Jasmonate activates secondary cell wall biosynthesis through MYC2-MYB46 module

Kyung Hwan Han[1], Jong Hee Im[1], Seungmin Son[2], Won-Chan Kim[3], Kihwan Kim[3], Nobutaka Mitsuda[4], and Jae-Heung Ko[5]

[1]Michigan State University Department of Horticulture
[2]National Institute of Agricultural Sciences Department of Agricultural Biotechnology
[3]Kyungpook National University College of Agriculture and Life Sciences
[4]National Institute of Advanced Industrial Science and Technology (AIST
[5]Kyung Hee University College of Life Sciences

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Abstract

The formation of secondary cell walls is tightly regulated spatio-temporally by various developmental and environmental signals. Successful fine-tuning of the trade-off between secondary cell wall biosynthesis and stress responses requires better understanding of how plant growth is regulated under environmental stress conditions. However, current understanding of the interplay between environmental signaling and secondary cell wall formation is limited. The lipid-derived plant hormone jasmonate (JA) and its derivatives are important signaling components involved in various physiological processes including plant growth, development, and abiotic/biotic stress response. Recent studies suggest that JA may be involved in secondary cell wall formation. We tested this hypothesis using the transcription factor MYB46, a master switch for secondary wall biosynthesis, and JA treatments. Both the transcripts and protein levels of MYB46 were significantly increased by the JA treatments, which also triggered the upregulation of MYB46 downstream genes with increased secondary wall formation. We then show that this JA-induced upregulation of MYB46 function was mediated by MYC2, a basic helix-loop-helix (bHLH) domain–containing transcription factor, which plays a pivotal role in the JA-mediated changes. We conclude that this MYC2-MYB46 module is a key component of the plant response to JA signaling.

1. Introduction

The lipid-derived plant hormone jasmonate (JA) and its derivatives, collectively called jasmonates (JAs), are important signaling components involved in various physiological processes including plant growth, development, and abiotic/biotic stress response. In the JA signaling, JA is first conjugated with amino acid leucine to form biologically active JA-Ile, which is then transported to nucleus by ATP-binding cassette G (ABCG) transporter that is localized in the nuclear envelop and acts as JA transporter (JAT; AtJAT1/AtABCG16) (Li et al., 2017). In the absence of JA, its signaling is repressed by JA zinc finger inflorescence meristem (ZIM)-domain (JAZ) proteins (Chini et al., 2007). The JAZs interact with MYC2, a central activator of JA signaling via ZIM domain, resulting in inactivation of MYC2 (Chini, Fonseca, Chico, Fernández-Calvo, & Solano, 2009; Kazan & Manners, 2013). In the presence of JA, JA-Ile drives the direct binding of JAZs with the F-box protein coronatine-insensitive 1 (COI1) through JAZ domain, which degrades the JAZs through ubiquitin-mediated proteasome pathway (Sheard et al., 2010; Thines et al., 2007; J. Yan et al., 2013). As a result, MYC2 is activated and regulates the expression of various target genes involved in JA signaling.

MYC2, first identified from the JA-insensitive mutant *jin1*(Lorenzo, Chico, Sanchez-Serrano, & Solano, 2004), is involved in various phytohormone crosstalk and signaling pathways related to JA (Son, Kwon, & Im,

2021). In its free form, MYC2 physically interacts with MED25, a subunit of the mediator transcriptional co-activator complex, to form a transcription activation complex that orchestrates JA-triggered transcriptional reprogramming (Cevik et al., 2012; H. Wang et al., 2019; Zhai, Deng, & Li, 2020). MYC2 has two close paralogs, MYC3 and MYC4, which are also directly regulated by JAZs (Fernandez-Calvo et al., 2011). Although some cases have shown their differential contributions, many reports revealed that MYC2, MYC3, and MYC4 have redundant function in a variety of regulatory processes including plant growth/development and metabolisms related to JA (Gao et al., 2016; Gasperini et al., 2015; Qi, Huang, Song, & Xie, 2015; Qi, Wang, et al., 2015; D.-D. Wang et al., 2021; H. Wang et al., 2017; Wasternack & Strnad, 2019). Therefore, they are considered as key synergistic regulators in the JA response (Chico et al., 2020; Van Moerkercke et al., 2019; C. Zhang, Lei, Lu, Wang, & Wu, 2020).

While primary cell wall exists in all plant cells, secondary cell wall (SCW) is formed in specialized cell types such as tracheary elements and fibers (R. Zhong, Cui, & Ye, 2019). SCW, located between primary cell wall and plasma membrane, is synthesized after cell expansion ceases (Nishitani & Demura, 2015). SCWs are mainly consisted with cellulose, hemicelluloses (e.g., xylan and glucomannan), and lignin. They are important both scientifically (i.e., growth and development of the plant) and economically (i.e., biomass production) (H. Z. Wang & Dixon, 2012). SCW synthesis is tightly regulated spatio-temporally by hierarchical transcriptional networks (Taylor-Teeples et al., 2015). Numerous transcription factors participate in the multi-faceted regulatory cascade controlling SCW formation (Kubo et al., 2005; Mitsuda, Seki, Shinozaki, & Ohme-Takagi, 2005; Ruiqin Zhong, Demura, & Ye, 2006; Ruiqin Zhong, Lee, & Ye, 2010). MYB46 and its paralog MYB83 are considered as master regulators of SCW biosynthesis (J.-H. Ko, Jeon, Kim, Kim, & Han, 2014). Promoters of *MYB46* and *MYB83* are activated by several secondary wall-associated NAC domain proteins (e.g., VND6, VND7, NST1, NST2, and NST3/SND1) (McCarthy, Zhong, & Ye, 2009; J. Zhang, Xie, Tuskan, Muchero, & Chen, 2018; Ruiqin Zhong, Richardson, & Ye, 2007). MYB46 and MYB83 transcription factors directly activate downstream regulators of the SCW transcriptional cascade including MYB20, MYB42, MYB43 and MYB85 as well as *cellulose synthase* (*CESA*) genes and a suit of lignin biosynthetic genes including *4-coumarate-CoA ligase 1* (*4CL1*) and*phenylalanine ammonia-lyase 4* (*PAL4*), resulting in transcriptional reprograming for the biosynthesis of SCW (J. H. Ko, Kim, & Han, 2009; Xiao, Zhang, Guo, Li, & Lu, 2021).

Several recent studies have shown that JA activates the expression of SCW biosynthesis-related genes (Didi, Jackson, & Hejatko, 2015; Xue, Tao, & Yang, 2008). However, the molecular mechanisms by which JA activates SCW formation are still poorly understood. Based on the observations in the literature and our preliminary studies, we hypothesized that JA activates SCW biosynthesis through MYC2-MYB46 module. Here, we provide experimental evidence in support of the hypothesis.

2. Materials and Methods

Plant growth conditions.

For experiment with seedlings, *Arabidopsis thaliana* Columbia (Col-0) plants were germinated and grown in 1/2 liquid MS media contained 1% sucrose in a growth room (14 h light; light intensity 150 μmol m^{-2} s^{-1}; humidity ~ 50%; 24°C). For stem staining, *Arabidopsis* plants were grown on soil for 8-9 weeks with 16 h light/8 h dark at 24°C. For protoplast generation, plants were grown in soil for 23 to 25 days with 13 h light/11 h dark at 23°C.

Methyl jasmonate treatment

Arabidopsis seeds were germinated and grown in liquid medium ($\frac{1}{2}$ MS plus 0.5% sucrose) for 7 days and added 1 μl of 50 mM methyl jasmonate (392707, Sigma-Aldrich, St Louis, MO, USA) to the media, and additionally grown for desired time points. For the treatment of mature plants, 32-day-old plants were sprayed with 1mL of 50 μM Methyl jasmonate every two days for desired time points.

Plasmid generation

GUS conjugated *MYB46* promoter was used as previously reported (Jong Hee Im et al., 2021). For generation

of *MYC2*overexpressing plant, cDNA of *MYC2* was cloned into pDONR/Zeo (Thermo Fisher Scientific) and LR reaction was carried out with pEarleygate201. This construct was introduced into *Agrobacterium tumefaciens* (GV3101) and transformed *Arabidopsis* with floral dip method. For transient promoter assay, *MYC2* , *MYC3* and*MYC4* were cloned into HBT vector (Im et al*.,* 2021) with YFP conjugation.

Yeast-one-hybrid screening

Yeast one-hybrid screening was performed as described previously (Mitsuda et al., 2010) with some modifications. Briefly, yeast strain YM4271 was employed for the screening of transcription factor(s) binding to *MYB46* promoter. The promoter region (ca. 1500 bp from translation start site) was cloned into pHISi2 vector in which extra start codons of pHISi (Clontech/Takara bio Inc.) residing within 5' untranslated region of the reporter gene *HIS3* are mutated. The promoter-cloned pHISi2 was linearized with *ApaI* restriction enzyme and promoter::*HIS3* fusion was integrated into YM4271 genome. 1,299 Arabidopsis transcription factor genes were cloned into pGAD424 vector (Clontech/Takara bio Inc.), divided into 348 mini pools and individual interactions between each promoter and mini pool were examined by the yeast growth on the selective media lacking histidine supplemented with 3-amino-1,2,4-triazole (3-AT) to suppress background growth. Second screening was performed for individual transcription factors from positive mini pools.

MYC2 expression and Electrophoretic Mobility Shift Assay (EMSA)

The full-length *MYC2* CDS was PCR-amplified from total cDNA of*Arabidopsis thaliana* and cloned into the bacterial expression vector pET28a (Novagen). The pET28a-MYC2 construct vector was transformed into *Escherichia coli* strain Rosetta-gami2 (DE3). The transformed cells were cultured in LB broth containing 50 μg/ml kanamycin at 37°C (OD_{600nm} = 0.5), then 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added and incubated at 16 °C for 16 h. For protein purification, cell pellets were collected and resuspended in binding buffer [500 mM NaCl, 20 mM Tris-HCl, pH 7.9, 5 mM imidazole] and sonicated. After the cell debris was removed by centrifugation (15,000 X g for 20 min at 4°C), His_6-MYC2 recombinant protein was isolated using Ni^{2+}-NTA (Qiagen) affinity chromatography according to the manufacturer's instructions. The His_6-MBP was purified in the same manner and used as the control.

The MYB46 promoter region, divided into seven fragments (Supplemental Figure 6), was amplified from leaf gDNA of *Arabidopsis thaliana*by PCR using gene-specific primers. The PCR fragments were biotin-labeled using Biotin 3' End DNA Labeling Kit (ThermoFisher Scientific). Briefly, the amplified PCR fragments were labeled with biotin-11-UTP using Terminal Transferase (TdT). The end-labeled fragments were purified using chloroform: isoamyl alcohol to extract the TdT. The purified end-labeled DNA fragments were incubated for 30 min with 200 ng of His_6-MYC2 recombinant protein in a binding buffer [10 mM Tris, pH 7.5, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM $MgCl_2$, 0.05% (v/v) NP-40, 50 μg/μl dI-dC]. Polyacrylamide gel electrophoresis (7% PAGE) was used to separate the His_6-MYC2 recombinant protein-labeled DNA fragments complex from the unbound ones. The 7% PAGE gel was transferred to a Nylon membrane to detect the chemiluminescent signal. The Nylon membrane was placed in a film cassette and exposed to X-ray film.

Protein blot analysis

In seedlings, total protein was isolated from 7-day-old seedlings using protein extraction buffer [50 mM Tris (pH 7.4), 1% NP-40, 150 mM NaCl, 1 mM EDTA with protease inhibitor cocktail]. The concentration of total protein was measured with Bradford assay (Bradford, 1976). In the protoplast, *MYC2, MYC3* and *MYC4* were respectively expressed in protoplast for 10 h and total protein was lysed using sodium dodecyl sulphate (SDS) protein sample buffer and heated at 95°C for 5 min. The protein was separated with SDS-PAGE system. After the protein was transferred to PVDF membrane, anti-MYB46 (Jong Hee Im et al., 2021) antibody or anti-GFP antibody (Thermo Fisher scientific) was used as primary antibody and HRP conjugated antibody was used for secondary antibody.

Generation of *Arabidopsis* mesophyll protoplasts and transient promoter assay.

Protoplast generation and transient promoter assay were performed as described previously (Jong Hee Im

& Yoo, 2014; Kim et al., 2013). For generation of effector protein, *MYC2* , *MYC3* and *MYC4* cDNA were cloned in *YFP* tagged *HBT* vector (J. H. Im et al., 2014). Plasmid DNA was prepared using a Plasmid Plus Maxi kit (QIAGEN, Valencia, CA, USA), and used for transfection. 8 μg of *GUS* conjugated *MYB46* promoter was co-expressed with 6 μg of *NAN* plasmid (Kirby & Kavanagh, 2002). Then, added 34 μg of effector plasmid or empty vector for control, then transfected to 200 μl of protoplasts, and incubated for 6 h. β-Glucuronidase and NAN enzyme assays were performed as described by (Kirby & Kavanagh, 2002). NAN and GUS activities were measured using MUN (Sigma-Aldrich Co.) and MUG (Sigma-Aldrich Co.) as substrates, respectively, against MU standards on a SpectraMax M2 Microplate Readers (excitation: 365 nm, emission: 455). The ratio of GUS and NAN activities is represented as relative GUS/NAN units.

Chromatin immunoprecipitation (ChIP)-qPCR

C-terminal *YFP* conjugated *MYC2* was expressed in protoplasts for 10 h. The DNA and protein were cross-linked with 1% formaldehyde for 20 min at room temperature. The cells were washed three times with cold WI buffer contained 125 mM glycine and protease inhibitor cocktail for 10 min. The cells were disrupted with SDS buffer (1% SDS, 1% Triton X-100, 10 mM EDTA, 50 mM Tris-HCl pH 8.0 and protease inhibitor cocktail) and sonicated, then centrifuged. The supernatant was mixed with ChIP dilution buffer (0.01% SDS, 1 mM EDTA, 20 mM Tris-HCl pH 8.0, 20 mM NaCl and protease inhibitor cocktail). The samples were equally divided and respectively added anti-HA antibody and anti-GFP antibody, then incubated for 3 h at 4 with 50 rpm in rotor. After added protein A-agarose and incubated for 5 h at 4, the samples were washed with low salt buffer (0.1% SDS, 2 mM EDTA, 2 mM Tris-HCl pH 8.0, 40 mM NaCl and protease inhibitor cocktail), high salt buffer (0.1% SDS, 2 mM EDTA, 2 mM Tris-HCl pH 8.0, 150 mM NaCl and protease inhibitor cocktail), and were washed two times with TE buffer (pH 8.0). DNA was eluted two times by 250 μl of elution buffer (0.1% SDS and 0.1 M $NaHCO_3$) and added 20 μl of 5M NaCl to the samples and incubated for 4 h at 65 for decross-linking. The samples were treated with protease K for 1 h at 45 . The DNA was purified with DNA purification kit (Promega) and used for qPCR with *MYB46* promoter specific primers.

Phloroglucinol-HCl staining.

Arabidopsis seedling root or rosette-level stem sections were stained with 2% phloroglucinol-HCl for 3 min and washed three times with distilled water and the image was taken by microscope (Diaphot Inverted ELWD0.3, Nikon).

Quantification of lignin by acetyl bromide method

Quantification of lignin was carried out as described previously (Moreira-Vilar et al., 2014). Briefly, Arabidopsis stems were harvested around 5 cm from the bottom of the stem and dried. The dried stems (˜ 0.3g) were then ground with mortar and pestle in liquid nitrogen and homogenized with 50 mM potassium phosphate buffer (pH 7.0). The samples were centrifuged at 2500 × *g* for 5 min at room temperature and washed twice with the 50 mM potassium phosphate buffer. Then, the samples were washed once with 1% Triton X-100 and washed twice with acetone. The samples were dried in oven for 24 h at 60 and cooled down.

For quantification of lignin, cell wall residues were mixed with 0.5 ml of 25% acetyl bromide in glacial acetic acid and incubated at 70 for 30 min. After cooled down on ice, the samples were subsequently mixed with 0.9 ml of 2 M NaOH, 0.1 ml of 5 M hydroxylamine-HCl and 4 ml of acetic acid. The samples were centrifuged at 2500 x *g* for 5 min at room temperature. The absorbance was measured at 280 nm using the supernatants of the samples with spectrophotometer (U-3000 Hitachi, Japan). Lignin content was calculated according to the method described previously (Barnes & Anderson, 2017).

RT-qPCR Analysis.

Total RNA was extracted from 7-day-old *Arabidopsis* or 8- or 9-week-old stem using RNeasy Plant Mini Kit (Qiagen). For cDNA synthesis, SuperScript II Reverse Transcriptase (Invitrogen) was used. qPCR was

carried out with specific primers on 7500 Real-Time PCR System (Applied Biosystems) using comparative Ct method with Fast SYBR Green Master Mix (Applied Biosystems).

3. Result

Jasmonate upregulates MYB46 activity

To see whether jasmonate (JA) transcriptionally regulates *MYB46* , we applied 50 μM Methyl jasmonate (MJA) treatment to 7-day-old seedlings in a time-dependent manner. The transcript level of *MYB46* was significantly increased after 6 h of the MJA treatment and peaked at 24 h. However, this JA-triggered upregulation of *MYB46* expression was not observed in a jasmonate-insensitive mutant *jar1-1* , which does not catalyze the formation of a biologically active jasmonyl-isoleucine (JA-Ile) conjugate (Staswick, Tiryaki, & Rowe, 2002) (Figure 1A). Next, we analyzed the protein level of MYB46 with the MJA treatments using anti-MYB46 antibody (Jong Hee Im et al., 2021). MYB46 protein level was first reduced after 3 h of the treatment but increased after 6 hour post treatment (hpt) and peaked at 24 hpt, similar to the *MYB46* gene expression response. (Figure 1B). To further confirm the JA-mediated up-regulation of MYB46, we analyzed the expression of MYB46 downstream target genes *CESA4* , *CESA8* ,*4CL1* and *PAL4* at 24 hpt. The expression of the four target genes was significantly increased by the MJA treatment at 24 hpt (Figure 1C), but such upregulation was not observed in the *jar1-1* plants (Supplemental Figure 1), indicating that the JA upregulates MYB46 activity.

In an effort to gain insights into the decrease of MYB46 protein at 3 hpt, we analyzed the expressions of *MYB46* and its downstream genes in 50 μM MJA treated Col-0 and *mpk6* seedlings since MPK6 is activated by JA and negatively regulates MYB46 (Jong Hee Im et al., 2021; Takahashi et al., 2007). Expressions of MYB46 downstream genes (*e.g.* , *4CL1* and *PAL4*) were significantly reduced in early time points of the MJA treatment (before 6 hpt) despite that*MYB46* gene expression was increasing (Supplemental Figure 2). However, this reduction in their gene expression was not observed in the MJA-treated *mpk6* mutant (Supplemental Figure 2), suggesting that the MYB46 reduction in early time point (3 hpt) by the MJA treatment may be due to negative regulation by MPK6.

JA upregulates SCW biosynthesis through MYB46

Since MJA treatment significantly increased *MYB46* gene expression (Figure 1A), and MYB46 is a master regulator of secondary cell wall (SCW) formation, we hypothesized that JA upregulates SCW formation. To test the hypothesis, we first carried out histological staining of lignin with MJA treatment, since lignin is a prominent feature of SCW. Seven-day-old seedlings were treated with 50 μM MJA and stained with phloroglucinol-HCl. The lignin staining (red color in Fig **2a**) was detected after 24 hpt and peaked at 36 hpt (Figure 2A). On the other hand, the lignin staining was not detected in the *myb46* mutant (Figure 2A). We then checked the expression of secondary wall biosynthetic genes (i.e., MYB46 downstream genes) using RT-qPCR after 24 h of the MJA treatment. The expression of *CESA4* , *CESA8* ,*4CL1* and *PAL4* was significantly increased in 50 μM MJA treated Col-0 seedlings but no significant change in the *myb46*plants (Figure 2B). However, it is notable that the MJA treatment did not affect the expression of *MYB83* , a paralog of MYB46 (Supplemental Figure 3).

Having confirmed the JA-mediated upregulation of SCW formation using seedlings, we wanted to further confirm this finding in the context of plant's life-long impact of JA using mature plants. To do this, we sprayed 32-day-old plants with 50 μM MJA every two days for two weeks. We first verified the impact of the MJA treatment by analyzing the expression of *MYC2* , which was significantly increased in the stem by the MJA treatment (Supplemental Figure 4). As expected, *MYB46*gene expression was significantly increased by the MJA spray (Figure 2C). As a visual confirmation of the JA-mediated up-regulation of SCW formation, we carried out phloroglucinol-HCl staining of the stem for lignin. The cross sections of the Col-0 stems treated with MJA showed ectopic lignification (Figure 2D), which is a characteristic feature of MYB46 overexpression (Ko et al., 2009). Indeed, the lignin contents were increased significantly by MJA treatment (Figure 2E). However, this ectopic lignification was not observed in the *myb46* stems (Figure 2D and 2E). This histological data corroborates the observations from gene expression analyses. Namely, the expression

of four MYB46 direct target genes was significantly increased in Col-0 stems but not in *myb46* (Figure 2F).

MYC2 binds to and activates *MYB46* promoter

In light of the JA-mediated upregulation of MYB46 function (Figure 1 and 2) and the fact that MYC2 is a central regulator of JA signaling (Kazan & Manners, 2013), we hypothesized that MYC2 may be an upstream regulator of MYB46. To test this hypothesis, we first carried out Yeast-1-Hybrid (Y1H) screening using *MYB46* promoter as bait. The Y1H screening identified seven putative upstream regulators including MYC2 (Table 1; Supplemental Figure 5). Direct binding of MYC2 to the promoter region of *MYB46* was then confirmed by Electrophoretic Mobility Shift Assay (EMSA) (Figure 3A; Supplemental Figure 6). It showed that MYC2 potentially binds to three parts of the promoter. To verify the binding, we then carried out chromatin immunoprecipitation (ChIP)-qPCR *in vivo* with the three potential binding parts. The ChIP-qPCR showed that MYC2 binds the most strongly to the *MYB46* promoter between -1100 and -900 bp area from the translation start site (TSS) (Figure 3B).

Having the binding of MYC2 to the *MYB46* promoter confirmed, we next tested whether MYC2 actually activates *MYB46* promoter activity. To do this, we expressed C-terminal *GUS* conjugated *MYB46* promoter (-1600 from TSS) in mesophyll protoplasts with or without MYC2 co-expression. As expected, the MYC2 co-expression significantly increased the GUS activity (Figure 3C). Serial deletions of the promoter were used to further pinpoint the MYC2 binding site in the promoter (Figure 3D). The MYC2-dependent *MYB46* promoter activity was significantly reduced when the upstream region between -1100 and -900 from the TSS was deleted (Figure 3E), corroborating the ChIP-qPCR results in Figure 3B suggesting the region between -1100 and -900 as the MYC2 binding site. The same pattern was also observed when tested in *nst1-1* mutant background, which was done to eliminate the influence of a known upstream regulator of *MYB46* (i.e., NST1) in the promoter analysis (Figure 3F). The MYC2 binding region of *MYB46* promoter contains two putative MYC2 binding motifs, G-box variant and Z-box (Figure 3D). In order to pinpoint the actual MYC2 binding motif(s), we used site-directed mutagenesis to generate G-box variant and Z-box mutants and carried out transient promoter assay using the mutants. As shown in Figure 3G, MYC2-dependent *MYB46* promoter activity was compromised in the G-box variant mutant but not in the Z-box mutant. Hence, we conclude that the G-box variant, not Z-box, of *MYB46* promoter is required for MYC2-dependent activation of its activity.

JA-dependent SCW formation is mediated by MYC2

With the conclusions that JA activates SCW formation (Figure 2) and MYC2 directly activates *MYB46* expression (Figure 3), we hypothesized that MYC2 plays a critical role in the JA-mediated SCW formation. To test this hypothesis, we observed MJA-induced increase in MYB46 protein level in the Col-0 plants but not in the *myc2-2* (Figure 4A). As expected, the expression of downstream SCW biosynthetic genes (i.e., *CESA4, CESA8, 4CL1, PAL4*) was significantly increased by the MJA treatment in the Col-0 seedlings but not in the *myc2-2* plants (Figure 4B). To phenotypically confirm this observation, we examined for lignin formation in the roots using phloroglucinol-HCl staining. JA-induced lignification was clearly observed in the Col-0 roots but not in the *myc2-2* (Figure 4C). Next, we wanted to confirm the seedling-based observations in mature plants. When 32-days-old mature plants were sprayed with 50 μM MJA every two days for two weeks (as in Figure 2), the MJA treatment significantly increased the expression of downstream SCW biosynthetic genes (i.e., *CESA4, CESA8, 4CL, PAL4*) in the Col-0 seedling but not in the *myc2-2* (Figure 4D). Likewise, the Col-0 stems showed ectopic lignification in the phloem region and increased lignin contents (Figure 4E and 4F), a typical phenotype of MYB46 overexpression (J. H. Ko et al., 2009). Based on these observations and prior knowledge that MYC2 is a central regulator in JA signaling, we conclude that MYC2 is necessary for the JA-activated SCW formation.

MYC2-MYB46 module in the regulation of SCW formation

Since JA increases SCW formation through MYB46 and MYC2 (Figure 2 and 4) and MYC2 is a direct upstream activator of *MYB46* , we hypothesize that MYC2-MYB46 regulatory module positively regulates SCW formation. To test this, we generated MYC2 overexpressing plants in the background of Col-0

(MYC2OX) and *myb46* (MYC2OX/*myb46*). We selected two independent individual lines, one each from MYC2OX and MYC2/*myb46* , having similar MYC2 expression levels (Supplemental Figure 7). We first verified that MYC2OX seedlings had increased MYB46 protein level (Figure 5A). As expected, the expression of four MYB46 target genes (i.e., *CESA4, CESA8, 4CL1,* PAL4) was significantly increased in the MYC2OX plants but not in the MYC2OX/*myb46* (Figure 5B), indicating that MYC2 activates SCW biosynthetic genes through MYB46.

To put the findings in plants' life-long context, we then analyzed SCW formation in the stems of 9-week-old MYC2OX and MYC2OX/*myb46* plants. We used 9-week-old plants, instead of 7-week, because *MYC2* over-expressing plant has delayed inflorescence phenotype and *myc2/3/4* triple mutant showing early inflorescence (H. Wang et al., 2017). As expected, the expression of the four SCW biosynthetic genes was significantly increased in the MYC2OX stems but not the MYC2OX/*myb46* stems (Figure 5C). When the stem cross sections were stained with phloroglucinol-HCl, both the MYB46OX and MYC2OX stems showed ectopic lignin formation but not the MYC2OX/*myb46* (Figure 5D). Lignin content was also increased in the MYC2OX plants but not in MYC2OX/*myb46* (Figure 5E). These data clearly support the hypothesis.

MYC3 and MYC4 activate MYB46 in JA signaling

Since MYC3 and MYC4 are functional paralogs of MYC2 (C. Zhang et al., 2020), we investigated whether MYC3 and MYC4 can activate *MYB46* promoter activity. We co-expressed MYC2, MYC3 and MYC4, respectively, with *pMYB46* ::*GUS* construct in mesophyll protoplasts (Figure 6A) and carried out transient promoter assay. The *MYB46* promoter activity was significantly increased by all three MYC proteins, albeit MYC2 had the highest increase (Figure 6B). In order to assess whether MYC3 and MYC4 are targeting the same binding site as MYC2, we carried out additional TA analysis with co-expression of G-box variant or Z-box mutants of *MYB46* promoter with MYC3 or MYC4. As shown in Figure 6C, both MYC3 and MYC4 activate *MYB46* promoter through G-box variant like MYC2. To further ascertain this *MYB46* activation by MYC3 and MYC4, we analyzed *MYB46* gene expression with MJA treatment in *myc2* , *myc3* , *myc4* and *mycT* (*myc2,3,4* triple mutant) mutant plants. MJA-dependent upregulation of *MYB46* expression was significantly compromised in *myc2* and *mycT* plants, while it was slightly reduced in the *myc3* or *myc4* single mutant plants (Figure 6D). These data suggest that MYC2 is the major activator of *MYB46* in JA signaling and that both MYC3 and MYC4 might have additive effect in the regulation. Additional studies are needed to define their exact roles in JA-mediated SCW formation.

4. Discussion

Plants as sessile organisms have to face changing environments throughout their life span. For their adaptation and survival, plants have evolved to balance the trade-off between stress-responses and growth (e.g., biomass formation). The cell walls are dynamically manipulated in composition and structure in response to internal and external stimuli to provide mechanical support and defense systems (Vaahtera, Schulz, & Hamann, 2019). The SCW biosynthesis is controlled by the metabolome and transcriptome regulated by phytohormone and environmental signaling (Bacete, Melida, Miedes, & Molina, 2018; Coleman, Brunner, & Tsai, 2021; Felten et al., 2018; Johnsson et al., 2019; Liu, Yu, Rao, Li, & Dixon, 2021). However, current understanding of SCW formation mechanisms and signaling components is limited.

The plant hormone JA and its derivatives are involved in various aspects of plant growth, such as biotic, abiotic stresses and development (J. Chen et al., 2020; Fugate et al., 2018; Hu et al., 2017; Seo, Sano, & Ohashi, 1999; Suzuki et al., 2021; Wojciechowska, Wilmowicz, Marzec-Schmidt, Ludwików, & Bagniewska-Zadworna, 2020). Recent studies showed that JA is also involved in SCW formation (Behr et al., 2019; Pauwels et al., 2008; Sehr et al., 2010). However, the signaling pathway from JA to SCW formation has not been studied yet. Thus, we attempted to gain insight into this signaling pathway using MYB46, a well-known master regulator of SCW formation. We show that MYB46 function is upregulated by JA. This was demonstrated by MJA treatment-induced increase in *MYB46* gene expression and protein level as well as its direct target genes in SCW biosynthesis (Figure 1).

It is notable that *MYB83* , a paralog of *MYB46* , was not regulated by the MJA treatment (Supplemental

Figure 3). While MYB46 and MYB83 are functionally redundant, their regulatory mechanism is not always the same as we demonstrated previously (Im et al., 2021). Moreover, this study revealed that JA- and MYC2-mediated SCW formation was inhibited significantly in *myb46* single mutant (Figure 2A, 2D, and 5D). These results suggested that MYB83 may not be a part of JA-mediated SCW formation due to non-activation by JA. Recent study reproted that short A/T-rich modules surrounding the MYC2 binding motif G-box, especially at position ±5, are necessary for the binding (López-Vidriero et al., 2021). Although both *MYB46* and*MYB83* have G-box motif in their promoter regions, *MYB46*promoter contains the necessary A/T-rich module near G-box variant, while *MYB83* promoter does not. It may result in non-activation of*MYB83* by JA signaling. However, since *MYB83* transcipts could be controlled by other mechanisms such as transcriptinoal repressor or post-transcriptional regulation, these possibilities must be addressed in future studies.

JA negatively regulates cell wall damage-dependent lignin formation (Denness et al., 2011). They showed that JA-mediated negative regulation on lignin formation was shown as early as 12 hpt. In our analysis, MYB46 protein stability was reduced at 3 hpt by MJA treatment (Figure 1B). MYB46 is negatively regulated by stress- and hormone-dependently activated MPK6 but MYB83, a paralog of MYB46, is not (Jong Hee Im et al., 2021), suggesting that MYB46 may be more responsive to hormone and environmental stresses than MYB83.

Since JA activates *MYB46* expression and increases SCW formation but not in *myb46* (Figure 2A and 2D), we carried out Y1H analysis in search of upstream transcriptional regulators of *MYB46* (Table 1) which led to the discovery of MYC2 as a potential direct regulator of MYB46. Among them, we tested the relationship between MYB46 and MYC2 because MYC2 is a major player of JA signaling. MYC2 was found to bind G-box variant of *MYB46* promoter and activated it (Figure 3E and 3G). Further, MYB46 protein level and expressions of MYB46 downstream genes were increased in the plant overexpressing *MYC2* (Figure 5). However, even JA treatment, SCW formation and expression of MYB46 downstream genes were not increased in the *myc2-2* mutant (Figure 4), and even *MYC2* was overexpressed, SCW biosynthesis also was not increased in *myb46* mutant (Figure 5). These findings suggest that JA-dependent SCW formation is regulated by MYC2-MYB46 module.

MKK3-MPK6-MYC2 module is activated by various signals including JA (Sethi, Raghuram, Sinha, & Chattopadhyay, 2014; Takahashi et al., 2007; Verma, Jalmi, Bhagat, Verma, & Sinha, 2020). In this signaling event, MYC2 inhibits MPK6 function in a negative feedback loop mechanism (Sethi et al., 2014; Verma et al., 2020). This dual function of MYC2, both an activator of *MYB46* expression and an inhibitor of MPK6 function, may explain the observed reduced expression of MYB46 direct target genes in early time point of MJA treatment that was observed (Supplemental Figure 2). The MYC2-mediated MPK6 inhibition may be important for MYB46 protein stability resulting in JA-induced SCW biosynthesis. This may explain why *myc2* single mutant alone is sufficient to inhibit JA-induced SCW biosynthesis (Figure 4C and 4E) and why MYC2 is more important than MYC3 and MYC4 (Figure 6B and 6D).

The Y1H analysis identified four ANACs, two MYCs and one DREB1D as putative *MYB46* upstream regulators (Table 1). ANACs are well known *MYB46/MYB83* upstream regulators (J.-H. Ko et al., 2014). MYC2 and MYC4 were identified as direct activators of *MYB46* in this study. However, the relationship between DREB1D and *MYB46* is not studied yet. *DREB1D* overexpression increases drought resistance in soybean and *Arabidopsis* (Guttikonda et al., 2014; Haake et al., 2002). Overexpression of MdMYB46, an apple ortholog of MYB46, enhances resistance against salt and osmotic stress (K. Chen et al., 2019). Low lignin content increases drought sensitivity in Cassava (Y. Yan et al., 2021). In light of these observations, it is prudent to think that MYB46 may also be involved in drought tolerance along with DREB1D.

Blue light activates SCW formation through MYC2-NST1-MYB46 regulatory module (Q. Zhang et al., 2018). Since NST1 is an upstream regulator of*MYB46* (Ruiqin Zhong et al., 2007), we wanted to confirm the MYC2-MYB46 direct activation module. To do this, we examined MYC2 overexpression phenotype in *nst1-1* mutant background (Supplemental Figure 8A). The MYC2OX/*nst1-1* plants had increased expression of MYB46 downstream target genes (Supplemental Figure 8B) and SCW formation in the stem (Supplemental Figure

8C), confirming that MYC2 can directly activates *MYB46* without the involvement of NST1. Furthermore, it appears that the two modules, MYC2-NST1-MYB46 and MYC2-MYB46, work through different regions of *MYB46* promoter (Figure 3E and 3F). The transient activity assay done in *myc2-2*background with serial deletions of *MYB46* promoter and MYC2 as effector showed residual activity of the promoter region without the MYC2 binding motif (i.e., under -900bp) (Figure 3E). However, the residual promoter activity was not observed when *nst1-1*background was used (Figure 3F).

Wound induces the expression of *4CL* , a key lignin biosynthetic gene (Soltani, Ehlting, Hamberger, & Douglas, 2006) and increases lignin deposition during the healing process (Lulai & Corsini, 1998; Ramamurthy, Ussuf, Nair, & Thomas, 2000). However, the signaling pathway is not well understood how wound signal is leading to lignin formation. Considering that JA is an important hormone involved in wound response (Koo & Howe, 2009), it is tempting to speculate that the JA-mediated MYC2-MYB46 module described in this report may be a part of the plant's response to wounding.

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Author Contributions

J.I., S.S., and K.H. conceptualized; J.I., S.S., W.K, K.K., and N.M. performed the experiments and data analysis; J.I., S.S., J.K., N.M., and K.H. interpret the data and wrote the manuscript; K.H. and J.K. supervised.

Conflict of interest

The authors declare no conflicts of interest.

Data availability

All data produced or analyzed in this study are included in the main text or the supplementary materials. Genes and the accession numbers used in this study are as follows: MYC2 (At1g32640), MYC3 (At5g46760), MYC4 (At4g17880), MYB46 (At5g12870), MYB83 (At3g08500), CESA4 (At5g44030), CESA8 (At4g18780), 4CL1 (At1g51680), PAL4 (At3g10340), and ACT (At3g12110). Sequence information was extracted from The Arabidopsis Information Resource (*http://www.arabidopsis.org/*).

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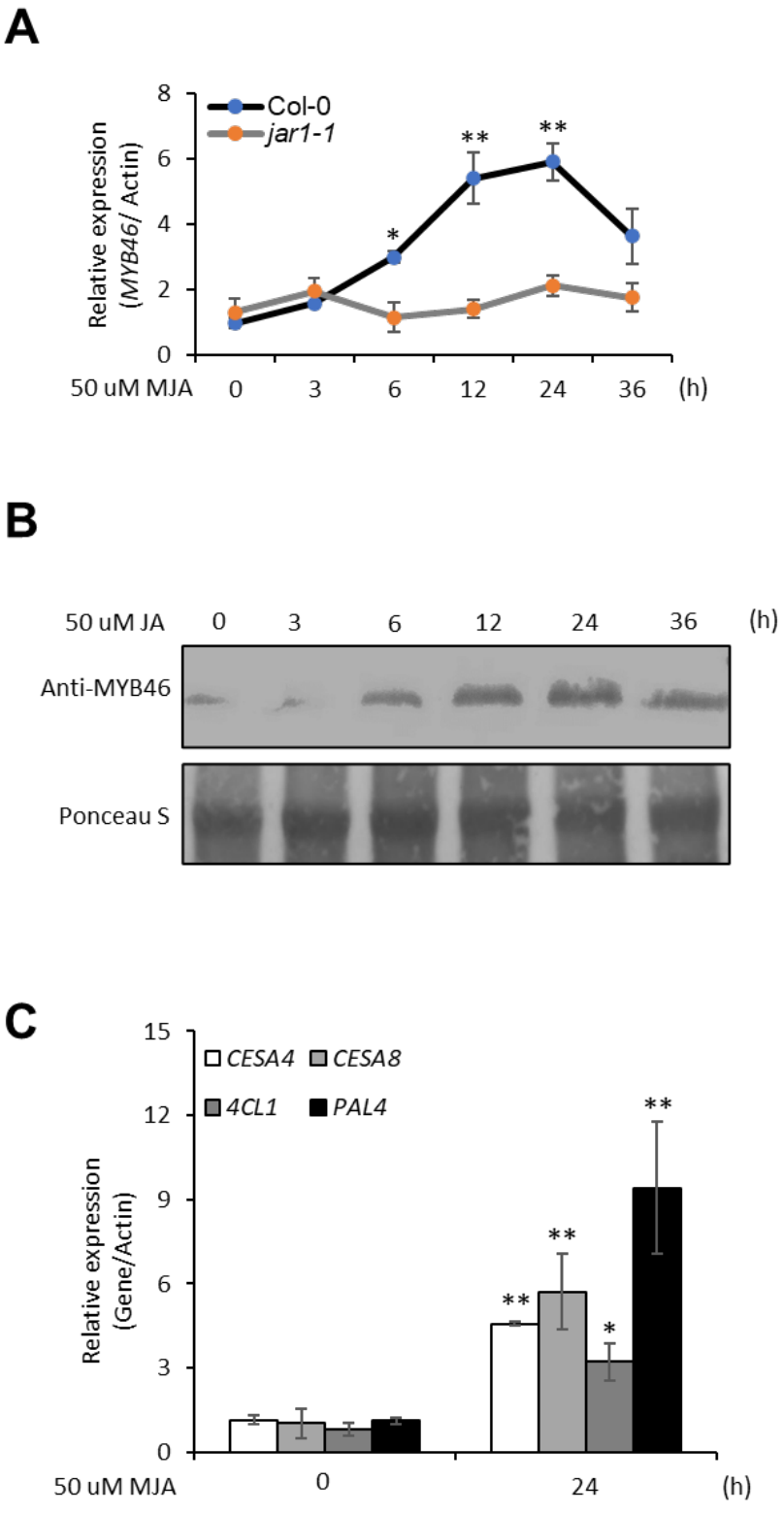

Figure 1 . Induction of MYB46 and its transcriptional target genes by MJA. (A) Gene expression of *MYB46* with 50 μM MJA treatment. Seven-day-old seedlings were treated with 50 μM MJA for the designated time points. Total RNA was isolated from the seedlings and used for cDNA synthesis. Values are means ± SD (*$P < 0.05$; **P <0.01). (B) Protein blot analysis from the samples in (A). Total protein was isolated and protein blot analysis was carried out with anti-MYB46 antibody. (C) Expression of MYB46 downstream genes with 50 μM MJA treatment. Seven-day-old seedlings were treated with 50 μM MJA for 24 h. Total RNA was isolated from the seedlings and used for cDNA synthesis. Values are means ± SD (*P < 0.05; **P <0.01).

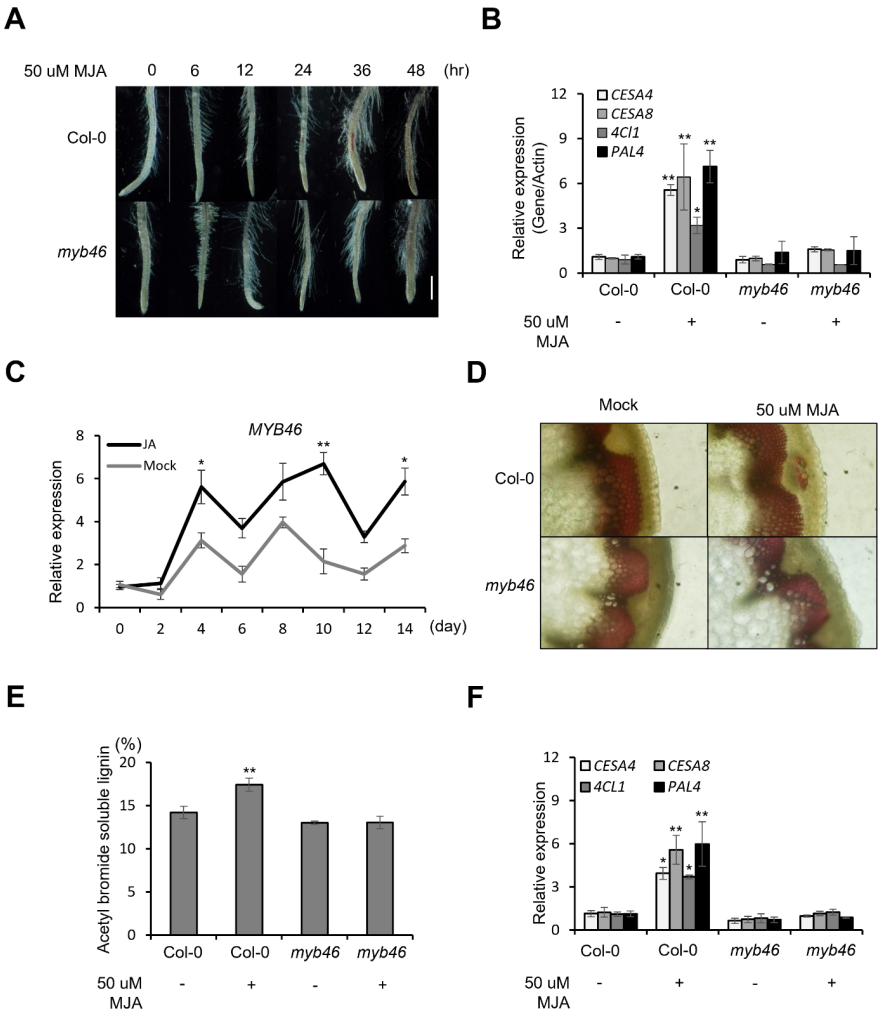

Figure 2. Jasmonate activates SCW biosynthesis through MYB46.(A) Phloroglucinol-HCl staining of the primary roots of MJA-treated seedlings. Seven-day-old Col-0 and *myb46* seedlings were treated with 50 μM MJA for the designated time points and stained with phloroglucinol-HCl. (B) Gene expression of MYB46 downstream genes in MJA-treated Col-0 and *myb46* seedlings. Seven-day-old Col-0 and*myb46* seedlings were treated with 50 μM MJA for 24 h. cDNA samples were prepared from the seedlings and used for RT-qPCR. Values are means ± SD (*$P < 0.05$; **P <0.01). (C) Gene expression of *MYB46* in MJA-treated mature stems. Thirty-two days-old Col-0 plants were treated with or without 50 μM MJA every two days for seven times. The stems were harvested after 12 h of each treatment. cDNA samples were prepared from the seedlings and used for RT-qPCR. Values are means ± SD (*$P < 0.05$; **P <0.01). (D) Phloroglucinol-HCl staining of MJA-treated mature stems. Thirty-two days-old Col-0 plants were treated

with 50 μM MJA every two days for seven times, then grown for four more weeks without MJA treatment. The stem cross-sections were stained with phloroglucinol-HCl. (E) Acetyl Bromide Soluble Lignin (ABSL) assay in the MJA treated Col-0 and*myb46* stems harvested from the experiment (D). (F) Expressions of MYB46 downstream genes in MJA-treated mature stems. Thirty-two days-old Col-0 and *myb46* plants were treated with 50 μM MJA every two days for 5 times. The stems were harvested after 12 h of each treatment. The gene expression was analyzed by RT-qPCR. Values are means ± SD (*$P < 0.05$; **$P < 0.01$).

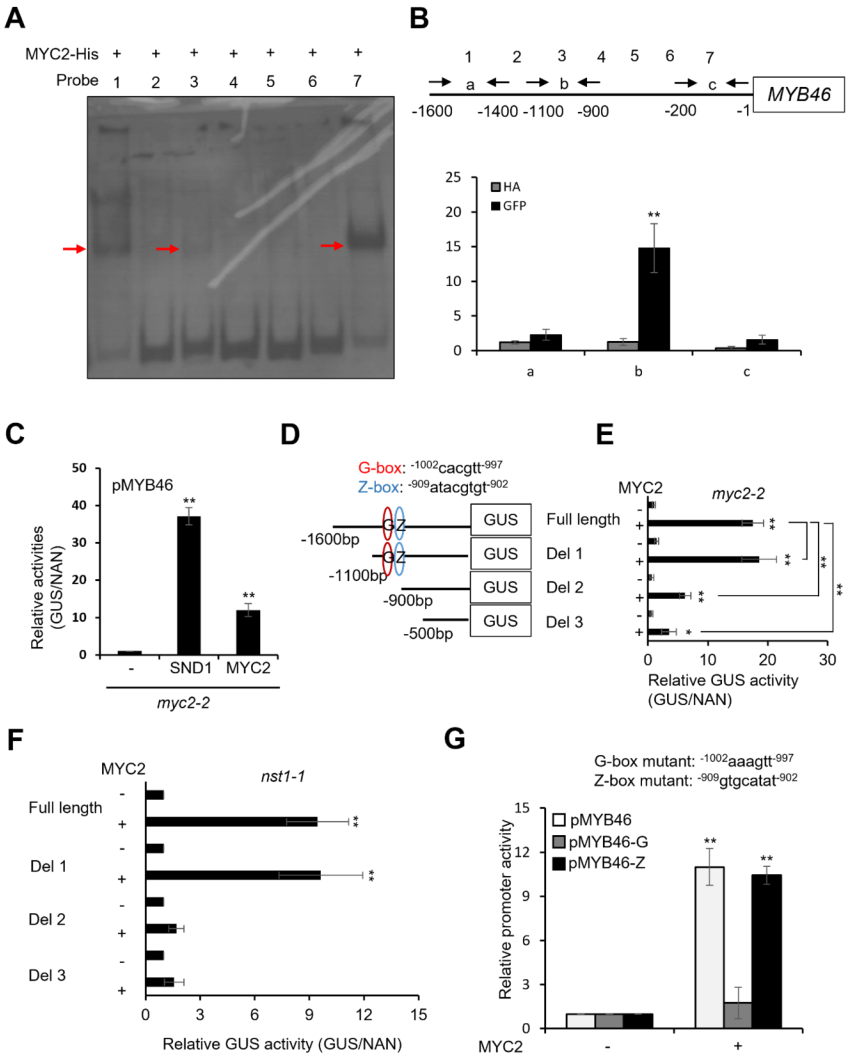

Figure 3. MYC2 directly binds and activates MYB46 promoter. (A) Electrophoretic Mobility Shift Assay (EMSA) confirms direct binding of MYC2 to the promoter region of *MYB46* . (B) Chromatin immunoprecipitation (ChIP)-qPCR of *MYB46* promoter.*GFP* -conjugated *MYC2* was expressed in *Arabidopsis*mesophyll protoplasts and immunoprecipitation was carried out with anti-GFP antibodies. After precipitation, qPCR was carried out with three primer sets of the *MYB46* promoter, covering MYC2 binding sites shown in the EMSA. Anti-HA was used as a control. (C) Promoter activity of *MYB46* with MYC2 co-expression. *MYB46* promoter was expressed in the mesophyll protoplasts isolated from *myc2-2*mutant with or without MYC2 co-expression. SND1 was used as a positive control. (D) Schematic drawing showing serial deletions of the*MYB46* promoter. (E and F) Activity of the truncated *MYB46*promoters. *GUS*

-conjugated truncated *MYB46* promoters were expressed with or without MYC2 co-expression in the protoplasts of*myc2-2* (E) and *nst1-1* (F), and GUS activities were measured. (G) Promoter activity of *MYB46* promoter and its mutants with MYC2 co-expression. The C-terminal *GUS* -conjugated*MYB46* promoter and its G-box variant mutant and Z-box mutant (G-box variant: $^{-1002}$aaagtt^{-997}, Z-box: $^{-909}$gtgcatat^{-902}) were expressed in the protoplasts with or without MYC2 co-expression.

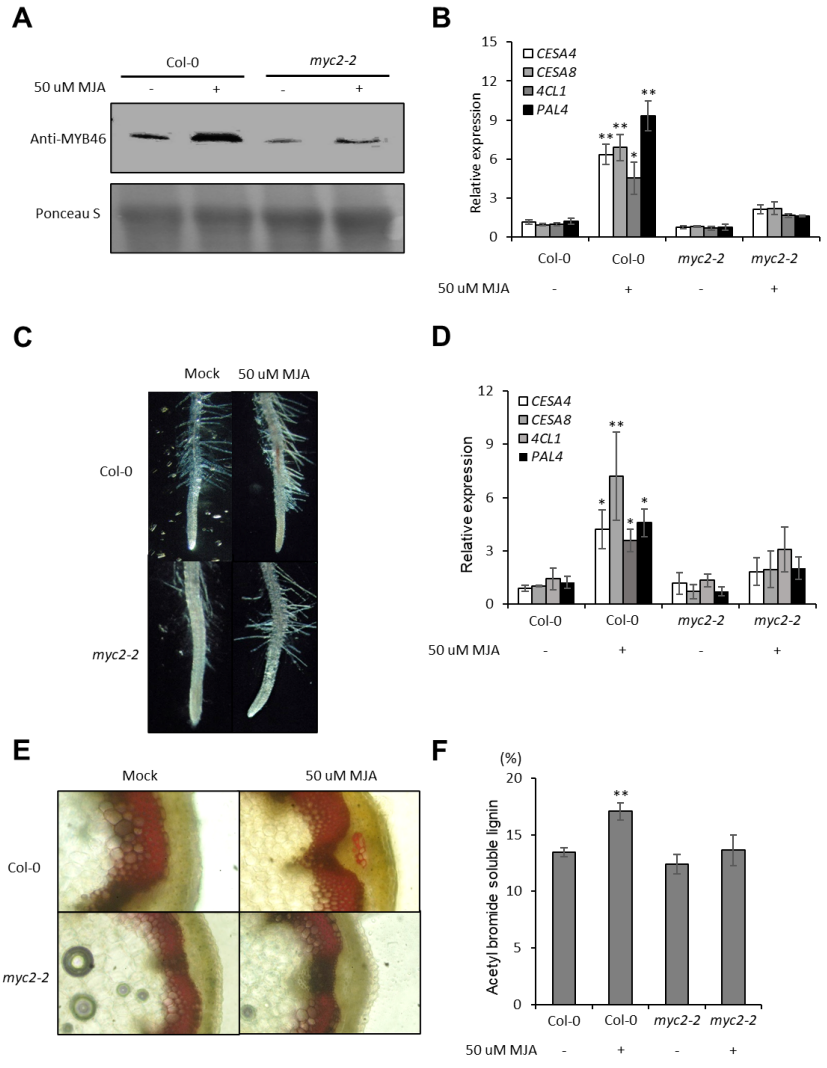

Figure 4. JA-dependent SCW formation is mediated by MYC2. (A) Protein blot analysis of MYB46 in Col-0 and *myc2-2* plants with or without 50 μM MJA treatment. Seven-days-old seedlings were treated with 50 μM MJA for 24 h and the protein blot analysis was carried out with anti-MYB46 antibodies. Ponceau S was used as a loading control. (B) Expression of MYB46 downstream target genes in MJA-treated Col-0 and*myc2-2* plants. Seven-day-old seedlings were treated with 50 μM MJA for 24 h. RT-qPCR was carried out with gene specific primers. Values are means ± SD ($*P < 0.05$; $**P < 0.01$). (C) Phloroglucinol-HCl staining of the primary roots of 7-day-old Col-0 and *myc2-2* seedlings, which were treated with 50 μM MJA for 36 h and stained with phloroglucinol-HCl. (D) Expressions of MYB46 downstream target genes in the mature stems of MJA-treated Col-0 and *myc2-2*plants. Thirty-two days-old plants were treated with 50 μM MJA every two days for 5 times. The stems were harvested after 12 h of each treatment and used to

prepare cDNA. RT-qPCR was carried out with gene specific primers. Values are means ± SD (*$P < 0.05$; **P <0.01). (E) Phloroglucinol-HCl staining of MJA-treated mature stems. (F) Acetyl Bromide Soluble Lignin (ABSL) assay in the MJA treated Col-0 and *myc2-2* stems harvested from the experiment (E). Thirty-two days-old Col-0 and *myc2-2* plants were treated with 50 μM MJA every two days for 7 times, then grown for four more weeks without MJA treatment. The stem cross-sections were stained with phloroglucinol-HCl.

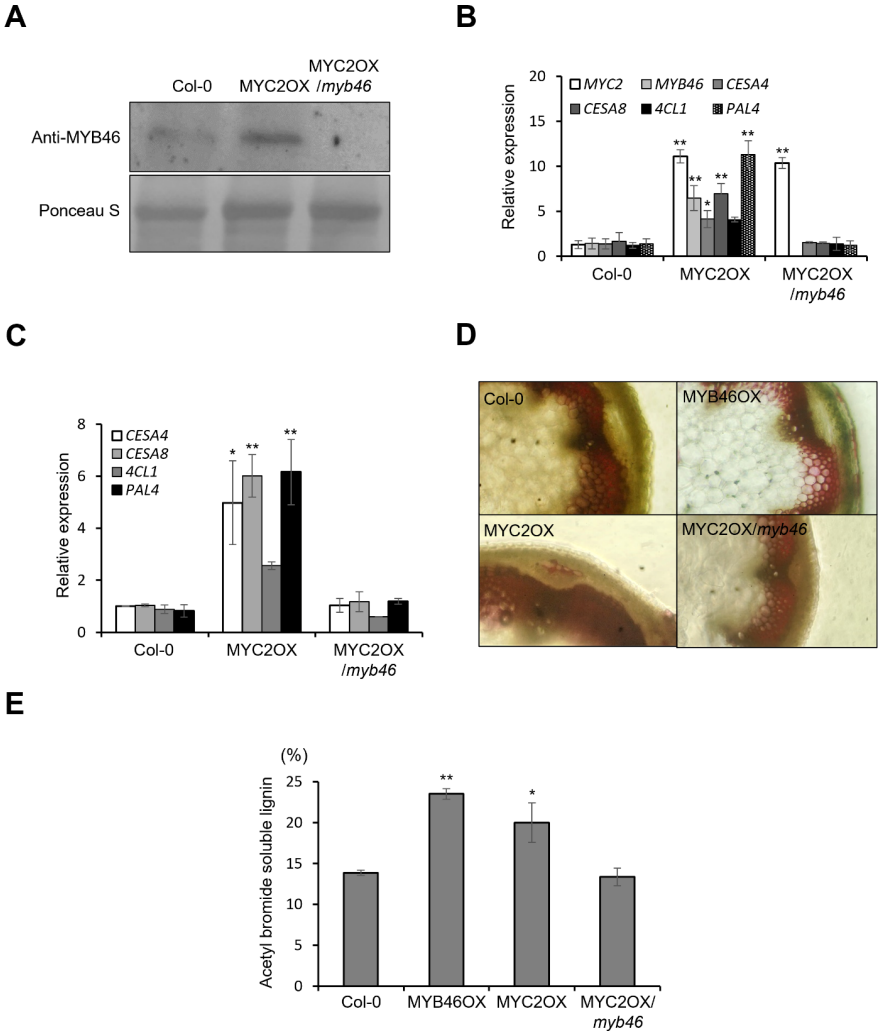

Figure 5. MYC2-MYB46 module in the regulation of SCW formation.(A) Protein blot analysis of MYB46 in Col-0, MYC2OX and MYC2OX/*myb46* plants. Total protein was extracted from seven-days-old seedlings and the protein blot analysis was carried out with anti-MYB46 antibodies. Ponceau S is used as a loading control. (B) Gene expressions of MYC2, MYB46 and its downstream target genes in Col-0, MYC2OX and MYC2OX/*myb46* seedlings. Total RNA was isolated from seven-days-old seedlings and used for cDNA synthesis. The RT-qPCR was carried out with gene specific primers. Values are means ± SD (*$P < 0.05$; **P <0.01). (C) Expressions of MYB46 downstream target genes in the stems of Col-0, MYC2OX and MYC2OX/*myb46* plants. Total RNAs and cDNAs were prepared from the six-weeks-old plants for the RT-qPCR with gene specific primers. Values are means ± SD (*$P < 0.05$; **P <0.01). (D) Phloroglucinol-HCl staining of mature stems. The stem cross sections were prepared from nine-weeks-old plants and stained with phloroglucinol-HCl. (E) Acetyl Bromide Soluble Lignin (ABSL) assay in Col-0, MYB46OX, MYC2OX and MYC2OX/*myb46* stems harvested from the experiment (D).

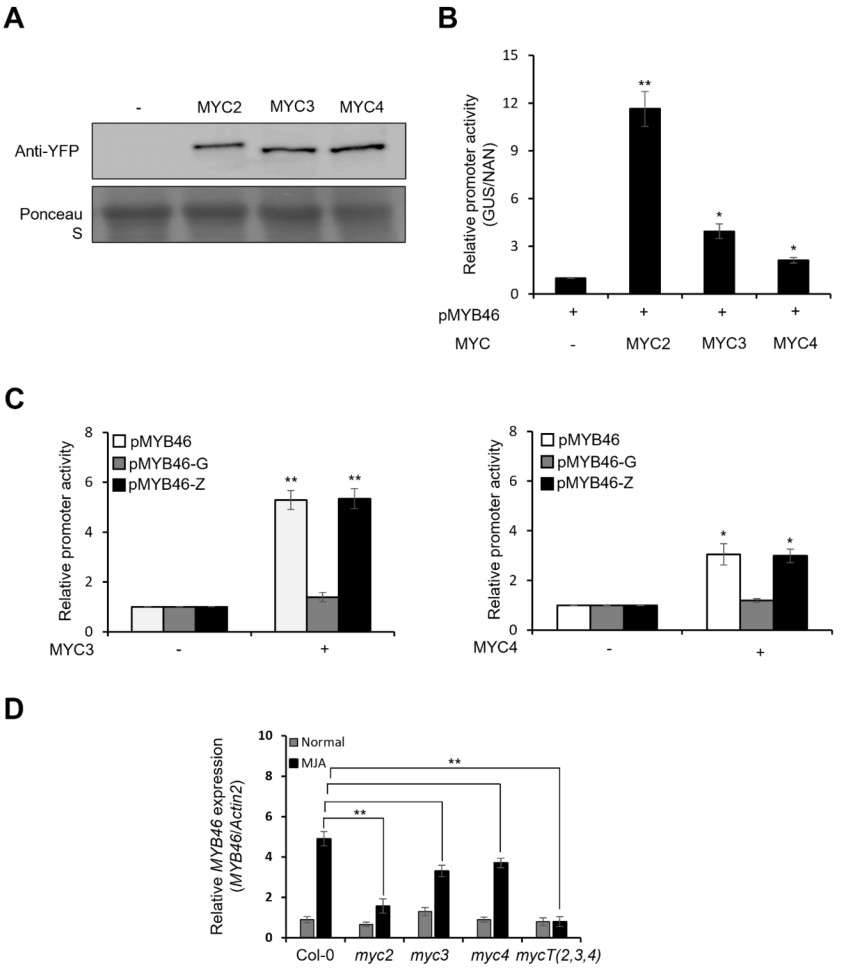

Figure 6. MYC3 and MYC4 induces *MYB46* expression. (A) Protein blot analysis of MYC2, MYC3 and MYC4. The C-terminal YFP-conjugated MYC2, MYC3 and MYC4 were expressed in the mesophyll protoplasts. The protein blot analysis was carried out with anti-GFP antibodies. Ponceau S was used as a loading control. (B) *MYB46*promoter activity with MYC2, MYC3 or MYC4 co-expression. The*GUS* -conjugated *MYB46* promoter was co-expressed in the mesophyll protoplasts with MYC2, MYC3 or MYC4 for GUS activity measurement. NAN was used as an expression control. Values are means ± SD (*$P < 0.05$; **P <0.01). (C) Promoter activities of *MYB46* and its mutants (G-box variant and Z-box mutant) with MYC3 or MYC4 co-expression. The GUS-conjugated *MYB46*promoter and its mutants (G-box variant and Z-box mutant) were expressed in the mesophyll protoplasts with or without MYC3 or MYC4 co-expression. GUS activity was measured after the expression. NAN was used as an expression control. Values are means ± SD (**P <0.01). (D) *MYB46* gene expression with MJA treatment in *myc2* ,*myc3* , *myc4* and *mycT* (*myc2,3,4* triple mutant) plants. MJA-dependent upregulation of *MYB46* expression was significantly compromised in *myc2* and *mycT* plants, while it was slightly reduced in the *myc3* and *myc4* plants.

Table 1. Result of yeast one hybrid screening: MYB46 promoter as bait

AGI code	Alias	TF class
AT5G51990	CBF4, DREB1D	AP2/ERF

AT1G32640	MYC2, JIN1, JAI1	bHLH
AT4G17880	MYC4	bHLH
AT4G10350	BRN2, ANAC070	NAC
AT1G33280	BRN1, ANAC015	NAC
AT5G46590	ANAC096	NAC
AT1G65910	ANAC028	NAC