines appeared to precede those in *LOX2*, a MYC2-regulated gene, during 3 d of MeJA treatment (Figure 7). We also show that the rapid induction of *MYC2* expression by MeJA (within 3 h) was reduced and enhanced in the *dof2.1*-KO and *Dof2.1*-OX leaves, respectively (Supplemental Figure 13), consistent with the modifications of wound-induced *MYC2* expression in the *dof2.1*-KO and *Dof2.1*-OX plants (Figure 5D).

Dof2.1 Directly Activates the *MYC2* Promoter

To further investigate the relationship between Dof2.1 function and *MYC2* expression, we first examined the relationship between the expression level of *Dof2.1* and *MYC2* in the *Dof2.1*-EI line (Figure 2E). In this line, expression of *MYC2* and a MYC2-regulated gene, *VSP1* (Berger et al., 1995), was elevated during 10 μM of estradiol treatment (Figure 8A), similar to elevation of *Dof2.1* expression shown in Figure 2E, indicating a positive relationship between *Dof2.1* and *MYC2* expression levels. Furthermore, the *MYC2* promoter region (−1,051 to +494 bp relative to the transcription start site) harbors a number of putative Dof binding motifs: 5′-(T/A)AAAG-3′ or its inverse sequence, 5′-CTTT(T/A)-3′ (Figure 8B; Yanagisawa and Schmidt, 1999). Thus, we divided the *MYC2* promoter into five regions and examined direct binding of Dof2.1 to these regions in vivo in a chromatin immunoprecipitation (ChIP) assay using *Dof2.1*-OX plants. Among the five regions in the *MYC2* promoter (Figure 8B), regions b and d were slightly or highly enriched in the immunoprecipitate, whereas other regions showed no enrichment (Figure 8C). Furthermore, cotransfection assays with reporter plasmids containing the luciferase (LUC) gene driven by the *35S* promoter (control) or *MYC2* promoter (Figure 8D) revealed that Dof2.1 activated the *MYC2* promoter but not the *35S* control promoter (Figure 8E). These results strongly suggest that Dof2.1 transactivates the *MYC2* promoter through its interaction with the Dof binding sites in regions b and d.

myc2 KO Mutation Is Epistatic to *Dof2.1* Overexpression in the Modulation of JA-Induced Leaf Senescence and Root Growth Inhibition

Genetic epistasis between *Dof2.1* and *MYC2* was analyzed to obtain evidence for the regulation of *MYC2* expression by Dof2.1 and to determine the physiological significance of this interaction. The *Dof2.1*-OX line was crossed to the *myc2*-KO mutant (SALK_083483), previously referred to as the *atmyc2-2* mutant (Boter et al., 2004). Then, phenotypic traits of plants homozygous for both the *atmyc2-2* mutation and *35S-Dof2.1-GFP* transgene (hereafter referred to as the *Dof2.1*-OX *atmyc2-2* line) were compared with those of wild-type, *Dof2.1*-OX, and *atmyc2-2* plants in the presence and absence of MeJA (Figure 8F; Supplemental Figure 15). In the absence of MeJA, no differences were detected in leaf color and F_v/F_m among the different genetic backgrounds. In the presence of MeJA, *Dof2.1*-OX leaves turned yellow much faster than wild-type leaves, whereas *atmyc2-2* mutant leaves remained green, similar to the result reported previously by Qi et al. (2015). Like *atmyc2-2* leaves, rosette leaves of the *Dof2.1*-OX *atmyc2-2* line also remained green (Figure 8F). We note that the expression level of *Dof2.1* was similar in the *Dof2.1*-OX and *Dof2.1*-OX *atmyc2-2* lines (Supplemental Figure 14). Furthermore, the effects of *Dof2.1* overexpression on the chlorophyll content and F_v/F_m of MeJA-treated leaves were abolished by the *atmyc2-2* mutation, and no difference was observed in the chlorophyll content and F_v/F_m between the *atmyc2-2* mutant and *Dof2.1*-OX *atmyc2-2* line (Figures 8G and 8H). Like *atmyc2-2* leaves, but unlike *Dof2.1*-OX leaves, *Dof2.1*-OX *atmyc2-2* leaves showed resistance to dark treatment (Supplemental Figure 15). Furthermore, *Dof2.1*-OX, *atmyc2-2*, and *Dof2.1*-OX *atmyc2-2* seedlings showed comparable root growth in the absence of MeJA; however, in the presence of MeJA, root growth of wild-type seedlings was better than that of *Dof2.1*-OX seedlings but worse than those of *atmyc2-2* and *Dof2.1*-OX *atmyc2-2* seedlings (Supplemental Figure 16). These results indicate that *MYC2* is necessary for the manifestation of effects by *Dof2.1* overexpression on JA-induced leaf senescence and root growth inhibition.

MYC2 Is Responsible for JA-dependent *Dof2.1* Expression

Expression of both *Dof2.1* and *VSP1* (a MYC2-regulated gene) was similarly induced by MeJA treatment (Figure 1A). Therefore, we investigated possible involvement of MYC2 in JA-inducible *Dof2.1* expression. We generated transgenic Arabidopsis lines

Figure 4. (continued).

additional 4 d (lower representations, 4 d after the beginning of MeJA treatment). Scale bars = 1 cm. DT, days after the beginning of MeJA treatment; WT, wild type.

(B) and **(C)** Primary root length **(B)** and fresh root weight **(C)** of wild-type, *dof2.1*-KO, and *Dof2.1*-OX (lines L1 and L2) at 0 d after the beginning of MeJA treatment. Data represent the mean ± SD of six biological replicates (one seedling per replicate), and the same letters above each bar indicate that means did not differ significantly at the 0.05 level in Tukey's multiple comparison test. DT, days after the beginning of MeJA treatment; WT, wild type.

(D) to **(G)** Primary root length **(D)**, fresh root weight **(E)**, lateral root number **(F)**, and lateral root length **(G)** of wild-type, *dof2.1*-KO, and *Dof2.1*-OX (lines L1 and L2) seedlings at 4 d after the beginning of MeJA treatment. Data represent the mean ± SD of six biological replicates (one seedling per replicate), and the same letters above each bar indicate that means did not differ significantly at the 0.05 level in Tukey's multiple comparison test. Each experiment was conducted twice with similar results. DT, days after the beginning of MeJA treatment.

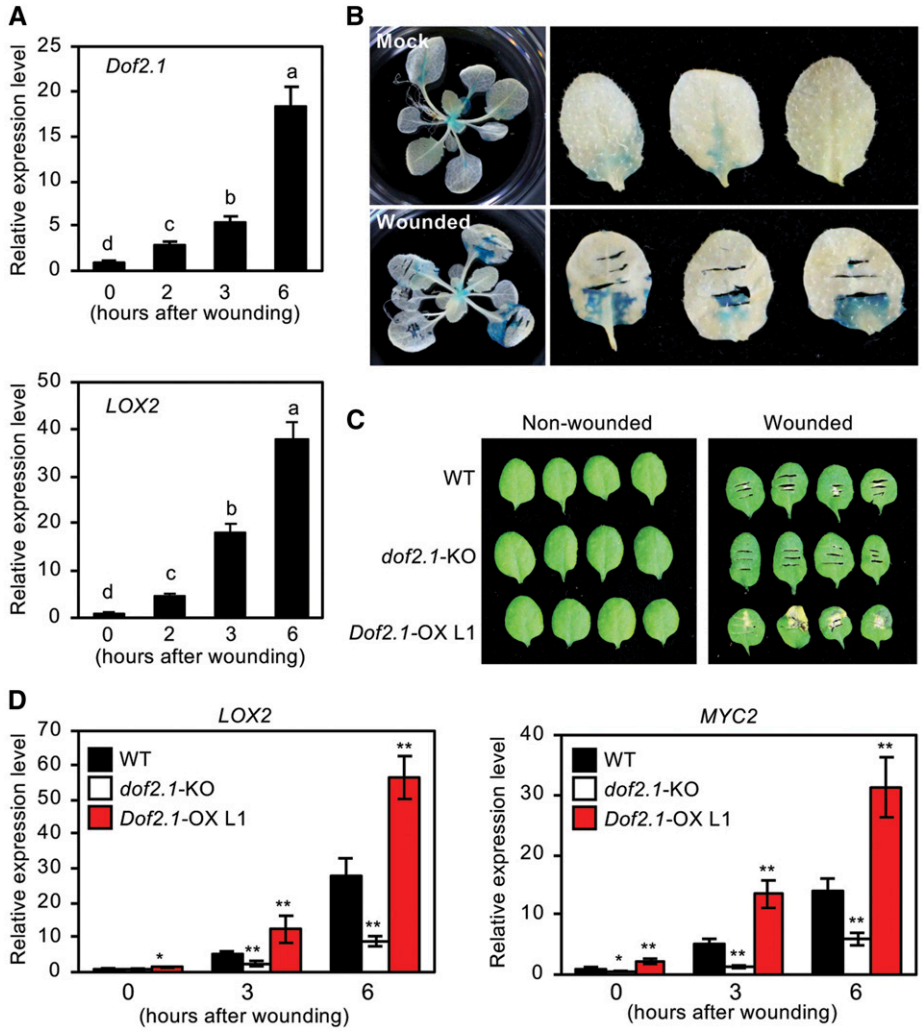

Figure 5. Dof2.1 Modulates Wounding Responses.

(A) The effect of wounding stress on expression of *Dof2.1* and *LOX2* in wild-type seedlings. Total RNA from rosette leaves of 3-week–old wild-type plants that were wounded and then incubated under continuous light condition for indicated periods was used for RT-qPCR analysis. Expression levels of *Dof2.1* and *LOX2* were normalized first against transcript levels of *ACT2* and then against the value obtained with rosette leaves before wounding treatment. Data represent the mean ± SD of four biological replicates (one rosette leaf per replicate), and the same letters above each bar indicate that means did not differ significantly at the 0.05 level in Tukey's multiple comparison test.

(B) *GUS* expression under the control of the *Dof2.1* promoter (−1,847 to +153 bp relative to the transcription start site) in 3-week–old plants subjected to wounding stress or no treatment (Mock) for 6 h. Wounding stress was applied by blades.

(C) Rosette leaves of 2.5-week–old wild-type, *dof2.1*-KO, and *Dof2.1*-OX L1 plants that were wounded by cutting with a blade and then incubated under continuous light condition for 5 d. WT, wild type.

(D) *LOX2* and *MYC2* expression in rosette leaves of 2.5-week–old wild-type, *dof2.1*-KO, and *Dof2.1*-OX L1 plants that were wounded and then incubated for the indicated periods. Expression levels of *LOX2* and *MYC2* were normalized first against transcript levels of *ACT2* and then against the value obtained from wild-type leaves at time zero. Data represent the mean ± SD of four biological replicates (one rosette leaf per replicate), and asterisks ($^{*}P < 0.05$; $^{**}P < 0.01$) indicate significant differences identified between wild type and other seedlings by Student's *t* test. Each experiment was conducted twice with similar results. WT, wild type.

overexpressing *MYC2* (*MYC2*-OX lines), and those showing >5-fold higher mRNA levels than wild-type plants were selected for subsequent analysis (Supplemental Figure 17). In the presence of MeJA, JA-induced *Dof2.1* expression was mostly diminished in the *atmyc2-2* mutant but enhanced in the *MYC2*-OX lines, suggesting that MYC2 is a regulator of *Dof2.1* expression (Figure 9A). In the absence of MeJA, *Dof2.1* expression was slightly but significantly higher in the *MYC2*-OX lines than in the *atmyc2-2* mutant (Figure 9A).

MYC2 binds to the G-box motif (CACGTG) and its variants such as the E-box (CANNTG), G/A box (CACGAG), and G/C box (CACGCG) to regulate expression of target genes (Dombrecht

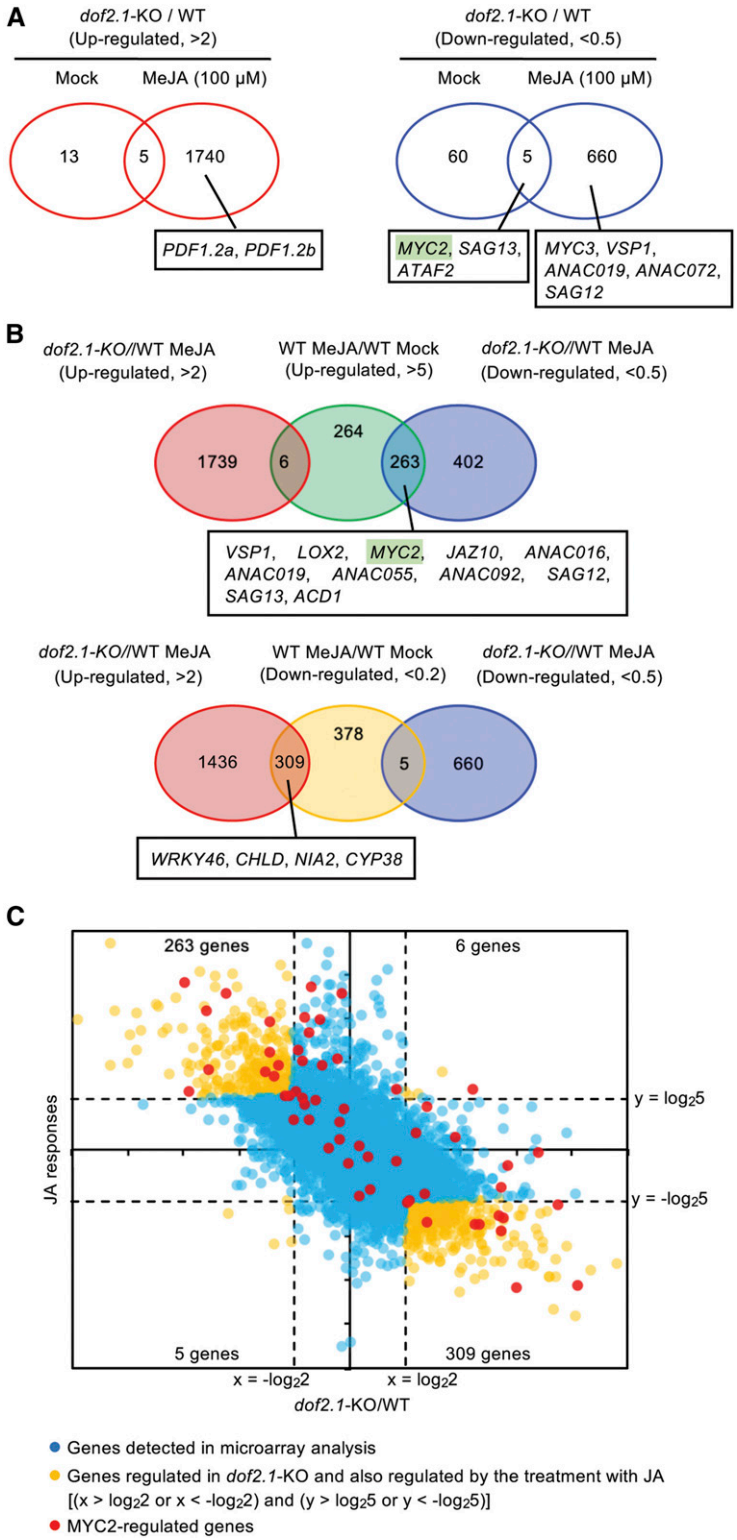

Figure 6. Overlap between JA-Responsive Genes and Dof2.1-Regulated Genes.

(A) Venn diagrams showing the overlap of upregulated (>2-fold) or downregulated (<0.5-fold) genes in the *dof2.1*-KO mutant in the presence of MeJA (100 μM) with those in the absence of MeJA (Mock). Numbers of genes are shown. Representative MeJA-inducible genes upregulated or downregulated in the *dof2.1*-KO mutant in both presence and absence of MeJA are also indicated. WT, wild type.

et al., 2007; Godoy et al., 2011). Because the *Dof2.1* promoter (−1,347 to +153 bp relative to the transcription start site) harbors two E-boxes (Figure 9B), we performed a ChIP assay using *MYC2*-OX plants to determine whether MYC2 binds directly to the *Dof2.1* promoter in vivo. The results indicated strong binding of MYC2 to region b in the *Dof2.1* promoter containing the second E-box motif (Figure 9C). Next, to investigate whether MYC2 activates the *Dof2.1* promoter, we performed protoplast cotransfection assays using an effector plasmid containing the *MYC2* gene and a reporter plasmid containing the *LUC* reporter gene under the control of the *Dof2.1* promoter (Figure 9D). In the protoplasts, *MYC2* expression increased activity of the *Dof2.1* promoter but not that of the *35S* promoter (control; Figure 9E), consistent with the effect of *MYC2* overexpression on *Dof2.1* expression in planta (Figure 9A).

Furthermore, two mutant *Dof2.1* promoters (Mut1 and Mut2), in which either one of the two E-box motifs was disrupted, were differentially activated by *MYC2* expression (Figure 9E). Although the disruption of the first E-box motif (−1,235 to −1,230 bp) did not affect transactivation of the *Dof2.1* promoter by MYC2, disruption of the second E-box motif (−1,016 to −1,011 bp) significantly decreased transactivation of the *Dof2.1* promoter by MYC2 (Figure 9E). This result suggests that MYC2 activates the *Dof2.1* promoter mainly by direct interaction with the second E-box motif in region b; these results were consistent with those of the ChIP assay.

The finding that MYC2 is a transcriptional activator responsible for JA-inducible expression of *Dof2.1* was further validated by protoplast transient assays using protoplasts prepared from wild-type and *atmyc2-2* leaves. In wild-type protoplasts, MeJA treatment strongly activated the *Dof2.1* promoter, and disruption of the second E-box motif significantly diminished JA response of the *Dof2.1* promoter (Figure 9F). However, in *atmyc2-2* protoplasts, MeJA treatment caused only minimal activation of any *Dof2.1* promoters (Figure 9F). Thus, the second E-box motif in the *Dof2.1* promoter mediates both transactivation by MYC2 and activation by JA.

The MYC2–Dof2.1–MYC2 Feedforward Transcriptional Loop Underlies the Dof2.1-Mediated Enhancement of JA-induced Leaf Senescence

Dof2.1 promoted *MYC2* expression and enhanced MYC2-controlled leaf senescence (Figure 8), whereas MYC2 mediated JA-dependent *Dof2.1* expression (Figures 9A to 9F). Thus, we hypothesized that a feedforward transcriptional loop consisting of Dof2.1 and MYC2 operates at the cellular level. To evaluate this hypothesis, we crossed the *dof2.1*-KO mutant with the *MYC2*-OX line and *atmyc2-2*. The *MYC2* expression level in *MYC2*-OX *dof2.1*-KO plants was similar to that in *MYC2*-OX plants (Supplemental Figure 18). Nevertheless, the effects of *MYC2*-OX on JA-induced promotion of leaf yellowing and reduction in chlorophyll content and F_v/F_m were attenuated in the *dof2.1* mutant background (Figures 9G to 9I). Furthermore, *dof2.1*-KO and *atmyc2-2* did not additively attenuate reductions in the chlorophyll content and inhibition of root development in the presence of MeJA (Supplemental Figures 19 and 20). These results support our hypothesis that activation of leaf senescence-associated genes by MYC2 is enhanced through the MYC2–Dof2.1–MYC2 loop. Additionally, the *dof2.1* KO mutation alleviated the effect of *MYC2* OX on root development (Supplemental Figure 21), suggesting that the MYC2–Dof2.1–MYC2 transcriptional loop may also be involved in JA response in roots.

DISCUSSION

Dof2.1 Acts as an Enhancer of JA-Induced Leaf Senescence

In this study, we showed that Arabidopsis Dof2.1 activates *MYC2* expression and thereby plays an important role in JA-induced and MYC2-regulated leaf senescence, thereby connecting a member of the Dof protein family and a plant-specific physiological process.

A recent study suggests that Dof2.1, together with its closely related Dof proteins (TMO6/Dof5.3 and DOF6/Dof3.2), redundantly controls cytokinin-dependent vascular cell proliferation (Smet et al., 2019). The *dof2.1 tom6/dof5.3 dof6/dof3.2* triple mutant showed a small number of cell files in the root meristem, although the *dof2.1* single mutant showed no difference in the number of cell files compared with the wild type (Smet et al., 2019). Because the *dof2.1*-KO mutant showed almost no changes in gene expression profiles in the absence of JA compared with the wild type (Figure 6A; Supplemental Data Sets 1 and 2), it is currently unclear how Dof2.1 contributes to vascular cell proliferation. However, it is possible that the *dof2.1* mutation assists in *tom6/dof5.3*-dependent and/or *dof6/dof3.2*-dependent changes in gene expression profiles. Although no correlation between JA signaling and vascular cell proliferation has been reported to date, an antagonistic interplay between JA and cytokinin has been implicated in a small number of studies (reviewed in O'Brien and Benková, 2013). Thus, disruption of *Dof2.1* might indirectly influence cytokinin signaling, resulting in phenotypic changes. The

Figure 6. (continued).

(B) Venn diagrams showing the overlap between MeJA-regulated genes and Dof2.1-regulated genes in the presence of MeJA. Numbers of genes are shown. Representative genes displaying both MeJA-induced upregulation (>5-fold) and *dof2.1* KO mutation-dependent downregulation (<0.5-fold), and genes displaying both MeJA-induced downregulation (<0.2-fold) and *dof2.1* KO mutation-dependent upregulation (>2-fold) are indicated. WT, wild type.

(C) Scatterplot showing effects of the *dof2.1* KO mutation on JA-induced or -reduced gene expression. The *y*-axis indicates MeJA response and the *x*-axis represents *dof2.1* KO mutation-dependent modulation of gene expression in the presence of MeJA. Yellow dots represent genes whose expression levels were modified in the *dof2.1*-KO mutant ($x > \log_2 2$ or $x < -\log_2 2$) and also regulated by MeJA treatment ($y > \log_2 5$ or $y < -\log_2 5$). Red dots represent all genes previously identified as MYC2-regulated genes by Dombrecht et al. (2007). Other genes detected in the microarray analysis of this study are indicated by blue dots.

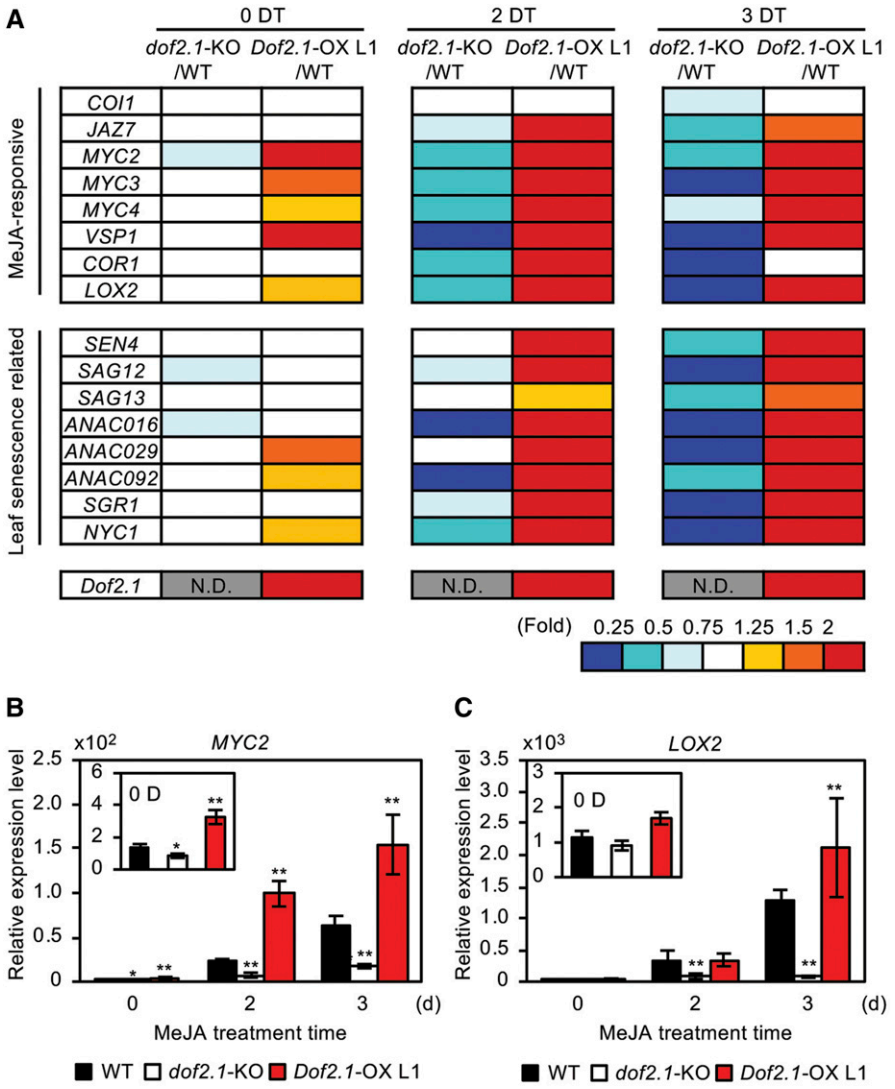

Figure 7. Opposite Effects of *Dof2.1* Overexpression and the *dof2.1* KO Mutation on the Expression of JA Response- and Leaf Senescence-Related Genes.

(A) Time-course analysis of the effects of the *dof2.1* KO mutation and *Dof2.1* overexpression on MeJA-inducible expression of JA response- and leaf senescence-related genes using rosette leaves detached from 3-week–old wild type, *dof2.1*-KO, and *Dof2.1*-OX (line L1) plants. Detached rosette leaves were floated on 3 mM of MES buffer containing 100 μM of MeJA or no MeJA for the indicated times. Expression levels of each gene were normalized first against expression levels of *ACT2* and then against expression levels in wild-type leaves. DT, days after the beginning of MeJA treatment.; WT, wild type. **(B)** and **(C)** Time-course analysis of *dof2.1* KO mutation- and *Dof2.1* overexpression-dependent changes in MeJA-induced *MYC2* **(B)** and *LOX2* **(C)** expression. Expression levels of *MYC2* and *LOX2* were normalized first against transcript levels of *ACT2* and then against the value obtained from wild-type leaves at time zero. The insets in **(B)** and **(C)** show expression levels of *MYC2* and *LOX2* at 0 D, respectively. Data represent the mean ± SD of four biological replicates (three rosette leaves per replicate), and asterisks ($^{*}P < 0.05$; $^{**}P < 0.01$) indicate significant differences identified between wild type and other seedlings using Student's *t* test. Each experiment was conducted twice with similar results. WT, wild type.

results in this study provided three lines of evidence, including (1) changes in gene expression caused by the *dof2.1* KO mutation; (2) phenotypes of the *dof2.1*-KO mutant and *Dof2.1*-OX lines; and (3) identification of *MYC2* as a direct target of Dof2.1, in that the role of Dof2.1 is associated with JA responses (however, it is possible to speculate that Dof2.1 might indirectly influence a variety of currently unpredictable processes through the modulation of expression of *MYC2* and other target genes).

MYC2–Dof2.1–MYC2 Feedforward Loop Acts as a Positive Regulatory Module for JA-Induced Leaf Senescence

Dof2.1 is a positive regulator of *MYC2* (Figures 7 and 8). MYC2 also activates *Dof2.1* transcription (Figure 9). Furthermore, analysis of epistasis between *Dof2.1* and *MYC2* (Figures 7 and 8) indicates that the *myc2* KO mutation is epistatic to overexpression of *Dof2.1* in controlling JA-induced leaf senescence, whereas the *dof2.1* KO mutation alleviated (but did not completely suppress) *MYC2*

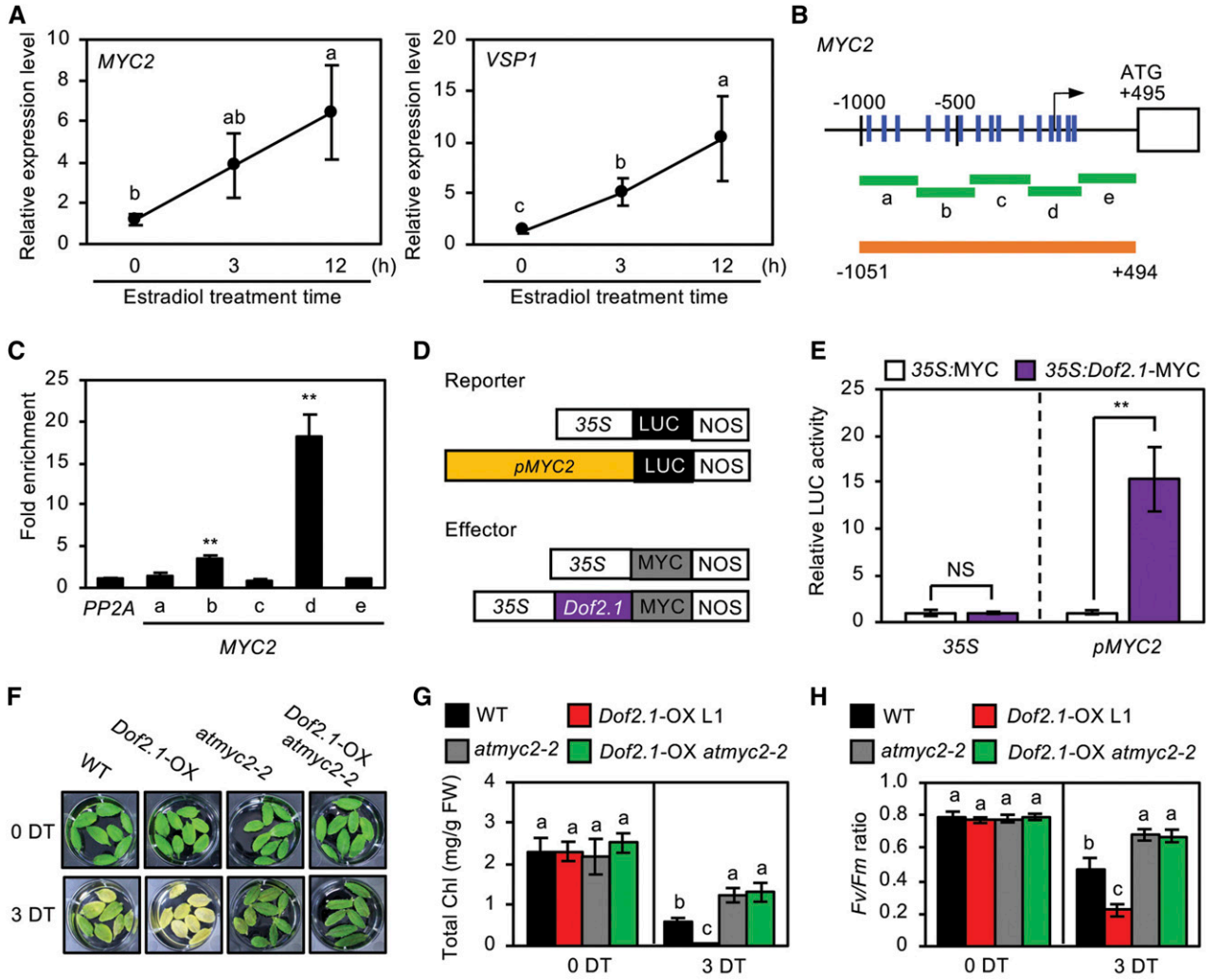

Figure 8. Direct Activation of the *MYC2* Promoter by Dof2.1 is Relevant to Leaf Senescence.

(A) Time-course analysis of Dof2.1-dependent *MYC2* and *VSP1* expression in 7-d–old *Dof2.1*-EI seedlings. To induce *Dof2.1* expression, leaves were exposed to 10 μM of estradiol for the indicated times. Expression levels of *MYC2* and *VSP1* were normalized first against transcript levels of *ACT2* and then against the value obtained from samples at time zero. Data represent the mean ± SD of six biological replicates (six seedlings per replicate), and the same letters above each bar indicate that means did not differ significantly at the 0.05 level in Tukey's multiple comparison test.

(B) Structure of the *MYC2* promoter. Regions amplified in the ChIP assay and used for transient expression-based transactivation assay are indicated using green and orange horizontal bars, respectively. Regions a, b, c, d, and e contained 3, 3, 4, 5, and 0 putative Dof binding sites indicated using vertical blue bars, respectively.

(C) ChIP assay to analyze binding of Dof2.1 to the *MYC2* promoter in 10-d–old *Dof2.1*-OX seedlings. Five regions were amplified by PCR with immunoprecipitated DNA. Enrichment of a promoter region of an unrelated gene, *PP2A*, served as a negative control. Data represent the mean ± SD of four biological replicates, and asterisks (**P < 0.01) above each bar indicate significant differences identified between *PP2A* and other samples using Student's *t* test.

(D) Reporter and effector constructs used in the transactivation assay.

(E) Activation of the *MYC2* promoter (−1,051 to +494 bp) by Dof2.1. An expression vector harboring *Dof2.1* fused to a MYC epitope tag (*35S:Dof2.1*-MYC) was cotransfected into protoplasts, together with a reporter plasmid containing the *MYC2* promoter or the *35S* promoter. The *35S* promoter and an empty expression vector harboring the MYC epitope tag alone (*35S:MYC*) were used as negative controls. Data represent the mean ± SD of four biological replicates, and asterisks (**P < 0.01) above the bar indicate significant differences identified between *35S:MYC* and *35S:Dof2.1-MYC* samples using Student's *t* test. NS, not significant.

(F) to **(H)** Change in color **(F)**, total chlorophyll content **(G)**, and F_v/F_m **(H)** of detached rosette leaves under MeJA treatment. Rosette leaves were detached from 3-week–old wild type, *Dof2.1*-OX (line L1), *atmyc2-2*, and *Dof2.1*-OX *atmyc2-2* plants and floated on 3 mM of MES buffer containing 100 μM of MeJA for 3 d. Data represent the mean ± SD of seven biological replicates (one rosette leaf per replicate), and the same letters above each bar indicate that means did not differ significantly at the 0.05 level in Tukey's multiple comparison test. Each experiment was conducted twice with similar results. DT, days after the beginning of treatment; FW, fresh weight; WT, wild type.

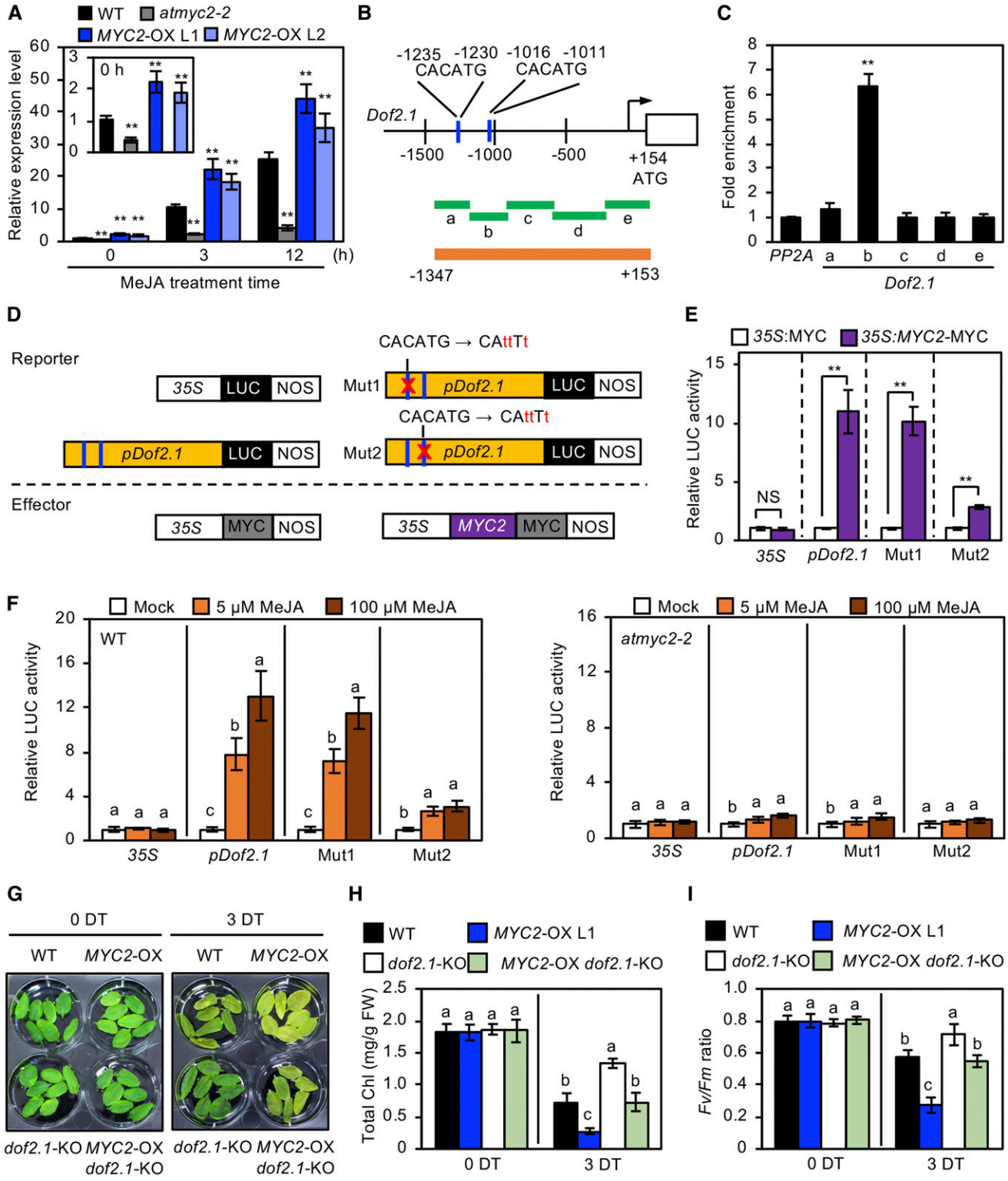

Figure 9. MYC2 Directly Activates the *Dof2.1* Promoter to Enhance JA Responses.

(A) Time-course analysis of MeJA-induced expression of *Dof2.1* in rosette leaves detached from 3-week–old wild type, *atmyc2-2*, and *MYC2*-OX (lines L1 and L2) plants. Detached rosette leaves were floated on 3 mM of MES buffer containing 100 μM of MeJA for the indicated times. Expression levels of *Dof2.1* were normalized first against transcript levels of *ACT2* and then against the value obtained from wild-type rosette leaves at time zero. The inset shows

overexpression-induced effects. Given that MYC2 directly regulates the expression of JA response-associated genes, our findings indicate that Dof2.1 and MYC2 form a MYC2–Dof2.1–MYC2 feedforward transcriptional loop for the enhancement of JA-induced leaf senescence. Thus, we propose a model for the enhancement of JA-induced leaf senescence in Arabidopsis (Figure 10). This model includes both the well-established posttranslational repression of MYC2 activity by the JAZ–NINJA–TOPLESS complex (Pauwels et al., 2010) and the MYC2–Dof2.1–MYC2 feedforward transcriptional loop-mediated enhancement of JA signaling identified in this study. In the absence of JA, MYC2 activity is repressed by the JAZ–NINJA–TOPLESS complex; therefore, *MYC2* expression is not enhanced via the MYC2–Dof2.1–MYC2 feedforward transcriptional loop. In the presence of JA, the SCF^{COI1} complexes interact with JAZ proteins, leading to degradation of JAZ proteins and induction of MYC2 activity. Then, MYC2 directly promotes expression of a number of genes associated with JA-induced leaf senescence as well as that of *Dof2.1*. Eventually, Dof2.1 promotes *MYC2* expression by directly binding to its promoter, thereby increasing MYC2 activity and enhancing JA-induced leaf senescence. In the *dof2.1*-KO mutant, *MYC2* expression is not strongly activated in response to JA, resulting in weak JA-induced leaf senescence.

The feedforward transcriptional loop in this model is a mechanism for positive autoregulation of MYC2. Positive autoregulation where a transcription factor enhances its own expression causes slower response time and enhanced variation, resulting in an S-shaped expression curve (Alon, 2007). Thus, responses on the basis of positive autoregulation are not apparent until signal intensity is beyond a threshold, while strong responses occur when the signal intensity exceeds the threshold. Because leaf senescence is an irreversible process after passing a checkpoint (Schildhauer et al., 2008), the MYC2–Dof2.1–MYC2 feedforward transcriptional loop may be relevant to the regulation of JA-induced leaf senescence.

It is noteworthy that overexpression of *Dof2.1* also enhanced JA-induced inhibition of root development and germination and wounding response, while the *dof2.1* KO mutation reduced them (Figures 4 and 5; Supplemental Figures 9 and 10). Accordingly, the MYC2–Dof2.1–MYC2 feedforward loop may be involved in other MYC2-regulated JA responses, which include pathogen defense responses and wounding responses (Chini et al., 2016; Zhang et al., 2017; Goossens et al., 2017). In agreement with this hypothesis, enhanced and reduced expression levels of *MYC2* during wounding responses were observed in the *Dof2.1*-OX and *dof2.1*-KO plants, respectively (Figure 5D). Further analyses of the MYC2–Dof2.1–MYC2 feedforward loop may reveal the connection of the loop with a variety of JA responses, leading to deeper understanding of the mechanism underlying JA responses.

Transcriptional Regulation of *MYC2*

The model in Figure 10 highlights the significance of transcriptional activation of *MYC2* in JA signaling and responses. Although the mitogen-activated protein kinase (MAPK) cascade involving MAPK kinase3 and MAPK6 was previously suggested to negatively affect *MYC2* expression (Takahashi et al., 2007), the physiological significance of the regulatory mechanism underlying JA-inducible *MYC2* expression remained elusive. Our findings clarify that JA-induced *MYC2* expression via the MYC2–Dof2.1–MYC2 feedforward loop is critical for JA-induced leaf senescence at the appropriate level. Because the importance of transcriptional regulation of *MYC2* in JA responses has been overlooked in the past, our findings provide new insights into the mechanism underlying JA responses.

However, these findings also raise new questions about the transcriptional regulation of *MYC2*. First, although *MYC2* expression was quickly accelerated by Dof2.1 in response to MeJA treatment (Figures 7A and 7B, Supplemental Figure 13A; also

Figure 9. (continued).

expression levels of *Dof2.1* at 0 h. Data represent the mean ± SD of six biological replicates (one rosette leaf per replicate). Asterisks (**$P < 0.01$) above each bar indicate significant differences identified between wild type and other samples using Student's *t* test. WT, wild type.

(B) Structure of the *Dof2.1* promoter. Two copies of the E-box (CACATG) are indicated using blue vertical bars. Regions amplified in the ChIP assay and used for transient expression assays are indicated using green and orange horizontal bars, respectively.

(C) ChIP assay. Binding of MYC2 to specific regions of the *Dof2.1* promoter was examined using 10-d–old *MYC2*-OX seedlings. Five regions were amplified by qPCR with immunoprecipitated DNA. Enrichment of a promoter region of an unrelated gene, *PP2A*, served as a negative control. Data represent the mean ± SD of four biological replicates, and asterisks (**P < 0.01) above each bar indicate significant differences identified between *PP2A* and other samples using Student's *t* test.

(D) Reporter and effector constructs used in the transactivation assay. In *Dof2.1* Mut1 and Mut2 promoters, either one of the two E-box motifs was changed to CAttTt. Two expression vectors containing *MYC2* fused to a MYC epitope or MYC epitope alone were used as effector plasmids. LUC, firefly luciferase gene; NOS, the transcriptional terminator of the nopaline synthase gene from *Agrobacterium tumefaciens*.

(E) Activation of the wild-type (*pDof2.1*) and mutated (Mut1 and Mut2) *Dof2.1* promoters (−1,347 to +153) by MYC2 in protoplasts. The *35S* promoter and an empty expression vector (*35S*:*MYC*) served as negative controls. Data represent the mean ± SD of four biological replicates. Asterisks (**P < 0.01) indicate significant differences identified between *35S*:*MYC* and *35S*:*Dof2.1-MYC* samples using Student's *t* test. NS, not significant.

(F) Activation of the *Dof2.1* promoter by MeJA in mesophyll protoplasts prepared from wild-type or *atmyc2-2* mutant plants. Protoplasts transfected with *pDof2.1-LUC* reporter construct were incubated in the protoplast culture solution in the presence or absence of 5 μM or 100 μM of MeJA. The *35S* promoter served as a negative control. Data represent the mean ± SD of four biological replicates, and the same letters above each bar indicate that means did not differ significantly at the 0.05 level in Tukey's multiple comparison test.

(G) to **(I)** Changes in color **(G)**, total chlorophyll content **(H)**, and F_v/F_m **(I)** due to MeJA treatment in rosette leaves detached from 3-week–old wild type, *MYC2*-OX (line L1), *dof2.1*-KO, and *MYC2*-OX *dof2.1*-KO plants. The leaves were floated on 3 mM of MES buffer containing 100 μM of MeJA. Data represent the mean ± SD of six biological replicates (one rosette leaf per replicate), and the same letters above each bar indicate that means did not differ significantly at the 0.05 level in Tukey's multiple comparison test. Each experiment was conducted twice with similar results. DT, days after the beginning of MeJA treatment; WT, wild type.

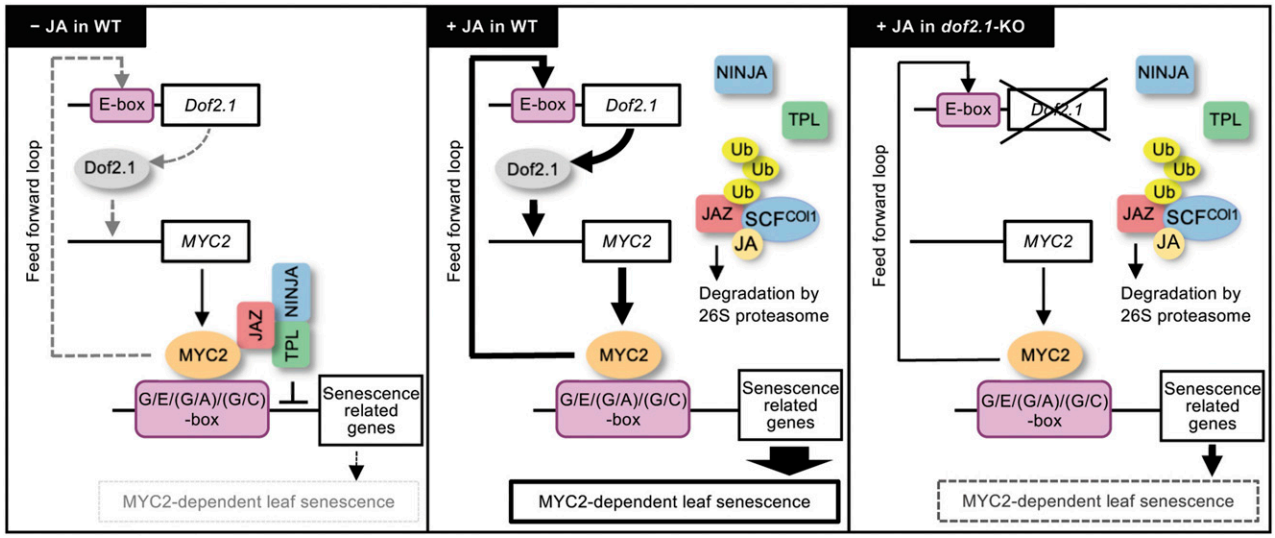

Figure 10. A Model for Dof2.1-Mediated Enhancement of JA-Induced Leaf Senescence in Arabidopsis.

In the absence of JA, MYC2 remains inactive because of its interaction with the JAZ–NINJA–TOPLESS complex. In the presence of JA, the interaction between JAZ and COI1, an F-box protein of the SCF complex, leads to ubiquitination of JAZ proteins by COI1-containing SCF complexes (SCF^{COI1}) and their degradation by the 26S proteasome system. Consequently, MYC2 promotes or represses the expression of genes associated with JA-induced leaf senescence, including *Dof2.1*, via interactions with G-, E-, (G/A)-, and (G/C)-boxes. Promotion of *Dof2.1* expression by MYC2 then enhances *MYC2* expression. Thereby, JA-induced leaf senescence is enhanced through a feedforward transcriptional loop involving MYC2 and Dof2.1. In the *dof2.1*-KO mutant, *MYC2* expression is not reinforced; thus, JA-induced leaf senescence is reduced. TPL, TOPLESS; Ub, ubiquitin; WT, wild type.

shown by Lorenzo et al., 2004), a previous report suggests that MYC2 negatively affects its own expression; this is based on the observation that the level of endogenous *MYC2* transcripts was significantly reduced when MYC2 was overexpressed in the *myc2*-KO mutant (Dombrecht et al., 2007). The reason for the seemingly irreconcilable observations is unclear at this stage; however, a possible explanation is that JA induces *MYC2* expression via the MYC2–Dof2.1–MYC2 transcriptional loop to enhance short-term responses to JA, while autorepression of *MYC2* may be effective only when MYC2 activity is overinduced by JA and associated with modulation of long-term responses to JA. Second, there are questions regarding the relationships between the MYC2–Dof2.1–MYC2 transcriptional loop-mediated transcription regulation of *MYC2* and different types of post-translational regulation of MYC2. Besides the complex formation with JAZ proteins that represses MYC2 activity in the absence of JA, MYC2 activity is also complicatedly regulated by other posttranslational mechanisms. MYC2 protein is stabilized by JA and light (Chico et al., 2014). On the other hand, a circadian-clock component TIME FOR COFFEE interacts with MYC2 and promotes MYC2 protein degradation (Shin et al., 2012). Phosphorylation-coupled proteolysis of MYC2 also affects its transcription activity (Zhai et al., 2013). Because the MYC2–Dof2.1–MYC2 transcriptional loop depends on MYC2 activity, the relationships with these posttranslational regulations for repression or induction of MYC2 activity are remaining questions. Third, transcriptome and RT-qPCR analyses revealed that expression of *MYC3* and *MYC4*, which redundantly regulate JA responses including both defense and senescence, together with *MYC2* (Fernández-Calvo et al., 2011; Goossens et al., 2017; Schweizer et al., 2013; Zhang et al., 2015), are also under the control of Dof2.1 (Figure 7A; Supplemental Figure 22A); however, a ChIP assay revealed that Dof2.1 did not bind to the promoters of *MYC3* and *MYC4* genes (Supplemental Figures 22B to 22D). Because the result of epistatic analysis suggested that the Dof2.1–MYC2 cascade is the main route for Dof2.1-dependent enhancement of JA-induced leaf senescence (Figure 8), Dof2.1 may activate these genes through MYC2 activity, although we cannot exclude the possibility that Dof2.1 promotes the expression of these genes through its interaction with Dof binding sites outside the analyzed regions. Thus, a new question associated with similarity and difference among transcriptional regulations of *MYC2*, *MYC3*, and *MYC4* is emerging. These examples of new questions emphasize the importance of further investigation of transcriptional regulation of *MYC2* expression to fully understand the mechanism of JA responses. Such analyses would help identify the currently unknown mechanisms of JA responses.

METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia (Col-0) was used as the wild type in all experiments, and all mutants and transgenic lines were generated in Col-0 background. Arabidopsis T-DNA insertion lines, GK-668G12-022986 (*dof2.1*-KO) and SALK_083483 (*atmyc2-2*), an estradiol-inducible *Dof2.1* transgenic line CS2104416 (*Dof2.1*-EI), and a T-DNA activation tagging line SAIL_1230_B08 (*dof2.1-D*) were obtained from the Arabidopsis Biological Resource Center. The *Dof2.1*-OX *atmyc2-2* line was generated by crossing the *atmyc2-2* mutant with *Dof2.1*-OX line L1. The *MYC2*-OX *dof2.1*-KO line was also generated by crossing the *dof2.1*-KO

mutant with *MYC2-OX* line L2. Plants homozygous for both the T-DNA insertion disrupting *Dof2.1* or *MYC2* and the introduced transgene were selected by PCR-based genotyping using primers listed in the Supplemental Table. Arabidopsis seeds were cold stratified at 4°C for 3 to 4 d. Arabidopsis seedlings were grown on half-strength Murashige and Skoog (MS) agar plates (half-strength MS salts [Murashige and Skoog, 1962], 0.8% [w/v] agar, 0.5% [w/v] Suc, 3 mM of MES-KOH at pH 5.8) or nutrient-supplemented peat moss (Jiffy, Sakata Seed) at 22°C under continuous light in a growth chamber equipped with cool white fluorescent light (70 μmol m^{-2} s^{-1}), unless stated otherwise.

Plasmid Construction and Plant Transformation

Dof2.1 and *MYC2* cDNAs were obtained by RT-PCR of RNA isolated from wild-type seedlings and cloned into the pGWB15 Gateway binary vector (Nakagawa et al., 2009) between the *35S* promoter and *GFP* gene to produce the *35S*:*Dof2.1-GFP* and *35S*:*MYC2-GFP* constructs, respectively. To construct the *proDof2.1*:*GUS* plasmid, the *Dof2.1* promoter (−1,847 to +153 bp relative to the transcription start site) was PCR-amplified from genomic DNA isolated from wild-type leaves and cloned into the pMDC164 Gateway binary vector. To construct the *proDof2.1*:*Dof2.1-MYC* plasmid, necessary for generating the *proDof2.1*:*Dof2.1*/*dof2.1*-KO complementation lines, the *Dof2.1* promoter and cDNA were inserted upstream of a sequence encoding four copies of MYC epitope tag in the pGWB16 Gateway binary vector. Sequences of all constructs were verified by sequencing. PCR primers used for cloning are listed in the Supplemental Table.

Agrobacterium-mediated transformation of Arabidopsis plants was performed using the *Agrobacterium tumefaciens* strain GV3101 and the floral-dip method (Zhang et al., 2006). Binary vectors containing the *35S*:*Dof2.1-GFP*, *35S*:*MYC2-GFP*, or *proDof2.1*:*GUS* construct were used for the transformation of wild-type plants to generate *Dof2.1*-OX, *MYC2*-OX, and *proDof2.1*:*GUS* lines, respectively, while vectors containing the *proDof2.1*:*Dof2.1-MYC* construct were used for transformation of *dof2.1*-KO plants to produce the transgenic complementation line, *proDof2.1*:*Dof2.1*/*dof2.1*-KO. Transgenic plants in the T2 generation with T-DNA insertion(s) at a single locus were selected, and T3 or T4 homozygotes were used for all analyses.

RNA Extraction and RT-qPCR Analysis

Total RNA was isolated from rosette leaves of Arabidopsis seedlings using the ISOSPIN Plant RNA Kit (Nippon Gene), according to the manufacturer's instructions. First-strand cDNA was synthesized using 1 μg of total RNA, SuperScript II reverse transcriptase, and oligo$(dT)_{15}$ primer (Invitrogen). Subsequently, RT-qPCR was conducted on the StepOnePlus instrument (Applied Biosystems) using the KAPA SYBR FAST qPCR Kit (KAPA Biosystems) and gene-specific primers (Supplemental Table). Transcript levels of each gene were normalized relative to those of the *ACTIN2* (*ACT2*) or the Glyceraldehyde-3-Phosphate Dehydrogenase gene. The number of biological replicates in each experiment is indicated in figure legends.

GUS Staining

Transgenic seedlings harboring the *proDof2.1*:*GUS* construct were fixed in 90% (v/v) acetone and then incubated in 5-bromo-4-chloro-3-indolyl-β-D-glucuronide solution (0.5 mg/mL of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 0.5 mM of potassium ferricyanide, 0.5 mM of potassium ferrocyanide, and 0.1 M of sodium phosphate, at pH 7.4) at 37°C for 1 h. Subsequently, seedlings were destained using 70% (v/v) ethanol. GUS staining was observed using a light microscope (MZ 16F; Leica Microsystems) equipped with a DXM 1200C digital camera (Nikon). Images were captured using the control software ACT-1C for DXM1200C (Nikon).

Phenotypic Analysis

To examine the MeJA-induced leaf senescence phenotype, rosette leaves were detached from 3-week–old plants and floated on 3 mM of MES-KOH buffer (pH 5.8) with or without 100 μM of MeJA at 22°C under continuous light for periods indicated in each figure. To examine the dark-induced leaf senescence phenotype, rosette leaves were detached from 3-week–old plants and floated on 3 mM of MES-KOH buffer (pH 5.8) in the dark, as described previously by Sakuraba et al. (2014a). To examine the phenotype of the *Dof2.1*-EI line, 10-d–old seedlings grown on agar plates under continuous light were treated with 10 μM of estradiol for 6 h in liquid medium containing half-strength MS salts, 0.5% (w/v) Suc, and 3 mM of MES-KOH (pH 5.8). Then, MeJA was added to the liquid medium to a final concentration of 100 μM, and seedlings were further incubated for 4 d. To investigate phenotypes associated with root development, seedlings were grown on vertically oriented agar plates under continuous light for 5 d and then further grown on agar plates supplemented with 5 μM of MeJA for 4 d. Root growth was also investigated by a different experimental approach as described previously by Chini et al. (2018). Seeds were sown on agar plates supplemented with 0, 10, or 50 μM of MeJA. After germination, seedlings were grown on the plates for 5 d, transferred onto new agar plates containing same concentrations of MeJA, and then further grown for 5 d to examine root development. To examine the MeJA-induced inhibition of seed germination, seeds were sown on agar plates supplemented with 0, 10, or 50 μM of MeJA and incubated at 22°C under continuous light for 5 d. The germination experiment was performed with six biological replicates, each of which contained 30 to 40 seeds from one individual plant. All the seeds used for each assay were harvested from plants grown at the same time and stored for at least two months at room temperature. To examine the response induced by mechanical wounding stress, rosette leaves of 2.5-week–old plants grown on peat moss under continuous light conditions were wounded with a blade. Then, plants were grown under the same growth conditions for 7 d for phenotypic characterization or for 2 h, 3 h, and 6 h for gene expression analysis. The number of biological replicates in each experiment is indicated in figure legends.

Quantification of Chlorophyll and Carotenoid Pigments

Chlorophyll pigments were extracted (using 80% [v/v] ice-cold acetone) from rosette leaves homogenized with zirconia beads (Nikkato). Absorbance of extracts was measured at 470, 647, and 664 nm, and chlorophyll and carotenoid contents were calculated as described previously by Porra et al. (1989). The number of biological replicates in each experiment is indicated in the figure legends.

Measurement of the F_v/F_m

Chlorophyll fluorescence and the F_v/F_m were measured using a kinetics multispectral fluorescence imaging system (FluorCam 800 MF; Photon System Instruments), according to the manufacturer's instructions. The number of biological replicates in each experiment is indicated in figure legends.

Immunoblot Analysis

Proteins were extracted from leaves ground in liquid nitrogen using SDS-PAGE loading buffer (10% [w/v] glycerol, 50 mM of Tris-HCl at pH 8.0, 2% [w/v] SDS, 6% [v/v] 2-mercaptoethanol, and 0.003% [v/v] bromophenol blue). Proteins were separated on a 12% (w/v) SDS-polyacrylamide gel, transferred onto an Immobilon-P transfer membrane (Merck Millipore), and detected using anti-PsbC (cat. no. AS111787; Agrisera), anti-Lhcb1 (cat. no. AS09522; Agrisera), anti-Lhcb4 antibodies (cat. no. AS04045; Agrisera), and anti-α tubulin antibody (cat. no. 10680; Agrisera), followed by an anti-rabbit IgG HRP-linked antibody (cat. no. 7074; Cell Signaling

Technology) and Supersignal West Dura Extended Duration Substrate (Thermo Fisher Scientific), according to the manufacturer's protocol. RbcL protein was detected by Coomassie Brilliant Blue staining.

DNA Microarray Analysis

Rosette leaves were detached from 3-week–old wild-type and *dof2.1*-KO plants grown on peat moss and then floated for 3 d on 3 mM of MES-KOH buffer (pH 5.8) with or without 100 μM of MeJA. Total RNA was extracted from leaf samples using the ISOSPIN Plant RNA Kit (Nippon Gene). Preparation of Cyanine-3–labeled cRNA using the Low Input Quick Amp Labeling Kit (One-Color; Agilent Technologies), hybridization with the Arabidopsis Oligo Microarray (V4; Agilent Technologies), and scanning of microarrays on the DNA Microarray Scanner (G2565BA; Agilent Technologies) were performed according to the manufacturer's instructions for "One-Color Microarray-Based Expression Analysis." Fluorescent signal intensities were detected with the software Scan Control (v7.0.03; Agilent Technologies). Data were extracted with the software Feature Extraction (v9.1; Agilent Technologies) and raw data were produced by calculating the average of three independent biological replicates. Welch's approximate Student's *t* test was used for the comparison of different groups and differences were considered significant at a P value < 0.05. Sample groups were compared in a pairwise manner to determine the differential expression between groups, which was calculated and expressed as a fold-change (FC). Hierarchical average linkage clustering analysis was performed using 2,464 genes differentially expressed between wild-type and *dof2.1*-KO mutant plants ($FC > 2$ or $FC < 0.5$). Hierarchical clustering was performed as implemented with the function "heatmap.2" in the gplots package of R (ver. 3.5.2).

ChIP Assay

Dof2.1-OX, *MYC2*-OX, and wild-type seedlings grown on agar plates for 10 d were crosslinked using 1% (v/v) formaldehyde for 30 min under vacuum. Then, nuclei were isolated and lysed, and chromatin complexes were isolated and sonicated, as described previously by Saleh et al. (2008). DNA was sonicated using a Bioruptor II (Cosmo Bio). Anti-GFP polyclonal antibody (cat. no. 290; Abcam) and protein A agarose beads (Merck Millipore) were used for immunoprecipitation. DNA recovered from agarose beads was purified using the DNeasy Plant Mini Kit (Qiagen). qPCR was performed using the KAPA SYBR FAST qPCR Kit (KAPA Biosystems) and gene-specific primers (Supplemental Table). Fold-enrichment was calculated as described previously by Sakuraba et al. (2014a). The number of biological replicates in each experiment is indicated in figure legends.

Protoplast Transient Assay

Reporter plasmids were constructed by the insertion of *Dof2.1* (−1,347 to +153 bp) and *MYC2* (−1,051 to +494 bp) promoters into the pJD301 vector (Luehrsen et al., 1992). Introduction of nucleotide substitutions in the *Dof2.1* promoter was performed using the Megaprimer PCR method (Ke and Madison, 1997). To construct effector plasmids, *Dof2.1* and *MYC2* cDNAs were cloned upstream of a sequence encoding four copies of a MYC epitope tag in the pGWB17 Gateway binary vector (Nakagawa et al., 2009). Arabidopsis mesophyll protoplasts were isolated from rosette leaves of 3-week–old wild-type or *atmyc2-2* plants grown on peat moss, as described previously by Wu et al. (2009). A reporter plasmid (2 μg) and an effector plasmid (4 μg) were cotransfected, together with 1 μg of an internal control plasmid (pUBQ10-GUS), into 5×10^4 protoplasts using the polyethylene glycol-mediated transfection method (Yoo et al., 2007). Transfected protoplasts were incubated in protoplast culture solution (0.4 M of mannitol, 15 mM of $MgCl_2$, and 4 mM of MES-KOH at pH 5.8) in the dark at room temperature for 16 h. To examine the effect of MeJA on the activity of the *Dof2.1* promoter, 5 or 100 μM of MeJA was added to the protoplast culture solution. LUC activity in each cell lysate was determined using the Luciferase Assay System Kit (Promega). LUC activity was normalized against GUS activity derived from the internal control plasmid. The number of biological replicates in each experiment is indicated in figure legends.

Accession Numbers

Sequence data generated in this study were deposited in the Arabidopsis Genome Initiative Database under the following accession numbers: ACT2 (At3g18780), ANAC016 (At1g34180), ANAC029 (At1g69490), ANAC092 (At5g39610), COI1 (At2g39940), COR1 (At1g19670), Dof2.1 (At2g28510), JAZ7 (At2g34600), LOX2 (At3g45140), MYC2 (At1g32640), MYC3 (At5g46760), MYC4 (At4g17880), NON-YELLOW COLORING1 (At4g13250), PDF1.2b (At2g26020), SAG12 (At5g45890), SAG13 (At2g29350), SEN4 (At4g30270), SGR1 (At4g22920), UBQ10 (At4g05320), VSP1 (At5g24780), and the glyceraldehyde-3-phosphate dehydrogenase gene (At1g16300). Microarray data generated in this study was deposited in the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE129682) under the accession number GSE129682.

Supplemental Data

Supplemental Figure 1. Phytohormone response of 24 Arabidopsis *Dof* genes.

Supplemental Figure 2. MeJA does not induce the expression of *Dof5.3*, *HCA2*/*Dof5.6*, and *DOF6*/*Dof2.3*.

Supplemental Figure 3. Characterization of the *dof2.1*-KO mutant.

Supplemental Figure 4. Characterization of *Dof2.1*-OX lines.

Supplemental Figure 5. Wild-type, *dof2.1*-KO, and *Dof2.1*-OX leaves floated on 3 mM of MES buffer in the absence of MeJA.

Supplemental Figure 6. Phenotypic characterization of *proDof2.1*:*Dof2.1*/*dof2.1*-KO complementation lines.

Supplemental Figure 7. Accelerated yellowing of *dof2.1-D* leaves in the presence of MeJA.

Supplemental Figure 8. Numbers of rosette leaves at bolting and the time to bolting of wild-type, *dof2.1*-KO, and *Dof2.1*-OX plants.

Supplemental Figure 9. Alleviated and enhanced inhibitory effects of MeJA on primary root elongation in *dof2.1*-KO and *Dof2.1*-OX seedlings that were grown from seeds sown on MeJA-containing plates.

Supplemental Figure 10. Alleviated and enhanced inhibitory effects of MeJA on germination in the *dof2.1*-KO and *Dof2.1*-OX lines.

Supplemental Figure 11. Hierarchical average linkage clustering of 2,464 genes showing differential expression between wild-type and *dof2.1*-KO mutant plants subjected to mock or MeJA treatment.

Supplemental Figure 12. Expression levels of MYC2-regulated genes in *myc2*-KO and *dof2.1*-KO mutants.

Supplemental Figure 13. Rapid MeJA-induction of *MYC2* and *LOX2* expression is modified in *dof2.1*-KO and *Dof2.1*-OX leaves.

Supplemental Figure 14. The *myc2-2* mutation does not affect the expression of *Dof2.1* in the *Dof2.1*-OX line.

Supplemental Figure 15. Effects of *Dof2.1* overexpression on dark-induced leaf senescence in the *atmyc2-2* background.

Supplemental Figure 16. Effects of *Dof2.1* overexpression on root growth in the *atmyc2-2* mutant background.

Supplemental Figure 17. Characterization of *MYC2*-OX lines.

Supplemental Figure 18. The *dof2.1* KO mutation does not affect the expression of *MYC2* in the *MYC2*-OX line.

Supplemental Figure 19. *dof2.1* KO and *atmyc2-2* mutations did not additively attenuate reductions in the chlorophyll content.

Supplemental Figure 20. Root growth of *dof2.1*-KO *atmyc2-2* seedlings in the presence of MeJA.

Supplemental Figure 21. Root growth of *MYC2*-OX *dof2.1*-KO seedlings.

Supplemental Figure 22. Dof2.1 affects *MYC3* and *MYC4* expression but does not bind to *MYC3* and *MYC4* promoters.

Supplemental Table. List of primers used in this study.

Supplemental Data Set 1. List of genes upregulated or downregulated in *dof2.1*-KO seedlings in the absence of MeJA.

Supplemental Data Set 2. List of genes upregulated or downregulated in *dof2.1*-KO seedlings in the presence of MeJA.

ACKNOWLEDGMENTS

We thank the Arabidopsis Biological Resource Center for providing seeds of the T-DNA insertion lines and Miyuki Tsumura in our laboratory for assistance in plant cultivation. This work was supported in part by the Core Research for Evolutional Science and Technology, Japan Science and Technology Agency (JPMJCR 15O5 to S.Y.) and by the Japan Society for the Promotion of Science KAKENHI (18H03940 to S.Y., and 17H05024 to Y.S).

AUTHOR CONTRIBUTIONS

M.Z., Y.S., and S.Y. designed the experiments; M.Z. and Y.S. performed the experiments and analyzed the data; M.Z., Y.S., and S.Y. wrote the article.

Received April 24, 2019; revised October 1, 2019; accepted October 21, 2019; published October 22, 2019.

REFERENCES

Alon, U. (2007). Network motifs: Theory and experimental approaches. Nat. Rev. Genet. **8:** 450–461.

Berger, S., Bell, E., Sadka, A., and Mullet, J.E. (1995). *Arabidopsis thaliana Atvsp* is homologous to soybean *VspA* and *VspB*, genes encoding vegetative storage protein acid phosphatases, and is regulated similarly by methyl jasmonate, wounding, sugars, light and phosphate. Plant Mol. Biol. **27:** 933–942.

Boter, M., Ruíz-Rivero, O., Abdeen, A., and Prat, S. (2004). Conserved MYC transcription factors play a key role in jasmonate signaling both in tomato and Arabidopsis. Genes Dev. **18:** 1577–1591.

Bueso, E., Muñoz-Bertomeu, J., Campos, F., Martínez, C., Tello, C., Martínez-Almonacid, I., Ballester, P., Simón-Moya, M., Brunaud, V., Yenush, L., Ferrándiz, C., and Serrano, R. (2016). Arabidopsis *COGWHEEL1* links light perception and gibberellins with seed tolerance to deterioration. Plant J. **87:** 583–596.

Chen, Q., et al. (2011). The basic helix–loop–helix transcription factor MYC2 directly represses *PLETHORA* expression during jasmonate-mediated modulation of the root stem cell niche in Arabidopsis. Plant Cell **23:** 3335–3352.

Chen, W., Chao, G., and Singh, K.B. (1996). The promoter of a H_2O_2 -inducible, *Arabidopsis* glutathione s-transferase gene contains closely linked OBF- and OBP1-binding sites. Plant J. **10:** 955–966.

Chico, J.M., Fernández-Barbero, G., Chini, A., Fernández-Calvo, P., Díez-Díaz, M., and Solano, R. (2014). Repression of jasmonate-dependent defenses by shade involves differential regulation of protein stability of MYC transcription factors and their JAZ repressors in *Arabidopsis*. Plant Cell **26:** 1967–1980.

Chini, A., Fonseca, S., Fernández, G., Adie, B., Chico, J.M., Lorenzo, O., García-Casado, G., López-Vidriero, I., Lozano, F.M., Ponce, M.R., Micol, J.L., and Solano, R. (2007). The JAZ family of repressors is the missing link in jasmonate signalling. Nature **448:** 666–671.

Chini, A., Gimenez-Ibanez, S., Goossens, A., and Solano, R. (2016). Redundancy and specificity in jasmonate signalling. Curr. Opin. Plant Biol. **33:** 147–156.

Chini, A., Monte, I., Zamarreño, A.M., Hamberg, M., Lassueur, S., Reymond, P., Weiss, S., Stintzi, A., Schaller, A., Porzel, A., García-Mina, J.M., and Solano, R. (2018). An OPR3-independent pathway uses 4,5-didehydrojasmonate for jasmonate synthesis. Nat. Chem. Biol. **14:** 171–178.

Dathe, W., Rönsch, H., Preiss, A., Schade, W., Sembdner, G., and Schreiber, K. (1981). Endogenous plant hormones of the broad bean, *Vicia faba* L. (−)-jasmonic acid, a plant growth inhibitor in pericarp. Planta **153:** 530–535.

Dave, A., Hernández, M.L., He, Z., Andriotis, V.M.E., Vaistij, F.E., Larson, T.R., and Graham, I.A. (2011). 12-oxo-phytodienoic acid accumulation during seed development represses seed germination in *Arabidopsis*. Plant Cell **23:** 583–599.

Dombrecht, B., Xue, G.P., Sprague, S.J., Kirkegaard, J.A., Ross, J.J., Reid, J.B., Fitt, G.P., Sewelam, N., Schenk, P.M., Manners, J.M., and Kazan, K. (2007). MYC2 differentially modulates diverse jasmonate-dependent functions in *Arabidopsis*. Plant Cell **19:** 2225–2245.

Fernández-Calvo, P., et al. (2011). The Arabidopsis bHLH transcription factors MYC3 and MYC4 are targets of JAZ repressors and act additively with MYC2 in the activation of jasmonate responses. Plant Cell **23:** 701–715.

Fonseca, S., Chini, A., Hamberg, M., Adie, B., Porzel, A., Kramell, R., Miersch, O., Wasternack, C., and Solano, R. (2009). (+)-7-iso-Jasmonoyl-L-isoleucine is the endogenous bioactive jasmonate. Nat. Chem. Biol. **5:** 344–350.

Fornara, F., Panigrahi, K.C.S.S., Gissot, L., Sauerbrunn, N., Rühl, M., Jarillo, J.A., and Coupland, G. (2009). *Arabidopsis* DOF transcription factors act redundantly to reduce *CONSTANS* expression and are essential for a photoperiodic flowering response. Dev. Cell **17:** 75–86.

Gabriele, S., Rizza, A., Martone, J., Circelli, P., Costantino, P., and Vittorioso, P. (2010). The Dof protein DAG1 mediates PIL5 activity on seed germination by negatively regulating GA biosynthetic gene *AtGA3ox1*. Plant J. **61:** 312–323.

Godoy, M., Franco-Zorrilla, J.M., Pérez-Pérez, J., Oliveros, J.C., Lorenzo, O., and Solano, R. (2011). Improved protein-binding microarrays for the identification of DNA-binding specificities of transcription factors. Plant J. **66:** 700–711.

Goossens, J., Mertens, J., and Goossens, A. (2017). Role and functioning of bHLH transcription factors in jasmonate signalling. J. Exp. Bot. **68:** 1333–1347.

Gualberti, G., Papi, M., Bellucci, L., Ricci, I., Bouchez, D., Camilleri, C., Costantino, P., and Vittorioso, P. (2002). Mutations in the Dof

zinc finger genes *DAG2* and *DAG1* influence with opposite effects the germination of Arabidopsis seeds. Plant Cell **14:** 1253–1263.

Guo, Y., Qin, G., Gu, H., and Qu, L.J. (2009). *Dof5.6/HCA2*, a Dof transcription factor gene, regulates interfascicular cambium formation and vascular tissue development in *Arabidopsis*. Plant Cell **21:** 3518–3534.

Ibáñez-Salazar, A., Rosales-Mendoza, S., Rocha-Uribe, A., Ramírez-Alonso, J.I., Lara-Hernández, I., Hernández-Torres, A., Paz-Maldonado, L.M.T., Silva-Ramírez, A.S., Bañuelos-Hernández, B., Martínez-Salgado, J.L., and Soria-Guerra, R.E. (2014). Over-expression of Dof-type transcription factor increases lipid production in *Chlamydomonas reinhardtii*. J. Biotechnol. **184:** 27–38.

Imaizumi, T., Schultz, T.F., Harmon, F.G., Ho, L.A., and Kay, S.A. (2005). FKF1 F-box protein mediates cyclic degradation of a repressor of CONSTANS in Arabidopsis. Science **309:** 293–297.

Jibran, R., A Hunter, D., and P Dijkwel, P. (2013). Hormonal regulation of leaf senescence through integration of developmental and stress signals. Plant Mol. Biol. **82:** 547–561.

Jung, C., Zhao, P., Seo, J.S., Mitsuda, N., Deng, S., and Chua, N.H. (2015). PLANT U-BOX PROTEIN10 regulates MYC2 stability in Arabidopsis. Plant Cell **27:** 2016–2031.

Kazan, K., and Manners, J.M. (2013). MYC2: The master in action. Mol. Plant **6:** 686–703.

Ke, S.H., and Madison, E.L. (1997). Rapid and efficient site-directed mutagenesis by single-tube 'megaprimer' PCR method. Nucleic Acids Res. **25:** 3371–3372.

Kim, H.S., Kim, S.J., Abbasi, N., Bressan, R.A., Yun, D.J., Yoo, S.D., Kwon, S.Y., and Choi, S.B. (2010). The DOF transcription factor Dof5.1 influences leaf axial patterning by promoting *Revoluta* transcription in Arabidopsis. Plant J. **64:** 524–535.

Konishi, M., and Yanagisawa, S. (2015). Transcriptional repression caused by Dof5.8 is involved in proper vein network formation in *Arabidopsis thaliana* leaves. J. Plant Res. **128:** 643–652.

Lorenzo, O., Chico, J.M., Sánchez-Serrano, J.J., and Solano, R. (2004). *JASMONATE-INSENSITIVE1* encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. Plant Cell **16:** 1938–1950.

Luehrsen, K.R., de Wet, J.R., and Walbot, V. (1992). Transient expression analysis in plants using firefly luciferase reporter gene. Methods Enzymol. **216:** 397–414.

Moreno-Risueno, M., Martínez, M., Vicente-Carbajosa, J., and Carbonero, P. (2007). The family of DOF transcription factors: From green unicellular algae to vascular plants. Mol. Genet. Genomics **277:** 379–390.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15:** 473–497.

Nakagawa, T., Ishiguro, S., and Kimura, T. (2009). Gateway vectors for plant transformation. Plant Biotechnol. **26:** 275–284.

Negi, J., Moriwaki, K., Konishi, M., Yokoyama, R., Nakano, T., Kusumi, K., Hashimoto-Sugimoto, M., Schroeder, J.I.I., Nishitani, K., Yanagisawa, S., and Iba, K. (2013). A Dof transcription factor, SCAP1, is essential for the development of functional stomata in *Arabidopsis*. Curr. Biol. **23:** 479–484.

O'Brien, J.A., and Benková, E. (2013). Cytokinin cross-talking during biotic and abiotic stress responses. Front. Plant Sci. **4:** 451.

Park, D.H., Lim, P.O., Kim, J.S., Cho, D.S., Hong, S.H., and Nam, H.G. (2003). The *Arabidopsis COG1* gene encodes a Dof domain transcription factor and negatively regulates phytochrome signaling. Plant J. **34:** 161–171.

Pauwels, L., et al. (2010). NINJA connects the co-repressor TOPLESS to jasmonate signalling. Nature **464:** 788–791.

Porra, R.J., Thompson, W.A., and Kriedemann, P.E. (1989). Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: Verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim. Biophys. Acta Bioenerg **975:** 384–394.

Qi, T., Wang, J., Huang, H., Liu, B., Gao, H., Liu, Y., Song, S., and Xie, D. (2015). Regulation of jasmonate-induced leaf senescence by antagonism between bHLH subgroup IIIe and IIId factors in Arabidopsis. Plant Cell **27:** 1634–1649.

Ravindran, P., Verma, V., Stamm, P., and Kumar, P.P. (2017). A novel RGL2–DOF6 complex contributes to primary seed dormancy in *Arabidopsis thaliana* by regulating a GATA transcription factor. Mol. Plant **10:** 1307–1320.

Reymond, P., Weber, H., Damond, M., and Farmer, E.E. (2000). Differential gene expression in response to mechanical wounding and insect feeding in Arabidopsis. Plant Cell **12:** 707–720.

Rueda-Romero, P., Barrero-Sicilia, C., Gómez-Cadenas, A., Carbonero, P., and Oñate-Sánchez, L. (2012). *Arabidopsis thaliana* DOF6 negatively affects germination in non-after-ripened seeds and interacts with TCP14. J. Exp. Bot. **63:** 1937–1949.

Sakuraba, Y., Jeong, J., Kang, M.-Y., Kim, J., Paek, N.-C., and Choi, G. (2014a). Phytochrome-interacting transcription factors PIF4 and PIF5 induce leaf senescence in *Arabidopsis*. Nat. Commun. **5:** 4636.

Sakuraba, Y., Park, S.-Y., Kim, Y.-S., Wang, S.-H., Yoo, S.-C., Hörtensteiner, S., and Paek, N.-C. (2014b). *Arabidopsis* STAY-GREEN2 is a negative regulator of chlorophyll degradation during leaf senescence. Mol. Plant **7:** 1288–1302.

Saleh, A., Alvarez-Venegas, R., and Avramova, Z. (2008). An efficient chromatin immunoprecipitation (ChIP) protocol for studying histone modifications in *Arabidopsis* plants. Nat. Protoc. **3:** 1018–1025.

Schildhauer, J., Wiedemuth, K., and Humbeck, K. (2008). Supply of nitrogen can reverse senescence processes and affect expression of genes coding for plastidic glutamine synthetase and lysine-ketoglutarate reductase/saccharopine dehydrogenase. Plant Biol (Stuttg) **10** (Suppl 1): 76–84.

Schweizer, F., Fernández-Calvo, P., Zander, M., Diez-Diaz, M., Fonseca, S., Glauser, G., Lewsey, M.G., Ecker, J.R., Solano, R., and Reymond, P. (2013). *Arabidopsis* basic helix–loop–helix transcription factors MYC2, MYC3, and MYC4 regulate glucosinolate biosynthesis, insect performance, and feeding behavior. Plant Cell **25:** 3117–3132.

Sheard, L.B., et al. (2010). Jasmonate perception by inositol-phosphate-potentiated COI1-JAZ co-receptor. Nature **468:** 400–405.

Shigyo, M., Tabei, N., Yoneyama, T., and Yanagisawa, S. (2007). Evolutionary processes during the formation of the plant-specific Dof transcription factor family. Plant Cell Physiol. **48:** 179–185.

Shin, J., Heidrich, K., Sanchez-Villarreal, A., Parker, J.E., and Davis, S.J. (2012). TIME FOR COFFEE represses accumulation of the MYC2 transcription factor to provide time-of-day regulation of jasmonate signaling in Arabidopsis. Plant Cell **24:** 2470–2482.

Skirycz, A., Jozefczuk, S., Stobiecki, M., Muth, D., Zanor, M.I., Witt, I., and Mueller-Roeber, B. (2007). Transcription factor At-DOF4;2 affects phenylpropanoid metabolism in *Arabidopsis thaliana*. New Phytol. **175:** 425–438.

Skirycz, A., Radziejwoski, A., Busch, W., Hannah, M.A., Czeszejko, J., Kwaśniewski, M., Zanor, M.I., Lohmann, J.U., De Veylder, L., Witt, I., and Mueller-Roeber, B. (2008). The DOF transcription

factor OBP1 is involved in cell cycle regulation in *Arabidopsis thaliana*. Plant J. **56:** 779–792.

Skirycz, A., Reichelt, M., Burow, M., Birkemeyer, C., Rolcik, J., Kopka, J., Zanor, M.I., Gershenzon, J., Strnad, M., Szopa, J., Mueller-Roeber, B., and Witt, I. (2006). DOF transcription factor AtDof1.1 (OBP2) is part of a regulatory network controlling glucosinolate biosynthesis in Arabidopsis. Plant J. **47:** 10–24.

Smet, W., et al. (2019). DOF2.1 controls cytokinin-dependent vascular cell proliferation downstream of TMO5/LHW. Curr. Biol. **29:** 520–529.e6.

Takahashi, F., Yoshida, R., Ichimura, K., Mizoguchi, T., Seo, S., Yonezawa, M., Maruyama, K., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2007). The mitogen-activated protein kinase cascade MKK3-MPK6 is an important part of the jasmonate signal transduction pathway in *Arabidopsis*. Plant Cell **19:** 805–818.

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., Nomura, K., He, S.Y., Howe, G.A., and Browse, J. (2007). JAZ repressor proteins are targets of the SCF(^{COI1}) complex during jasmonate signalling. Nature **448:** 661–665.

Vijayan, P., Shockey, J., Lévesque, C.A., Cook, R.J., and Browse, J. (1998). A role for jasmonate in pathogen defense of *Arabidopsis*. Proc. Natl. Acad. Sci. USA **95:** 7209–7214.

Ward, J.M., Cufr, C.A., Denzel, M.A., and Neff, M.M. (2005). The Dof transcription factor OBP3 modulates phytochrome and cryptochrome signaling in Arabidopsis. Plant Cell **17:** 475–485.

Wasternack, C. (2007). Jasmonates: An update on biosynthesis, signal transduction and action in plant stress response, growth and development. Ann. Bot. **100:** 681–697.

Wasternack, C., and Hause, B. (2013). Jasmonates: Biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in *Annals of Botany*. Ann. Bot. **111:** 1021–1058.

Wei, P.C., Tan, F., Gao, X.Q., Zhang, X.Q., Wang, G.Q., Xu, H., Li, L.J., Chen, J., and Wang, X.C. (2010). Overexpression of AtDOF4.7, an Arabidopsis DOF family transcription factor, induces floral organ abscission deficiency in Arabidopsis. Plant Physiol. **153:** 1031–1045.

Weidhase, R.A., Lehmann, J., Kramell, H., Sembdner, G., and Parthier, B. (1987). Degradation of ribulose-1,5-bisphosphate carboxylase and chlorophyll in senescing barley leaf segments triggered by jasmonic acid methylester, and counteraction by cytokinin. Physiol. Plant. **69:** 161–166.

Woo, H.R., Kim, H.J., Lim, P.O., and Nam, H.G. (2019). Leaf senescence: Systems and dynamics aspects. Annu. Rev. Plant Biol. **70:** 347–376.

Wu, F.H., Shen, S.C., Lee, L.Y., Lee, S.H., Chan, M.T., and Lin, C.S. (2009). Tape-*Arabidopsis* Sandwich—a simpler *Arabidopsis* protoplast isolation method. Plant Methods **5:** 16.

Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M., and Turner, J.G. (1998). COI1: An Arabidopsis gene required for jasmonate-regulated defense and fertility. Science **280:** 1091–1094.

Yadav, V., Mallappa, C., Gangappa, S.N., Bhatia, S., and Chattopadhyay, S. (2005). A basic helix–loop–helix transcription factor in Arabidopsis, MYC2, acts as a repressor of blue light-mediated photomorphogenic growth. Plant Cell **17:** 1953–1966.

Yan, Y., Stolz, S., Chételat, A., Reymond, P., Pagni, M., Dubugnon, L., and Farmer, E.E. (2007). A downstream mediator in the growth repression limb of the jasmonate pathway. Plant Cell **19:** 2470–2483.

Yanagisawa, S. (1995). A novel DNA-binding domain that may form a single zinc finger motif. Nucleic Acids Res. **23:** 3403–3410.

Yanagisawa, S. (1996). Dof DNA-binding proteins contain a novel zinc finger motif. Trends Plant Sci. **1:** 213–214.

Yanagisawa, S. (2015). Structure, function, and evolution of the Dof transcription factor family. In Plant Transcription Factors: Evolutionary, Structural and Functional Aspects, D. Gonzalez, ed (New York: Elsevier), pp. 183–197.

Yanagisawa, S. (2002). The Dof family of plant transcription factors. Trends Plant Sci. **7:** 555–560.

Yanagisawa, S., and Schmidt, R.J. (1999). Diversity and similarity among recognition sequences of Dof transcription factors. Plant J. **17:** 209–214.

Yanagisawa, S., and Sheen, J. (1998). Involvement of maize Dof zinc finger proteins in tissue-specific and light-regulated gene expression. Plant Cell **10:** 75–89.

Yoo, S.D., Cho, Y.H., and Sheen, J. (2007). *Arabidopsis* mesophyll protoplasts: A versatile cell system for transient gene expression analysis. Nat. Protoc. **2:** 1565–1572.

Zhai, Q., Yan, L., Tan, D., Chen, R., Sun, J., Gao, L., Dong, M.Q., Wang, Y., and Li, C. (2013). Phosphorylation-coupled proteolysis of the transcription factor MYC2 is important for jasmonate-signaled plant immunity. PLoS Genet. **9:** e1003422.

Zhang, F., et al. (2015). Structural basis of JAZ repression of MYC transcription factors in jasmonate signalling. Nature **525:** 269–273.

Zhang, L., Zhang, F., Melotto, M., Yao, J., and He, S.Y. (2017). Jasmonate signaling and manipulation by pathogens and insects. J. Exp. Bot. **68:** 1371–1385.

Zhang, X., Henriques, R., Lin, S.S., Niu, Q.W., and Chua, N.H. (2006). *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method. Nat. Protoc. **1:** 641–646.

Zhu, X., Chen, J., Xie, Z., Gao, J., Ren, G., Gao, S., Zhou, X., and Kuai, B. (2015). Jasmonic acid promotes degreening via MYC2/3/4- and ANAC019/055/072-mediated regulation of major chlorophyll catabolic genes. Plant J. **84:** 597–610.

Zou, H.F., et al. (2013). The transcription factor AtDOF4.2 regulates shoot branching and seed coat formation in *Arabidopsis*. Biochem. J. **449:** 373–388.

Zuo, J., Niu, Q.W., and Chua, N.H. (2000). Technical advance: An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. Plant J. **24:** 265–273.