The MIEL1 E3 Ubiquitin Ligase Negatively Regulates Cuticular Wax Biosynthesis in Arabidopsis Stems

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Cuticular wax is an important hydrophobic layer that covers the plant aerial surface. Cuticular wax biosynthesis is shaped by multiple layers of regulation. In particular, a pair of R2R3-type MYB transcription factors, MYB96 and MYB30, are known to be the main participants in cuticular wax accumulation. Here, we report that the MYB30-INTERACTING E3 LIGASE 1 (MIEL1) E3 ubiquitin ligase controls the protein stability of the two MYB transcription factors and thereby wax biosynthesis in Arabidopsis. MIEL1-deficient miel1 mutants exhibit increased wax accumulation in stems, with upregulation of wax biosynthetic genes targeted by MYB96 and MYB30. Genetic analysis reveals that wax accumulation of the miel1 mutant is compromised by myb96 or myb30 mutation, but MYB96 is mainly epistatic to MIEL1, playing a predominant role in cuticular wax deposition. These observations indicate that the MIEL1–MYB96 module is important for balanced cuticular wax biosynthesis in developing inflorescence stems.

Keywords: Arabidopsis • Cuticular wax • MIEL1 • MYB96 • Ubiquitination.

Abbreviations: ABC, ATP-binding cassette; AP2, APETALA 2; ATT1, ABERRANT INDUCTION OF TYPE THREE 1; BiFC, bimolecular fluorescence complementation; CER, eceriferum; DEWAX, DECREASE WAX BIOSYNTHESIS; ECR, enoyl-CoA reductase; ER, endoplasmic reticulum; FAE, fatty acid elongase; FAR3, fatty acyl-CoA reductase; FDH, FIDDLEHEAD; HCD, β-hydroxyceryl-CoA dehydratase; HUB, HISTONE MONOUBIQUITINATION; KCR, β-oxoacyl-CoA reductase; KCS, β-ketoacyl-CoA synthase; LACS, long-chain acyl-CoA synthase; MAH1, midchain alkane hydroxylase 1; MIEL1, MYB30-INTERACTING E3 LIGASE 1; PAS2, PASTICCINO 2; PTGS, post-transcriptional gene silencing; RT–qPCR, quantitative real-time reverse transcription–PCR; tasiRNA, transacting small interfering RNA; VLCFA, very-long-chain fatty acid; WRI, wrinkled; WSD1, bifunctional wax synthase/acyl-CoA:diacylglycerol acyltransferase; YFP, yellow fluorescent protein; Y2H, yeast-two-hybrid.

Introduction


In the process of cuticular wax biosynthesis, C16 and C18 fatty acids are synthesized and activated into C16- and C18-CoA at the plastid envelope, and the activated forms are transported to the cytoplasm (Schuur et al. 2014, Lu et al. 2009, Weng et al. 2010, Li et al. 2013). The C16- and C18-CoAs are further elongated into VLCFAs in the endoplasmic reticulum (ER) membranes. Repeated addition of C2 unit finally synthesizes C20–C34 VLCFAs, and the process is accomplished primarily by a multi-enzyme complex, fatty acid elongases (FAEs), which perform four-step reactions (Millar et al. 1999, Hooker et al. 2002, Kunst and Samuels 2003, Joubert et al. 2008, Yeats and Rose 2013): condensation by a β-ketoacyl-CoA synthase (KCS), reduction of β-ketoacyl-CoA by a β-ketoacyl-CoA reductase (KCR), dehydration of β-hydroxyacyl-CoA by a β-hydroxyacyl-CoA dehydratase (HCD) and additional reduction of enoyl-CoA to a C2 unit extended acyl-CoA (ECR) (Millar et al. 1999, Zheng et al. 2005, Bach et al. 2008, Beaudoin et al. 2009, Kunst and Samuels 2009, Kim et al. 2013). In addition to the FAE core complex, elongation from C28 to C34 requires additional components, ECERIFERUM 2 (CER2) and CER2-like-1 and CER2-like-2, which share sequence homology with BAHD acyltransferase (Haslam et al. 2012, Haslam et al. 2015).

The VLCFAs are then modified through two independent pathways: alcohol-forming and alkane-forming pathways. The alcohol-forming pathway produces primary alcohol and wax esters (Kunst and Samuels 2003, Rowland et al. 2006). The FATTY ACYL-COA REDUCTASE 3/ECERIFERUM 4 (FAE3/
CER4) protein catalyzes primary alcohol synthesis (Rowland et al. 2006), and the resultant products are further processed into wax esters by the bifunctional wax synthase/acyl-CoA:diacylglycerol acyltransferase (WSD1) enzyme (Li et al. 2008). Meanwhile, the alkane-forming pathway produces aldehydes, alkanes, secondary alcohols and ketones (Kunst and Samuels 2003, Greer et al. 2007). Alkane derivatives are synthesized by a protein complex consisting of CER1, CER3/WAX2 and Cyt b6 isofrom (Rowland et al. 2007, Bourdenez et al. 2011, Bernard et al. 2012). The MIDCHAIN ALKANE HYDROXYLASE 1 (MAH1) protein catalyzes oxidation of alkane products to form secondary alcohols and ketones (Greer et al. 2007). Cuticular wax components generated in the ER are finally transported on the plant surface by ATP-binding cassette (ABC) transporters and glycosylphosphatidylinositol anchored lipid transfer proteins (LTPCs) (Bird et al. 2007, Debono et al. 2009, Lee et al. 2009, McFarlane et al. 2010, Kim et al. 2012).

Although cuticular wax biosynthetic enzymes have been well characterized, their regulatory mechanisms are still largely unknown. Several lines of evidence have demonstrated that transcriptional regulation is a crucial scheme for wax biosynthesis control in Arabidopsis. The APETALA 2 (AP2)/ethylene-response element-binding factor (ERF) transcription factor WAX INDUCER 1 (WIN1)/SHINE 1 (SHN1) triggers cutin accumulation by directly binding to cutin biosynthesis genes and also exerts indirect roles in cuticular wax biosynthesis (Aharoni et al. 2004, Broun et al. 2004, Kannangara et al. 2007, Shi et al. 2011). In addition, the AP2/ERF transcription factor DECREASE WAX BIOSYNTHESIS (DEWAX) negatively modulates wax accumulation by repressing FAR6, CERT, LONG-CHAIN ACYL-COA SYNTHETASE 2 (LACS2) and ECR in the darkness and also contributes to organ-specific wax loads (Go et al. 2014, Suh and Go 2014). A pair of R2R3-type MYB transcription factors are also unequivocally involved in cuticular wax biosynthesis. MYB30 regulates wax biosynthesis through direct activation of FAE complex genes in response to pathogen attacks (Raffaele et al. 2008). The MYB96 transcription factor is another crucial regulator of cuticular wax accumulation under drought conditions. This protein stimulates cuticular wax biosynthesis by directly activating KCS1, KCS2, KCS6, KCR1, CER3 and WSD1 genes (Seo et al. 2011). Consistently, MYB96-overexpressing activation-tagged myb96-TD plants show elevated levels of total wax loads with enhanced drought tolerance, whereas MYB96-deficient mutants are susceptible to drought and exhibit reduced wax deposition in vegetative tissues (Seo et al. 2009, Seo et al. 2011, Guo et al. 2013, Lee and Seo 2016, Lee et al. 2016).

Covalent attachment of ubiquitin protein is a representative way of modulating protein turnover. The ubiquitination process is mediated by the sequential action of three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-ligase (E3). The Arabidopsis genome encodes 2 E1, >37 E2 and >1,500 E3 enzymes (Mazzucotelli et al. 2006, Lee and Kim 2011). Consistent with the large number, the ubiquitination process underlies diverse aspects of plant growth and development (Moon et al. 2004, Santner et al. 2010). In this study, we demonstrate that cuticular wax biosynthesis is also controlled at the post-translational level. The RING-type E3 ligase MIEL1 is known to stimulate ubiquitination of MYB96 and MYB30 and lead them to be susceptible to 26S proteasome-dependent protein degradation (Marino et al. 2013, Lee and Seo 2016). In agreement with the fact that the two R2R3-MYB transcription factors are the pivotal transcriptional regulators of cuticular wax biosynthesis (Raffaele et al. 2008, Seo et al. 2011), MIEL1 negatively regulates cuticular wax accumulation. MIEL1 affects a majority of genes in VLCFA biosynthesis and modification, which are targeted by MYB96 and MYB30. While MIEL1 has biochemical functions in regulating the protein stability of both MYB96 and MYB30, genetic analysis supports that MIEL1 function is highly dependent on MYB96. This genetic interaction is attributable, in part, to predominant accumulation of MYB96, relative to MYB30, in vegetative tissues. The MIEL1–MYB96 module is a key player in cuticular wax biosynthesis, conferring balanced wax accumulation in developing stems under normal growth conditions.

### Results

#### Cuticular wax accumulation is elevated in miel1 mutants

The MIEL1 E3 ubiquitin ligase stimulates protein turnover of both MYB96 and MYB30, establishing a signaling cross-talk between abiotic and biotic stress responses (Lee and Seo 2016). Since the two MYB transcription factors are key transcriptional regulators of cuticular wax biosynthesis (Raffaele et al. 2008, Seo et al. 2011), we hypothesized that MIEL1 may be a pivotal player that determines cuticular wax accumulation in Arabidopsis.

To test this hypothesis, we first analyzed transcript accumulation of MIEL1 in various tissues, including roots, leaves, stems, stem epidermis and flowers. Quantitative real-time reverse transcription–PCR (RT–qPCR) analysis revealed that transcript accumulation of MIEL1 was higher in roots than in aerial tissues (Fig. 1A). In particular, stem epidermis, where cuticular wax is mainly synthesized (Suh et al. 2005, Samuels et al. 2008), showed relatively low accumulation of MIEL1 transcripts.

We employed MIEL1-deficient mutants (miel1-1 and miel1-2) and measured their total cuticular wax contents. Since cuticular wax deposition mainly occurs in Arabidopsis stems (Jenk et al. 2002, Suh et al. 2005, Go et al. 2014), we harvested stem tissues to analyze wax accumulation in wild-type and miel1 mutant plants. Total wax measurement showed that miel1 mutants displayed increased cuticular wax accumulation (Fig. 1B). Quantitative analysis supported that total wax accumulation increased by 10–20% in miel1 mutants compared with wild-type plants (Fig. 1B). Furthermore, major chemical components, including primary alcohols, alkanes and ketones, were increased in stem cuticular wax of miel1 mutant plants (Fig. 1C). We observed allelic differences of total wax load and wax composition, which might be due to the differences in the extent of the lesion. These results suggest that MIEL1 negatively regulates cuticular wax accumulation in stems under normal growth conditions.
Core wax biosynthetic genes are regulated by MIEL1

We wanted to determine the molecular networks underlying MIEL1-regulated wax deposition. It needs to be clarified whether MIEL1 has additional targets regulating wax biosynthesis, in addition to MYB96 and MYB30. Since the biochemical roles of E3 ubiquitin ligases require protein–protein interactions (Jackson et al. 2000, Ardley and Robinson 2005, Deshaies and Joazeiro 2009), we investigated possible interactions of MIEL1 with transcriptional regulators involved in cuticular wax, cutin and suberin biosynthesis, including MYB41, MYB94, DEWAX and WIN1 (Aharoni et al. 2004, Broun et al. 2004, Kannangara et al. 2007, Cominelli et al. 2008, Shi et al. 2011, Go et al. 2014, Kosma et al. 2014; Lee and Suh 2015b, Lee et al. 2016). Yeast-two-hybrid (Y2H) analysis showed that MIEL1 strongly interacts with MYB96 and MYB30, while the other transcription factors were not preferred as interaction partners, except for DEWAX (Supplementary Fig. S1). However, considering the negative role of MIEL1 in cuticular wax deposition, we can rule out the possibility that MIEL1 primarily stimulates DEWAX degradation in wax biosynthesis.

In line with this, transcript accumulation of MYB96 and MYB30 was also elevated in miel1 mutants (Supplementary Fig. S2), supporting the negative roles of MIEL1 in the control of MYB96 and MYB30 activities.

To examine the functional association of MIEL1 with MYB96 and MYB30, we analyzed expression of genes involved in cuticular wax biosynthesis, modification and transport, which are targeted either by MYB96 or by MYB30. Two MYB transcription factors are known to regulate the cuticular wax biosynthetic process with activation of overlapping as well as unique target genes: MYB30 regulates expression of KCS1, KCS2, KCR1, GLYCEROL-3P ACYL TRANSFERASE 4 (G PAT4), FIDDL EHEAD (FDH), CER10, ABERRANT INDUCTION OF TYPE THREE 1 (ATT1), 3-HYDROXYACYL-COA DEHYDRATASE 1 (HCD1), CER2, PASTICCINO 2 (PAS2) and WAX2/CER3 (Raffaele et al. 2008), whereas MYB96 binds to promoters of KCS1, KCS2, KCS6, KCR1, WAX2/CER3 and WSD1 genes to activate their expression and also indirectly regulates CER1 and LTP3 (Seo et al. 2011, Guo et al. 2013).
RT–qPCR analysis showed that all of the genes examined were up-regulated in *miel1* mutants (Fig. 2). Most genes involved in VLCFA and wax biosynthesis and wax transport were significantly increased in *miel1* mutants (Fig. 2). In addition, several cutin metabolic genes, including *GPAT4*, *ATT1* and *LACS3*, were also misregulated in *miel1* mutants (Fig. 2), which may result in cutin accumulation in a balance with enhanced wax load.
The up-regulation of key wax biosynthetic genes in miel1 mutants was observed in other aerial tissues (Supplementary Fig. S3), which means that MIEL1 is implicated generally in wax accumulation, rather than tissue-specific wax load. These observations suggest that MIEL1 negatively regulates accumulation of both MYB96 and MYB30 (Lee and Seo 2016), and thus suppresses cuticular wax biosynthesis in aerial vegetative tissues.

**Mutual interactions of MYB96 and MYB30 in the control of wax biosynthesis**

MIEL1 controls expression of genes encoding core enzymes of cuticular wax biosynthesis probably through protein degradation of MYB96 and MYB30. Given that they not only share regulatory targets but also exclusively regulate different sets of target genes (Raffaele et al. 2008, Seo et al. 2011, Guo et al. 2013), the two MYB proteins probably play either independent or interdependent roles in the control of cuticular wax biosynthesis, rather than simply being functionally redundant. A plausible explanation would be the dynamic interactions between MYB96 and MYB30.

To examine this possibility, we performed bimolecular fluorescence complementation (BiFC) assays using Arabidopsis protoplasts. The MYB96 and MYB30 cDNA sequences were fused in-frame to the 5′ end of gene sequences encoding the N-terminal half of yellow fluorescent protein (nYFP) as well as the C-terminal half of YFP (cYFP). Possible combinations of fusion constructs were then transiently co-expressed in Arabidopsis protoplasts. As a result, yellow fluorescence was observed in the nucleus in MYB96–MYB96, MYB96–MYB30 and MYB30–MYB30 combinations (Fig. 3). Dynamic physical interactions probably diversify target selection and transcriptional strength, ensuring delicate control of wax biosynthesis.

**MYB96 is mainly epistatic to MIEL1 in the control of cuticular wax biosynthesis**

To estimate genetic interactions among MIEL1, MYB96 and MYB30, we analyzed transcript accumulation of wax biosynthetic genes in wild-type, miel1-2, myb96-1, myb30-1, miel1-2myb96-1, miel1-2myb30-1 and miel1-2myb96-1myb30-1 plants. RT–qPCR analysis revealed that cuticular wax biosynthetic genes were up-regulated in miel1 mutants, but some of them were slightly down-regulated in myb96-1 and/or myb30-1 (Fig. 4). A majority of the genes examined were dependent on both MYB96 and MYB30. The increased expression in miel1-2 was compromised in miel1-2myb96-1 and miel1-2myb30-1 mutants (Fig. 4). However, the degree of gene suppression, in many cases, was more obvious in miel1-2myb96-1 compared with miel1-2myb30-1 (Fig. 4), supporting the prevalent role of MYB96 in MIEL1-regulated wax deposition.

The MIEL1 function in cuticular wax biosynthesis most probably depends on MYB96. To confirm this result, we analyzed cuticular wax accumulation in wild-type, miel1-1, miel1-2, myb96-1, myb30-1, miel1-1myb96-1, miel1-2myb96-1, miel1-2myb30-1 and miel1-2myb96-1myb30-1 stems. Total wax measurement showed that a substantial increase of cuticular wax accumulation was observed in miel1-2 stems, whereas myb96-1 and myb30-1 stems exhibited reduced wax deposition (Fig. 5A), as previously examined (Fig. 1B) (Raffaele et al. 2008, Seo et al. 2011). Notably, total wax accumulation of miel1-
and miel1-2myb30-1 mutants was equivalent to that of myb96-1, while the miel1-2myb30-1 mutant showed intermediate levels compared with miel1-2 and myb30–1 (Fig. 5A). Moreover, cuticular wax deposition of miel1-2myb96-1myb30-1 was also comparable with that of myb96-1 (Fig. 5A). Wax composition analysis supported that accumulation of individual components, particularly alkanes, primary and secondary alcohols and ketones, was changed accordingly in the mutants (Fig. 5B). These observations indicate that MYB96 is epistatic to MIEL1 and plays a fundamental role in cuticular wax accumulation in developing stems under normal growth conditions.

MYB96 plays a prevalent role in wax biosynthesis in aerial tissues

Although MIEL1 simultaneously triggers protein turnover of both MYB96 and MYB30 (Lee and Seo 2016), its function in wax accumulation was largely dependent on MYB96. These results raised the possibility that MYB96 plays a prevalent role in cuticular wax biosynthesis under normal growth conditions. To examine this hypothesis, we determine the relative expression levels of MYB96 and MYB30 in various vegetative tissues. MYB96 transcript levels were ubiquitously expressed in aerial tissues (Supplementary Fig. S4), and transcript accumulation of MYB30 also showed similar expression patterns (Supplementary Fig. S4). Notably, expression of MYB96 was approximately 4- to 8-fold higher in all aerial tissues examined, relative to MYB30, suggesting that MYB96 plays a predominant role in cuticular wax accumulation (Supplementary Fig. S4). However, we cannot rule out the possibility that the affinity of the different transcription factors for the promoters as well as the hierarchy of these transcription factors could also be of importance. Taken together, MIEL1 shapes cuticular wax accumulation, and its function is dependent largely on MYB96, ensuring proper levels of cuticular wax accumulation in aerial vegetative tissues under normal growth conditions.
Transcriptional regulation is a key regulatory scheme of cuticle deposition (see the Introduction). However, recent advances in signaling networks underlying cuticular wax biosynthesis underscore that multiple regulatory mechanisms add complexity to this biological process. Changes in chromatin structure that potentiate accessibility of transcriptional regulatory components are associated with cuticular wax biosynthesis. The RING-containing E3 ubiquitin ligases, HISTONE MONOUBIQUITINATION 1 (HUB1) and HUB2 that catalyze H2B monoubiquitination (Cao et al. 2007, Liu et al. 2007, Zhou et al. 2008), trigger active chromatin formation allowing transcriptional activation of cutin and wax biosynthetic genes (Ménard et al. 2014). Genetic mutants having defects in either hub1 or hub2 show disorganized cuticle layers with alterations in the amount and composition of cutin and wax components (Ménard et al. 2014). Consistently, the cutin and wax biosynthetic genes including LACS2, ATT1, HOTHEAD (HTH) and CER1 are down-regulated in hub mutants, with impairment of histone H2B monoubiquitination at corresponding loci (Ménard et al. 2014).

Production of trans-acting small interfering RNAs (tasiRNAs) is also implicated in balanced wax accumulation. Genetic mutants of CER7/AtRRP45B encoding a putative 3′-to-5′ exoribonuclease, a core subunit of the exosome (Hooker et al. 2007), display wax deficiency with reduced CER3 expression (Lam et al. 2012). However, introduction of the rna-dependent rna polymerase 1 (rdr1), rdr6, suppressor of gene silencing 3 (sgs3), silencing defective 5 (sde5), dicer-like 4 (dcl4), hua enhancer 1 (hen1) or argonaute 1 (ago1) mutation, which results in defects in functional tasiRNA production (Peragine et al. 2004, Vazquez et al. 2004, Allen et al. 2005, Baumberger and Baulcombe 2005, Gasciolli et al. 2005, Li et al. 2005, Xie et al. 2005a, 2005b, Yoshikawa et al. 2005, Zhang et al. 2013), rescues the cer7-associated wax deficiency (Lam et al. 2012). These results indicate that some tasiRNAs, which are particularly relevant in the cer7 mutant, serve as direct effectors of CER3 (Lam et al. 2012). In detail, connections between exosome-dependent degradation pathway and tasiRNA-mediated post-transcriptional gene silencing (PTGS) define optimal wax deposition. Bidirectional cytoplasmic RNA decay, 3′-to-5′ exosome and 5′-to-3′ XRN4 exoribonuclease, eliminates undesirable mRNAs (Souret et al. 2004, Valencia-Sanchez et al. 2006). Impairment of either decay pathway triggers siRNA-mediated PTGS to maintain proper levels of target transcripts (Gazzani et al. 2004, Gy et al. 2007, Gregory et al. 2008). In other words, disruption of 3′-to-5′ decay of CER3 transcripts stimulates tasiRNA production to activate PTGS (Zhao and Kunst 2016).
Controlled protein turnover is another important way of regulating cuticular wax biosynthesis. The RING-containing E3 ubiquitin ligase CER9 negatively regulates VLCFA biosynthesis, although the working mechanism has not been fully elucidated (Lü et al. 2012). In addition, MIEL1 stimulates protein turnover of both MYB96 and MYB30 (Marino et al. 2013, Lee and Seo 2016), which are unequivocal regulators of cuticular wax accumulation (Raffaele et al. 2008, Seo et al. 2011). Indeed, MIEL1 is responsible for maintaining proper wax levels in aerial tissues. Notably, MYB96 is genetically epistatic to MIEL1 in the control of cuticular wax accumulation of developing stems. Cuticular wax levels of miei1-2myb96-1 are nearly equivalent to those of myb96-1. Furthermore, wax-deficient phenotypes of miei1-2myb96-1myb30-1 are also comparable with myb96-1, accounting for the importance of the MIEL1–MYB96 module in balanced wax accumulation.

The trio MIEL1, MYB96 and MYB30 are dynamically regulated upon diverse environmental challenges. They are responsive to drought stress and pathogen attacks, and are properly regulated lipid metabolic pathways in accordance with environmental situations (Raffaele et al. 2008, Seo et al. 2011, Okazaki et al. 2014). While MIEL1 is highly dependent on MYB96 under normal growth conditions, MIEL1 may mainly depend on MYB30 in other environmental situations, such as pathogen attack. This would be attributable to absolute levels of target transcripts/proteins or molecular behaviors, such as dimer formation. Overall, MIEL1 is a key regulator of cuticular wax accumulation, which attenuates MYB96 and MYB30 activities. Complementary accumulation of MIEL1 and MYB proteins ensures balanced wax accumulation and would elaborately set up physical barriers against environmental risks.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana (Columbia-0 ecotype) was used for all experiments unless otherwise specified. Plants were grown under long-day conditions (16 h light/8 h dark cycles) with cool white fluorescent light (100 μmol photons m⁻² s⁻¹) at 23 °C. The myb96-1 mutant (GABI_120B015) was previously reported (Seo et al. 2009, Seo et al. 2011). The miei1-1 (SALK-097638) and miei1-2 (SALK-041369) mutants were isolated from a T-DNA insertion mutant pool deposited in the Arabidopsis Biological Resource Center (ABRC, https://abrc.osu.edu/). Single mutant plants were crossed to generate higher order mutants.

Quantitative real-time RT–PCR analysis

Total RNA was extracted using TRI reagent (TAKARA BIO) according to the manufacturer’s recommendations. Reverse transcription was performed using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Dr. Protein, Seoul, South Korea) with oligo(dT)₁₈ to synthesize first-strand cDNA from 2 μg of total RNA. Total RNA samples were pre-treated with an RNase-free DNase. cDNAs were diluted to 100 μl with TE buffer, and 1 μl of diluted cDNA was used for PCR amplification.

RT–qPCRs were performed in 96-well blocks using the Step-One Plus Real-Time PCR System (Applied Biosystems). The PCR primers used are listed in Supplementary Table S1. The values for each set of primers were normalized relative to the EUKARYOTIC TRANSLATION INITIATION FACTOR 4A1 (eIF4A) gene (At3g13920). All RT–qPCRs were performed with biological triplicates, using total RNA samples extracted from three independent replicate samples. The comparative ΔΔCt method was employed to evaluate relative quantities of each amplified product in the samples. The threshold cycle (Ct) was automatically determined for each reaction with the analysis software set using default parameters. The specificity of the RT–qPCRs was determined by melt curve analysis of the amplified products using the standard method employed by the software.

Cuticular wax analysis

Arabidopsis wild type and mutants were transplanted to soil after 10 d of growth on half-strength Murashige and Skoog (MS) plates containing 1% sucrose. Four weeks later, inflorescence stems were used to measure wax content and composition. Stem waxes were extracted by dipping in chloroform for 30 s, and three internal standards (γ-octanal, docosenoic acid and 1-tricosanol) were added into the wax extracts. After evaporation of wax extracts under nitrogen gas, 100 μl of pyridine and 100 μl of N-O-Bis(trimethylsilyl) trifluoroacetamide were added and incubated at 95 °C for 30 min. The reactants were evaporated again and then dissolved in hexane:toluene (1:1, v/v) solvent. Finally, wax content and composition analyses were performed using gas chromatography (GC) and GC–mass spectrometry as described previously (Seo et al. 2011).

Y2H assays

Y2H assays were performed using the BD Matchmaker system (Clontech). The pGADT7 vector was used for the GAL4 AD fusion, and the pGBK7 vector was used for GAL4 BD fusion. The yeast strain AH109 harboring the LacZ and His reporter genes was used. PCR products were subcloned into the pGBK7 and pGADT7 vectors. The expression constructs were co-transformed into yeast AH109 cells and transformed cells were selected by growth on SD/-Leu/-Trp/-His/-Ade. Interactions between proteins were analyzed by measuring the β-galactosidase activity using o-nitrophenyl-β-d-galactopyranoside as a substrate.

BiFC assays

The MYB96 and MYB30 genes were fused in-frame to the 5’ end of a gene sequence encoding the C-terminal half of EYFP in the pSATN-cEYFP-C1 vector (E3082) or the 5’ end of a gene sequence encoding the N-terminal half of EYFP in the pSATN-nEYFP-C1 vector (E3081). Expression constructs were co-transformed into Arabidopsis protoplasts. Expression of the fusion constructs was monitored by fluorescence microscopy using a Zeiss LSM510 confocal microscope (Carl Zeiss).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

References


