

The MYB96 Transcription Factor Regulates Cuticular Wax Biosynthesis under Drought Conditions in *Arabidopsis*^W

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Drought stress activates several defense responses in plants, such as stomatal closure, maintenance of root water uptake, and synthesis of osmoprotectants. Accumulating evidence suggests that deposition of cuticular waxes is also associated with plant responses to cellular dehydration. Yet, how cuticular wax biosynthesis is regulated in response to drought is unknown. We have recently reported that an *Arabidopsis thaliana* abscisic acid (ABA)-responsive R2R3-type MYB transcription factor, MYB96, promotes drought resistance. Here, we show that transcriptional activation of cuticular wax biosynthesis by MYB96 contributes to drought resistance. Microarray assays showed that a group of wax biosynthetic genes is upregulated in the activation-tagged *myb96-1D* mutant but downregulated in the MYB96-deficient *myb96-1* mutant. Cuticular wax accumulation was altered accordingly in the mutants. In addition, activation of cuticular wax biosynthesis by drought and ABA requires MYB96. By contrast, biosynthesis of cutin monomers was only marginally affected in the mutants. Notably, the MYB96 protein acts as a transcriptional activator of genes encoding very-long-chain fatty acid-condensing enzymes involved in cuticular wax biosynthesis by directly binding to conserved sequence motifs present in the gene promoters. These results demonstrate that ABA-mediated MYB96 activation of cuticular wax biosynthesis serves as a drought resistance mechanism.

INTRODUCTION

Drought or water deficit conditions profoundly affect plant growth and productivity and reduce crop yield worldwide. Therefore, plants have evolved versatile mechanisms to cope with drought stress. Molecular and cellular responses to drought stress and underlying regulatory mechanisms have been extensively studied via molecular genetic and physiological approaches and gene expression studies.

Gene transcriptional regulation is the most important step during plant adaptation to environmental fluctuations (Zhu, 2002; Shinozaki et al., 2003; Chinnusamy et al., 2004; Yamaguchi-Shinozaki and Shinozaki, 2006). Particularly, roles of various transcription factors, such as basic leucine zipper, MYC, MYB, and NAC (NAM, ATAF1/2, CUC2) members, have been widely documented in drought stress response and signaling cascades (Kang et al., 2002; Abe et al., 2003; Fujita et al., 2004; Tran et al., 2004; Seo et al., 2009). The transcription factors interact with specific *cis*-acting elements existing in target gene promoters and regulate elaborate networks of drought-responsive genes

and associated physiological responses (Yamaguchi-Shinozaki and Shinozaki, 2006).

Plant resistance responses to drought conditions are exerted via several distinct mechanisms, such as synthesis of osmoprotectants and antioxidants, maintenance of root growth and water uptake, and adjustments of aerial development (Schroeder et al., 2001; Seki et al., 2007; Mouillon et al., 2008). Previous studies have shown that stomatal regulation of transpiration is a primary defense mechanism that prevents water loss under drought conditions (Schroeder et al., 2001; Sirichandra et al., 2009). Abscisic acid (ABA) is a key component of signaling networks that induce stomatal closure, minimizing water transpiration from the leaves (Christmann et al., 2006; Sirichandra et al., 2009). In addition, a number of transcription factors and their target genes are involved in mediating ABA perception and signaling and have been shown to regulate drought stress responses by modulating stomatal movement (Kang et al., 2002; Abe et al., 2003).

Notably, recent studies strongly support the idea that cuticular wax accumulation is also closely associated with drought resistance responses (Aharoni et al., 2004; Zhang et al., 2005, 2007; Kosma et al., 2009). The cuticle is an outermost thin hydrophobic layer that covers the surface of aerial plant parts, such as leaves and stems. It protects plants from nonstomatal water loss (Riederer and Schreiber, 2001), pathogen infection (Barthlott and Neinhuis, 1997), insect attack (Eigenbrode and Espelie, 1995), and UV radiation (Reicosky and Hanover, 1978; Solovchenko and Merzlyak, 2003). The cuticle framework is provided by cutin, a plant-specific polyester that consists of C16 and C18 hydroxy

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and epoxy-hydroxy fatty acids and glycerol monomers (Kolattukudy, 2001; Nawrath, 2006; Pollard et al., 2008).

Within the cuticle framework, the cutin matrix is surrounded by intracuticular waxes and overlaid with epicuticular waxes, which consist mainly of very-long-chain fatty acids (VLCFAs; C20 to C34) and their derivatives, such as alcohols, aldehydes, alkanes, ketones, and wax esters (Post-Beittenmiller, 1996; Kunst and Samuels, 2003; Samuels et al., 2008). The C16 and

C18 fatty acids synthesized in the plastids are exported to the cytoplasm, where they are further elongated to VLCFAs through sequential addition of two-carbon units in a reaction catalyzed by fatty acid elongase complexes at the endoplasmic reticulum (Millar et al., 1999; Hooker et al., 2002; Joubès et al., 2008; Samuels et al., 2008). Fatty acid elongation involves a series of four reactions: condensation of the two-carbon units to acyl-CoA by 3-ketoacyl-CoA synthetases (KCSs), reduction of 3-ketoacyl-CoA

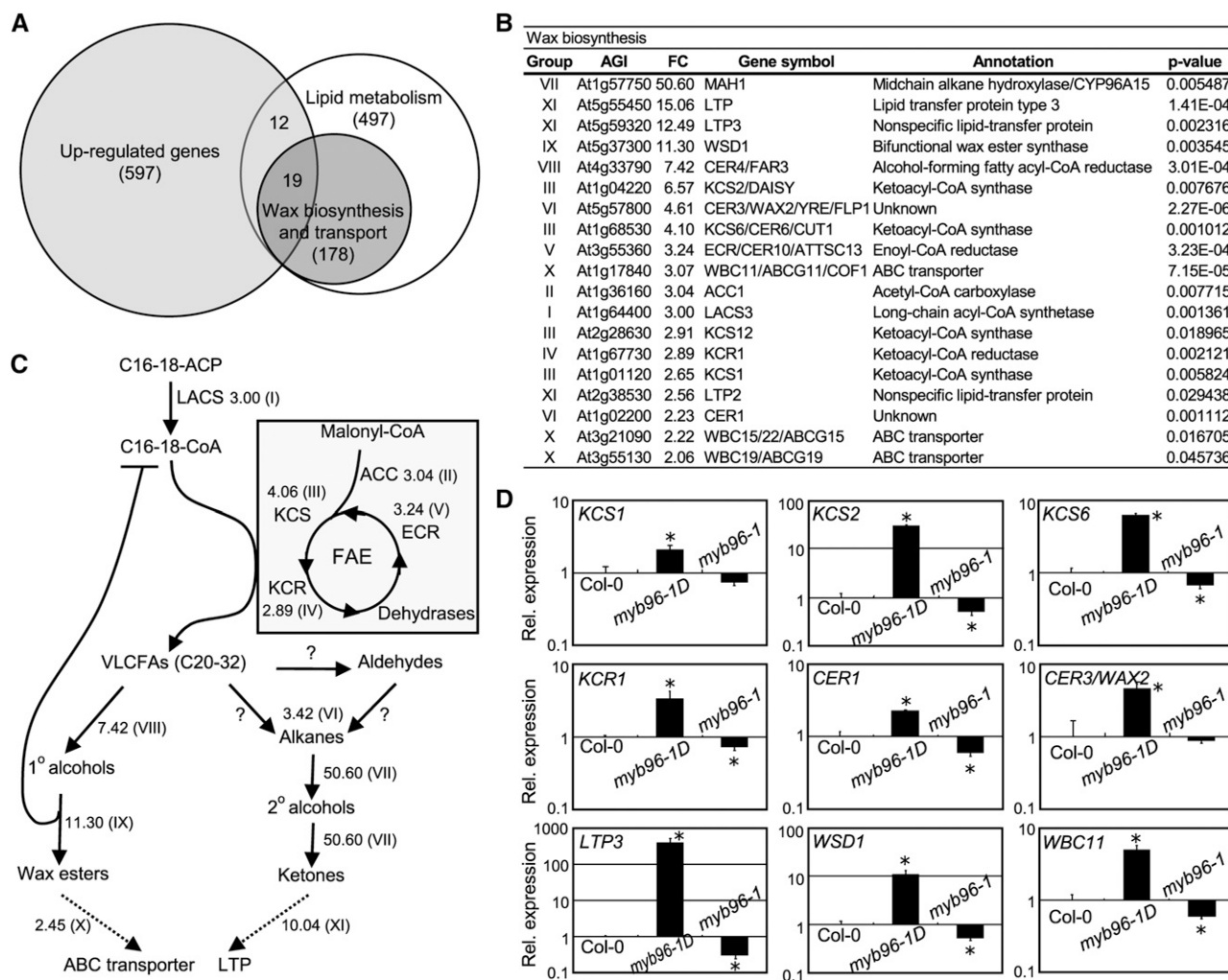


Figure 1. Upregulation of Cuticular Wax Biosynthetic Genes in *myb96-1D*.

(A) Venn diagrams showing the distribution of overlapping and nonoverlapping genes encoding lipid metabolic and wax biosynthetic enzymes with genes upregulated in *myb96-1D*. Three independent experiments were performed. The microarray data set was deposited into the ArrayExpress database with accession number E-MEXP-2965 at <http://www.ebi.ac.uk/at-miamexpress>.

(B) List of wax biosynthetic genes upregulated in *myb96-1D*. Genes upregulated >2-fold in the *myb96-1D* mutant compared with the wild type are listed in Supplemental Data Set 1A online. The P values were corrected for multiple testing using FDR methodology. The group of genes was classified based on their biochemical functions. AGI, Arabidopsis Genome Initiative number; FC, fold changes.

(C) Simplified cuticular wax biosynthetic pathway. Adapted from Kunst and Samuels (2009). Numbers indicate mean fold change of the genes belonging to individual gene groups (I to XI), as marked in **(B)**. The question marks and dotted lines indicate unidentified enzymes and processes, respectively. FAE, fatty acid elongation.

(D) qRT-PCR of wax biosynthetic gene expression. Total RNAs were extracted from 2-week-old whole plants grown on MS-agar. Transcript levels were examined via qRT-PCR. Biological triplicates were averaged and statistically analyzed using a student *t* test (**P* < 0.01). Bars indicate SE of the mean. The vertical axis is displayed on a logarithmic scale to obtain better comparison of transcript levels.

by 3-ketoacyl-CoA reductases (KCRs), dehydration of 3-hydroxyacyl-CoA by 3-hydroxyacyl-CoA dehydratases, and reduction of *trans*-2,3-enoyl-CoA by *trans*-2-enoyl-CoA reductases (Todd et al., 1999; Fiebig et al., 2000; Kunst and Samuels, 2003, 2009; Beaudoin et al., 2009). Cuticular wax biosynthesis is regulated by diverse environmental signals, and few transcription factors have been implicated in cuticular wax biosynthesis and accumulation (Aharoni et al., 2004; Zhang et al., 2005, 2007; Cominelli et al., 2008). However, little is known about how cuticular wax biosynthesis is regulated, particularly under drought stress conditions.

We recently reported that the MYB96 transcription factor plays a role in drought stress responses (Seo et al., 2009). MYB96 mediates ABA signaling in the modulation of lateral root development. It regulates lateral root meristem activation under drought conditions via an ABA-auxin signaling crosstalk. In this signaling scheme, the MYB96-mediated ABA signals are incorporated into an auxin signaling pathway that involves a subset of *GH3* genes encoding auxin-conjugating enzymes (Seo et al., 2009). MYB96 also regulates the *RD22* gene, possibly in modulating stomatal movement. Whereas an activation-tagged *myb96-1D* mutant exhibits enhanced drought resistance, the MYB96-deficient mutant (*myb96-1*) is susceptible to drought stress (Seo et al., 2009). It has been proposed that the MYB96 transcription factor contributes to optimization of plant growth and development under drought conditions, although the underlying molecular mechanisms have been unexplored.

In this study, we demonstrate that the MYB96 transcription factor confers drought resistance by modulating cuticular wax biosynthesis. A microarray assay revealed that whereas expression of a group of genes encoding cuticular wax biosynthetic enzymes was greatly upregulated in the *myb96-1D* mutant, they were downregulated in the *myb96-1* mutant. Accordingly, epicuticular waxes accumulated to a high level in *myb96-1D* leaves and stems, but epicuticular wax deposition was significantly reduced in *myb96-1* leaves and stems. Notably, the MYB96 transcription factor binds directly to the promoters of genes encoding enzymes involved in VLCFA biosynthesis, demonstrating that MYB96-mediated cuticular wax biosynthesis is intimately associated with drought resistance responses.

RESULTS

Wax Biosynthetic Genes Are Upregulated in *myb96-1D*

The MYB96 transcription factor promotes drought resistance: whereas the *myb96-1D* mutant is resistant to drought, the *myb96-1* mutant is susceptible to drought (Seo et al., 2009). Accordingly, stomatal aperture is slightly altered in the mutants. However, we expected that additional traits would also contribute to drought resistance. To obtain clues as to how MYB96 promotes drought resistance, we performed microarray assays using the Affymetrix GeneChip representing ~24,000 genes, and differentially expressed genes were identified after statistical analysis (>2-fold change, $P < 0.05$). The P values were corrected for multiple testing using false discovery rate (FDR) methodology (see Methods).

Approximately 600 genes were upregulated in the *myb96-1D* mutant (Figure 1A; see Supplemental Data Set 1A online). A major functional category of the upregulated genes included those encoding a subset of wax biosynthetic enzymes (Figure 1B; see Supplemental Data Set 1B online), such as KCS1, KCS2, KCS6, KCR1, ECERIFERUM1 (CER1), and CER3 (Aarts et al., 1995; Todd et al., 1999; Fiebig et al., 2000; Rowland et al., 2007; Beaudoin et al., 2009; Lee et al., 2009b). Genes encoding putative wax transporters were also upregulated in the mutant (Figures 1B and 1C), suggesting that cuticular wax biosynthesis and transport are broadly influenced in the *myb96-1D* mutant. The microarray data were verified by quantitative real-time RT-PCR (qRT-PCR). Whereas the wax biosynthetic genes were upregulated in the *myb96-1D* mutant, they were downregulated in the *myb96-1* mutant (Figure 1D). Furthermore, MYB96 was expressed to a high level in stem epidermal cells (see Supplemental Figure 1 online), where cuticular waxes are synthesized (Suh et al., 2005), supporting the idea that the MYB96 transcription factor is related to cuticular wax biosynthesis.

Epicuticular Wax Deposition Is Altered in *myb96-1D* and *myb96-1* Mutants

We found that expression of cuticular wax biosynthetic genes was altered in *myb96-1D* and *myb96-1* mutants. We therefore

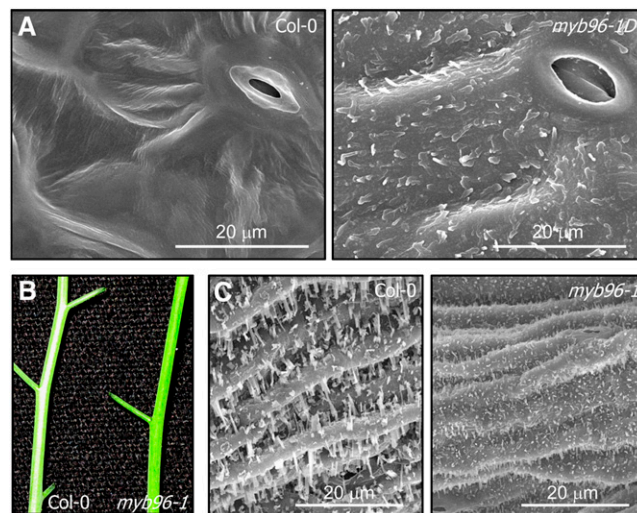


Figure 2. Scanning Electron Microscopy Analysis of Epicuticular Wax Deposition on the Surfaces of Plant Organs in *myb96-1D* and *myb96-1* Mutants.

(A) Scanning electron microscopy images of epicuticular wax crystals on the leaves. Rosette leaves of 4-week-old wild-type (Col-0) and *myb96-1D* plants grown in soil were used for scanning electron microscopy analysis. Bars = 20 μ m.

(B) Glossy appearance indicating wax deficiency of the *myb96-1* stem. Inflorescence stems of 5-week-old wild-type (Col-0) and *myb96-1* plants grown in soil were photographed.

(C) Scanning electron microscopy images of epicuticular wax crystals on the stems. Inflorescence stems of 5-week-old wild-type (Col-0) and *myb96-1* plants grown in soil were subject to scanning electron microscopy. Bars = 20 μ m.

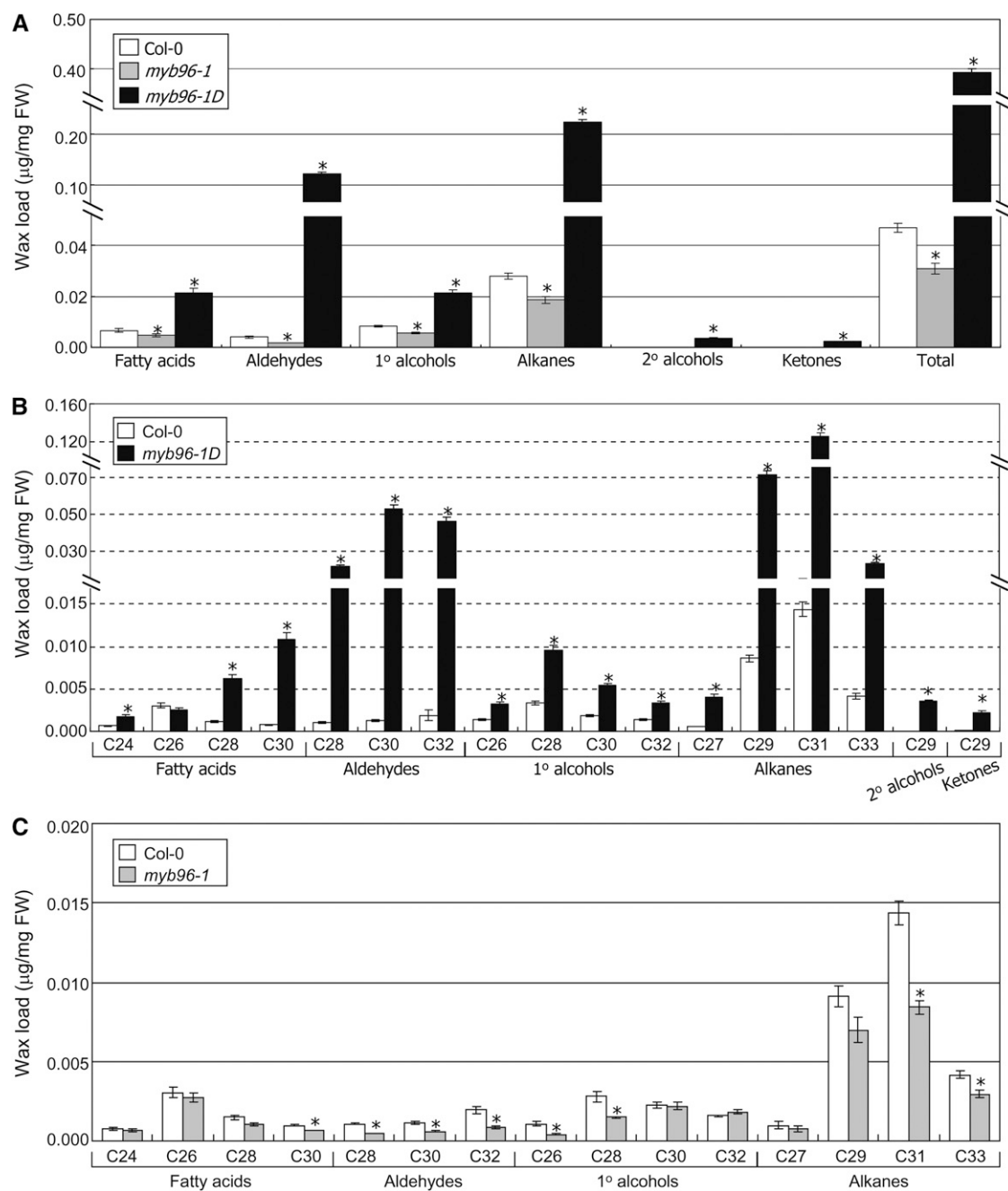


Figure 3. Cuticular Wax Amount and Composition in *myb96-1D* and *myb96-1* Leaves.

Rosette leaves of 4-week-old wild-type (Col-0) and mutant plants grown in soil were used for analysis of cuticular wax composition and loads. Biological triplicates, each with technical duplicates, were averaged and statistically analyzed using a student *t* test (**P* < 0.01). Bars indicate SE of the mean. FW, fresh weight.

(A) Cuticular wax amount in *myb96-1D* and *myb96-1* leaves compared with the wild type.

(B) and **(C)** Cuticular wax composition and loads in the leaves of the *myb96-1D* **(B)** and *myb96-1* mutants **(C)** compared with the wild type. The loads of cuticular waxes of the *myb96-1* mutant were also expressed as μg/cm² in Supplemental Figure 3 online.

analyzed deposition of epicuticular wax crystals on the leaf surface by scanning electron microscopy. Strikingly, a large amount of epicuticular wax crystals was observed on *myb96-1D* leaves, unlike wild-type leaves (Figure 2A). By contrast, they were considerably reduced on *myb96-1* stems. The whitish appearance observed in the wild type largely disappeared on *myb96-1* stems (Figure 2B). Scanning electron microscopy and Nile red staining revealed that epicuticular wax crystals were reduced accordingly on mutant stems (Figure 2C; see Supplemental Figure 2 online).

Measurements of cuticular wax content and composition by gas chromatography with flame ionization detection and gas chromatography with mass spectrometry, respectively, showed that the total wax load was elevated 8.6-fold in *myb96-1D* leaves (Figure 3A). Cuticular wax composition was also altered in mutant leaves (Figure 3B). Alterations in the contents of aldehydes and alkanes were the most prominent changes (Figures 3A and 3B). By contrast, the total wax load was decreased by ~34% in *myb96-1* leaves (Figure 3C; see Supplemental Figure 3 online). Similar to the alterations in cuticular wax contents and compositions in the leaves, the total wax load was increased ~1.6-fold in *myb96-1D* stems (Figure 4A), whereas it was decreased ~25% in *myb96-1* stems (Figure 4B; see Supplemental Figure 4 online).

However, deposition and composition of cutin monomers, which are the major components of the cuticle (Pollard et al., 2008), and expression of cutin biosynthetic genes (Wellesen et al., 2001; Schnurr et al., 2004; Xiao et al., 2004) were not discernibly altered in *myb96-1D* leaves (see Supplemental Figures 5 and 6 online), indicating that MYB96 specifically regulates biosynthesis and accumulation of cuticular waxes.

Chlorophyll leaching assays, which are frequently used to examine cuticular defects in leaves (Chen et al., 2003), showed that chlorophyll leaching occurred more slowly in *myb96-1D* leaves but more rapidly in *myb96-1* leaves in comparison to wild-type leaves (see Supplemental Figure 7 online), which corresponds with the differential accumulation of epicuticular wax crystals. Collectively, these observations support the role of MYB96 in biosynthesis and accumulation of cuticular waxes.

Drought Induction of Cuticular Wax Biosynthetic Genes Requires MYB96

The MYB96 transcription factor induces drought resistance via an ABA signaling pathway (Seo et al., 2009). Therefore, we asked whether the selected wax biosynthetic genes are influenced by drought and ABA. The *KCS1*, *KCS2*, *KCS6*, *CER1*, *LIPID TRANSFER PROTEIN3 (LTP3)*, *WAX ESTER SYNTHASE/ACYL-COA*:

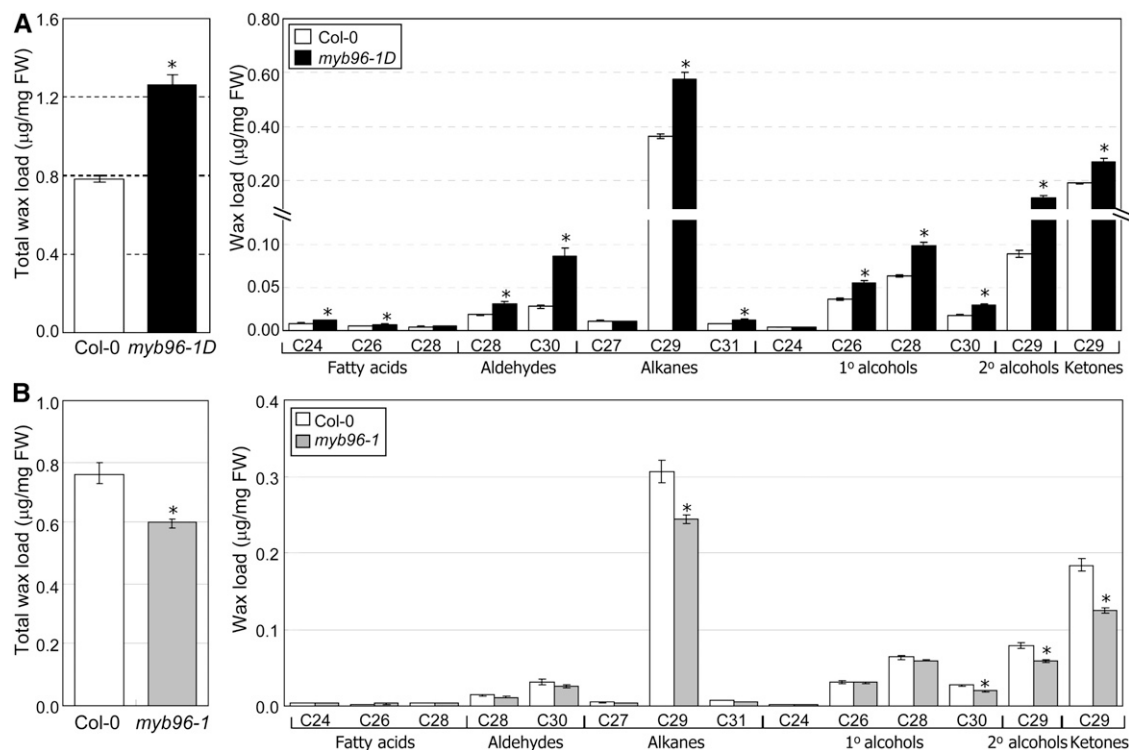


Figure 4. Cuticular Wax Composition in *myb96-1D* and *myb96-1* Stems.

Inflorescence stems of 5- to 10-week-old wild-type and mutant plants grown in soil were used for analysis of cuticular wax composition and loads. Biological triplicates, each with technical duplicates, were averaged and statistically analyzed using a Student's *t* test (* $P < 0.01$). Bars indicate SE of the mean. Cuticular wax composition and loads in the stems of *myb96-1D* (A) and *myb96-1* mutants (B). The loads of cuticular waxes of the *myb96-1* mutant were also expressed as $\mu\text{g}/\text{cm}^2$ in Supplemental Figure 4 online. FW, fresh weight.

DIACYLGLYCEROL ACYLTRANSFERASE1 (WSD1), and *WHITE-BROWN COMPLEX HOMOLOG PROTEIN11 (WBC11)* genes were significantly induced by exogenous application of ABA and exposure to drought (Figure 5A). *KCR1* and *CER3* were also induced by ABA but not by drought. Furthermore, the effects of ABA on wax biosynthetic gene expression were greatly reduced in the *myb96-1* mutant (Figure 5B; see Supplemental Figure 8A online), showing that ABA induction of the genes is at least partially dependent on MYB96. However, *PHOSPHOLIPASE C1* and *HYDROXYSTEROID DEHYDROGENASE1* encoding lipid biosynthetic enzymes were induced normally by ABA in the *myb96-1* mutant (see Supplemental Figure 8B online; Sanchez and Chua, 2001; Li et al., 2007), indicating that the ABA induction of these genes is independent of MYB96.

We next examined whether drought induces cuticular wax biosynthesis. Consistent with the ABA induction of wax biosynthetic genes, the total cuticular wax load was elevated ~ 4 -fold

in wild-type leaves under drought conditions (Figure 6A). However, the positive effects of drought on wax accumulation were significantly reduced in the leaves of the drought-susceptible *myb96-1* mutant, which is in agreement with the notion that cuticular wax accumulation confers drought resistance (Aharoni et al., 2004; Zhang et al., 2005; Seo et al., 2009). These observations indicate that MYB96 regulates, at least in part, drought stress induction of cuticular wax accumulation. The composition of cuticular waxes was also significantly altered in the mutant leaves. Under drought conditions, whereas the levels of VLCFAs, aldehydes, primary alcohols, and alkanes were elevated in wild-type leaves, accumulation of aldehydes and alkanes was considerably reduced in *myb96-1* leaves (Figure 6B).

To examine more directly the role of MYB96 in cuticular wax biosynthesis, we produced transgenic *Arabidopsis thaliana* plants expressing the *MYB96* gene under the control of a β -estradiol-inducible promoter and examined deposition of epicuticular

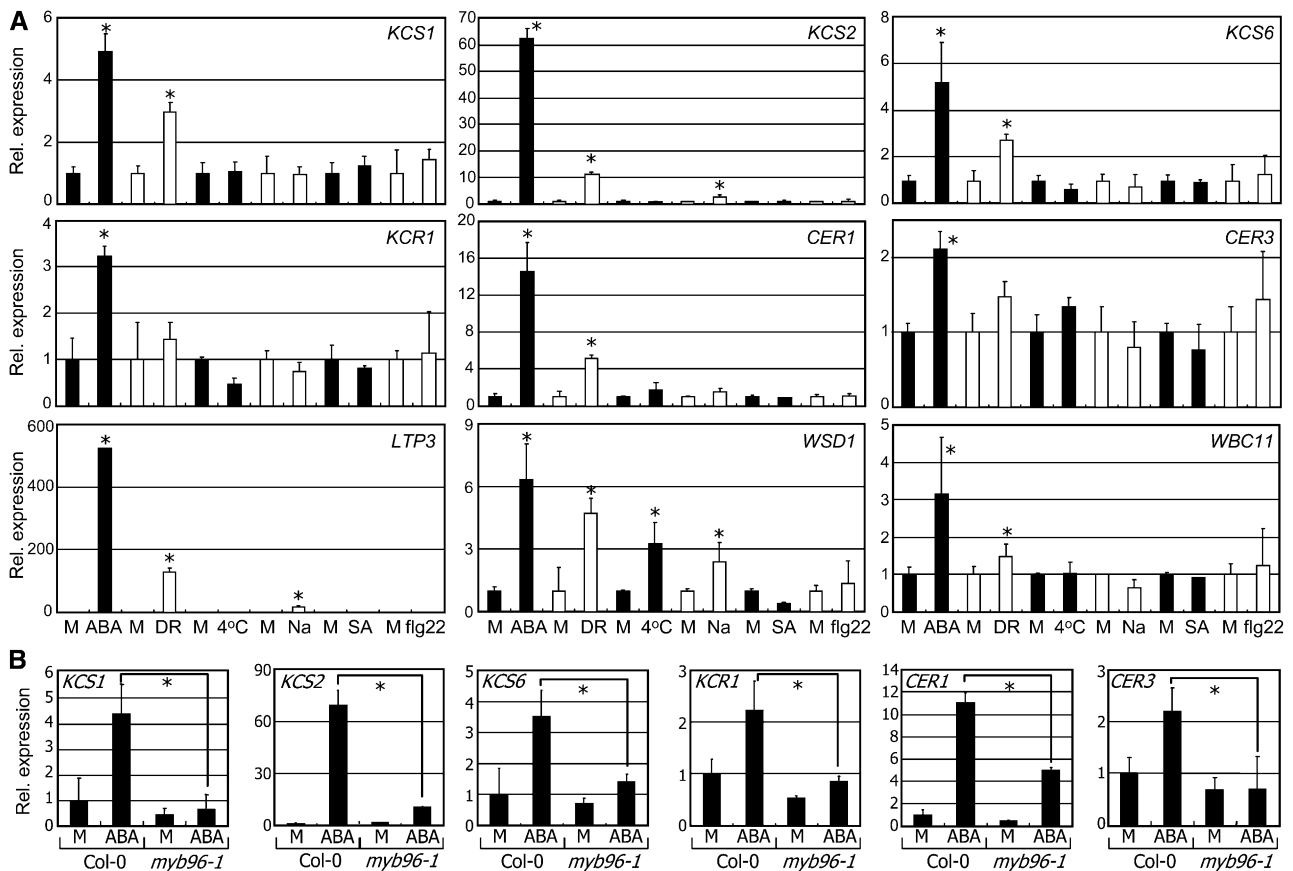


Figure 5. Drought Induction of Cuticular Wax Biosynthetic Genes.

Two-week-old plants grown on MS-agar plates were treated with growth hormones, such as 20 μ M ABA (6 h) and 100 μ M SA (6 h), and stress conditions, including cold (4°C, 24 h), 150 mM NaCl (Na, 6 h), and 5 μ M flagellin22 (flg22, 24 h), before whole-plant materials were harvested for extraction of total RNAs. Drought stress (DR) was induced in 20-d-old plants grown in soil by halting watering. Transcript levels were examined as described in Figure 1D. Bars indicate SE of the mean (*t* test, **P* < 0.01). M, mock treatment.

(A) Effects of growth hormones and stresses on gene expression. Statistical significance of the measurements was determined using a Student's *t* test by comparing with mock treatment.

(B) Effects of ABA on gene expression in *myb96-1*. Statistical significance of the measurements was determined using a Student's *t* test by comparing Col-0 and mutant plants treated with ABA.

waxes on the leaves. Genes involved in VLCFA biosynthesis, such as *KCS1*, *KCS2*, *KCS6*, and *KCR1*, were induced within 6 h after the inducer was added (Figure 7A; see Supplemental Figure 9 online), confirming that MYB96 regulates the transcription of these genes. However, the *CER1*, *WBC11*, *WSD1*, and *LTP3* genes were induced after 6 h, suggesting that the inductive effects of MYB96 may be secondary responses (see Supplemental Figure 9 online). Accordingly, deposition of epicuticular wax crystals was increased on the transgenic leaves only after induction of the *MYB96* gene (Figure 7B). These observations demonstrate that the induction of cuticular wax biosynthesis and accumulation by drought is mediated by *MYB96*.

Cuticular Wax Accumulation Contributes to Drought Resistance

Our data support the idea that MYB96 promotes drought resistance by inducing cuticular wax biosynthesis and accumulation. We examined more directly whether cuticular wax accumulation is linked with drought resistance response by measuring cuticular transpiration and chlorophyll leaching. Four-week-old plants grown under either normal or drought conditions were dark acclimated for 6 h to assure stomatal closure and soaked in water for 1 h in the dark. The leaves were subject to water loss measurements and chlorophyll leaching assays (Kosma et al., 2009). The results showed that cuticular transpiration occurred more slowly in *myb96-1D* leaves but more rapidly in *myb96-1* leaves compared with the wild type (Figure 8A), demonstrating that cuticular wax accumulation contributes to drought resistance.

Chlorophyll leaching assays further supported the linkage between cuticular wax accumulation and drought resistance. In wild-type leaves, chlorophylls were extracted more slowly from drought-treated leaves (Figure 8B), certainly due to cuticular wax accumulation. It was also found that whereas chlorophyll extrac-

tion occurred more slowly from *myb96-1D* leaves, it occurred more rapidly from *myb96-1* leaves, regardless of drought treatments. These observations indicate that MYB96-mediated cuticular wax accumulation is linked with the drought resistance response.

MYB96 Binds to Consensus Motifs in the Promoters of Wax Biosynthetic Genes

Transcriptional control of cuticular lipid biosynthesis has been documented (Zhang et al., 2005, 2007; Kannangara et al., 2007; Cominelli et al., 2008; Raffaele et al., 2008). However, no specific transcription factors have been unequivocally proven to bind directly to the biosynthetic gene promoters. We therefore asked whether the MYB96 transcription factor regulates directly the cuticular wax biosynthetic genes identified from the microarray assays.

Sequence analysis revealed that promoters of the genes that are related to wax biosynthesis, such as *KCS1*, *KCS2*, *KCS6*, *KCR1*, *CER1*, *CER3*, *LTP3*, *WSD1*, and *WBC11*, contain conserved sequence motifs (Figure 9A; see Supplemental Figure 10A online), which are analogous to the MYB binding consensus sequence (BS) (Urao et al., 1993; Abe et al., 2003). Electrophoretic mobility shift assays using a recombinant maltose binding protein (MBP)-MYB96 fusion protein produced in *Escherichia coli* cells showed that the MYB96 protein bound specifically to the BS sequence motifs of *KCS1*, *KCS2*, *KCS6*, *KCR1*, *CER3*, and *WSD1* gene promoters (see Supplemental Figure 10B online). The MBP protein alone did not bind to the consensus sequences (see Supplemental Figure 11 online). Whereas the MYB96 binding of these genes was considerably reduced in the presence of excess amounts of unlabeled BS fragments, it was reduced to a lesser degree by mutated DNA fragments (mBS), supporting the specific binding of MYB96 to the BS sequences (Figure 9B).

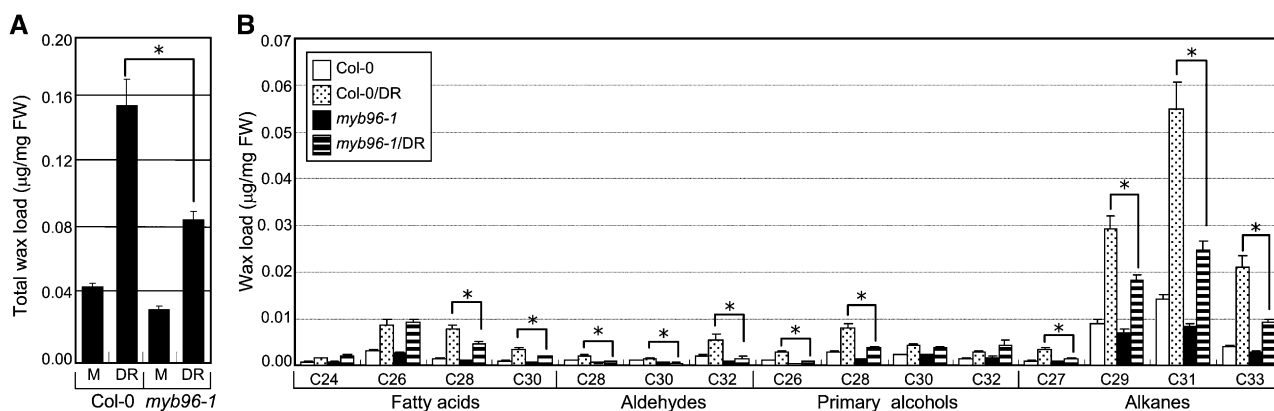


Figure 6. Cuticular Wax Accumulation in the Leaves under Drought Conditions.

(A) Accumulation of cuticular waxes under drought. Biological triplicates, each with technical duplicates, were averaged. Statistical significance of the measurements was determined using a Student's *t* test (*t* test, **P* < 0.01) by comparing Col-0 and mutant plants treated with drought (DR). Bars indicate SE of the mean. FW, fresh weight; M, mock.

(B) Cuticular wax composition in the leaves under drought. Three-week-old leaves were used. Biological triplicates, each with technical duplicates, were averaged. Statistical significance of the measurements was determined using a Student's *t* test (*t* test, **P* < 0.01) by comparing Col-0 and mutant plants treated with drought (DR). Bars indicate SE of the mean.

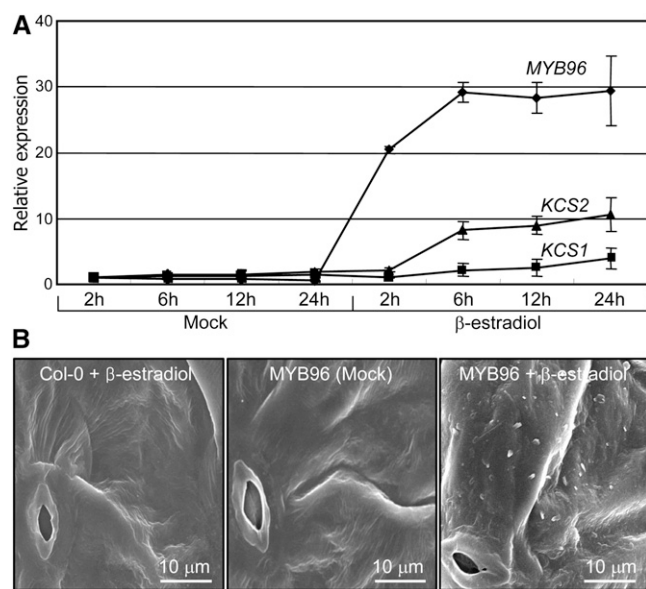


Figure 7. Effects of *MYB96* Induction on Epicuticular Wax Deposition.

(A) Induction of wax biosynthetic genes. Two-week-old transgenic plants expressing the *pER8-MYB96* gene fusion under the control of a β -estradiol-inducible promoter were incubated in MS liquid cultures supplemented with $10 \mu\text{M}$ β -estradiol. Whole plants were harvested at the indicated time points (h) after β -estradiol application for total RNA extraction. Transcript levels were examined by qRT-PCR. Biological triplicates were averaged. Bars indicate SE of the mean.

(B) Elevation of epicuticular wax crystals after β -estradiol induction of *MYB96*. One-week-old plants grown in soil were sprayed every 3 d with $10 \mu\text{M}$ β -estradiol solution. Epicuticular wax crystals were examined by scanning electron microscopy 3 weeks after induction. Bars = $10 \mu\text{m}$.

Chromatin immunoprecipitation (ChIP) assays were employed to confirm the binding of *MYB96* to the gene promoters in 35S:96-*MYC* transgenic plants. Total proteins extracted from 35S:96-*MYC* transgenic plants were immunoprecipitated with an anti-*MYC* antibody. Quantitative real-time ChIP-PCR assays showed that the *MYB96* protein binds to the gene promoters in plants in identical patterns to those observed in the electrophoretic mobility shift assays (Figure 9C; see Supplemental Figure 12 online).

We next performed transient β -glucuronidase (*GUS*) expression assays in *Arabidopsis* protoplasts to investigate further the *MYB96* regulation of wax biosynthetic genes. *KCS1*-BS and -mBS DNA fragments were transcriptionally fused to a cauliflower mosaic virus (CaMV) 35S minimal promoter in the pCAM-BIA1305.1 plasmid containing the *GUS* reporter gene, resulting in p*KCS*-P or p*KCS*-mP (Figure 9D). The reporter plasmids and an effector plasmid, p35S-*MYB96*, were cotransformed into *Arabidopsis* protoplasts. A vector construct containing the luciferase gene was included to monitor transformation efficiencies (Miura et al., 2007). The cotransformation of p35S-*MYB96* with p*KCS*-P was found to elevate the reporter gene expression >6-fold (Figure 9E). By contrast, cotransformation with p*KCS*-mP did not elevate the reporter gene expression, confirming that *MYB96* acts as a transcriptional activator of the *KCS* genes.

Altogether, our data demonstrate that the *MYB96* transcription factor regulates directly the genes involved in cuticular wax biosynthesis under drought (Millar and Kunst, 1997; Kunst and Samuels, 2009), thus providing resistance to drought (Figure 10). This signaling pathway is distinct from the ABA signaling pathways governing stomatal regulation (Zhang et al., 2005; Cominelli et al., 2008; Kosma et al., 2009), although it is still possible that the *MYB96* transcription factor contributes at least in part to stomatal regulation via the *RD22* gene (Seo et al., 2009).

DISCUSSION

It has been documented that biosynthesis and accumulation of cuticular waxes are closely linked with drought resistance responses in many plant species. Water deficit and osmotic

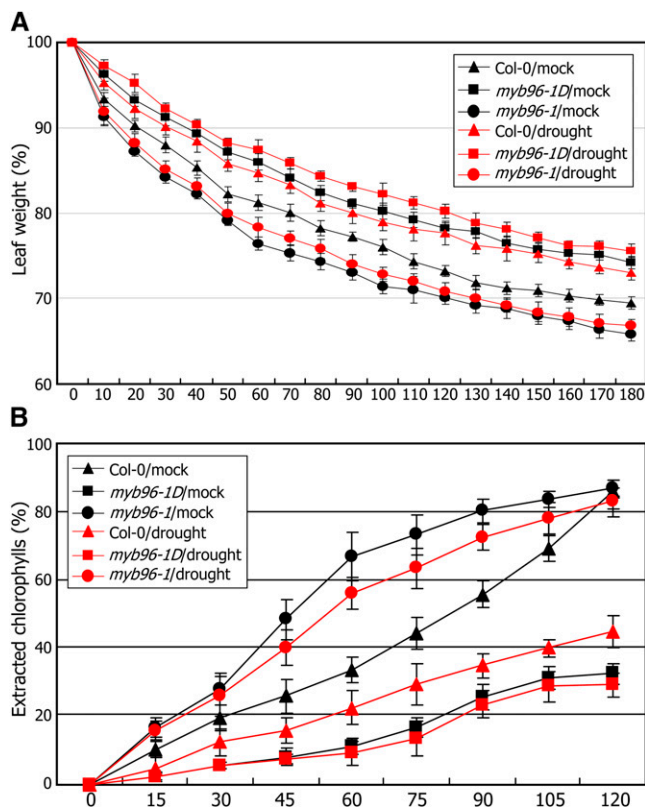


Figure 8. Cuticular Transpiration in *myb96-1D* and *myb96-1* Leaves.

Four-week-old plants grown under either normal (mock) or drought conditions were acclimated in the dark for 6 h and used for subsequent treatments.

(A) Water loss assays. Whole rosettes of the dark-acclimated plants were excised and soaked in water for 60 min in the dark. They were dried and weighed at the indicated time points. Three measurements were averaged at each time point. Bars indicate SE of the mean.

(B) Chlorophyll leaching assays. Whole rosettes of the dark-acclimated plants were soaked in 80% ethanol for the indicated time periods (min). Extracted chlorophyll contents at individual time points were expressed as percentages of that at 24 h after initial immersion. Three measurements were averaged. Bars indicate SE of the mean.

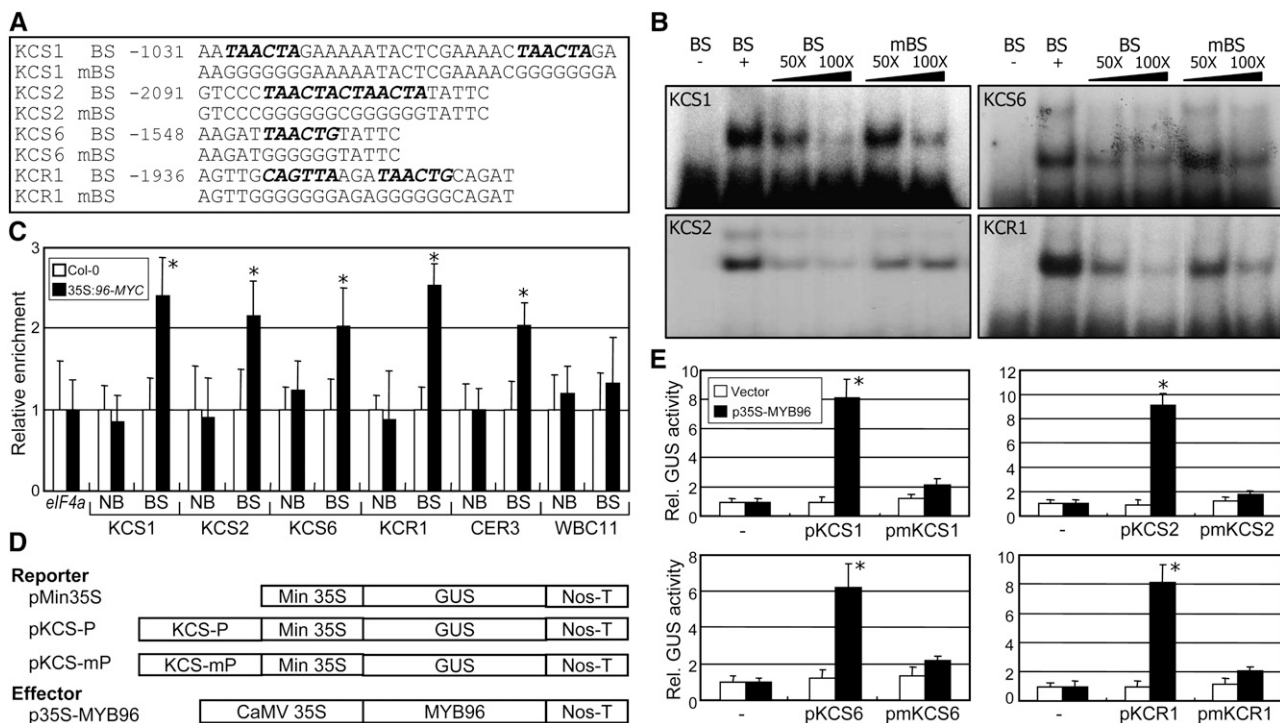


Figure 9. Binding of MYB96 to Consensus Motifs in the Promoters of Wax Biosynthetic Genes

(A) MYB binding consensus sequences (BSs). Core binding sequences, marked in bold, were mutated, resulting in mBSs, to verify specific binding.

(B) In vitro binding of MYB96 to the consensus sequences. The (–) lanes are controls without recombinant MBP-MYB96 proteins. Excess amounts (50× or 100×) of unlabeled BS or mBS DNA fragments were added as competitors.

(C) ChIP assays. Total protein extracts from 35S:96-MYC transgenic plants grown on MS-agar plates for 2 weeks were immunoprecipitated with an anti-MYC antibody. Fragmented genomic DNA was eluted from the protein-DNA complexes and subjected to quantitative PCR analysis. Three measurements were averaged for individual assays. Statistical significance of the measurements was determined using a Student's *t* test (*t* test, **P* < 0.01) by comparison with the value of Col-0. Bars indicate SE of the mean. In each measurement, the measurement values in Col-0 were set to 1 after normalization against *elF4a* for quantitative PCR analysis. NB, nonbinding sequence.

(D) Expression constructs used. The promoter sequence elements of the KCS genes listed in **(A)** were fused to minimal 35S promoter (Min 35S) and GUS reporter gene. In the effector construct, the *MYB96* gene was fused to the CaMV 35S promoter. Nos-T, Nos terminator.

(E) Transcriptional activation activity assays in *Arabidopsis* protoplasts. The expression constructs in **(D)** were expressed transiently in *Arabidopsis* protoplasts, and GUS activities were determined fluorimetrically. Luciferase gene expression was used to normalize the GUS activities. Three measurements were averaged (*t* test, **P* < 0.01). Bars indicate SE of the mean.

stresses induce significant increases in the amount of leaf cuticular lipids (Kosma et al., 2009). Cuticular wax accumulation is elevated by ~75% under osmotic stress conditions, primarily by accumulation of wax alkanes, in *Arabidopsis* (Kosma et al., 2009). Accumulation of cuticular waxes under osmotic stress or water deficit conditions have also been observed in other plant species, such as tobacco (*Nicotiana tabacum*), sesame (*Sesamum indicum*), and soybean (*Glycine max*) (Cameron et al., 2006; Kim et al., 2007a, 2007b), suggesting that cuticular wax accumulation is an important stress adaptation strategy that minimizes cellular and organismal dehydration under drought stress conditions in plants (Kosma et al., 2009).

To date, ~190 genes have been implicated in cuticular wax biosynthesis and processing in *Arabidopsis* (Beisson et al., 2003; Li-Beisson et al., 2010), and some of them have been biochemically characterized in several plant species (Aarts et al., 1995; Todd et al., 1999; Fiebig et al., 2000; Rowland et al., 2007;

Samuels et al., 2008; Beaudoin et al., 2009; Lee et al., 2009b). In addition, several transcription factors have been implicated in the functional relationship between cuticle formation and leaf surface permeability. It has been observed that *Arabidopsis* transgenic plants overexpressing a plant-specific AP2/EREBP transcription factor gene *SHINE1/WIN1* accumulate cuticular waxes and show enhanced drought resistance (Aharoni et al., 2004). Furthermore, leaf ultrastructures, such as epidermal cell structure, trichome number, and stomatal index that contribute to drought resistance, have also been altered in the transgenic plants. The soybean AP2/EREBP transcription factors *WXP1* and *WXP2* also affect cuticular wax accumulation. Cuticular waxes accumulate to a high level in *Arabidopsis* transgenic plants overexpressing either *WXP1* or *WXP2* that exhibit a drought-resistant phenotype (Zhang et al., 2005, 2007). A MYB transcription factor has been also implicated in cuticle-mediated drought resistance. Overexpression of *MYB41* influences leaf surface

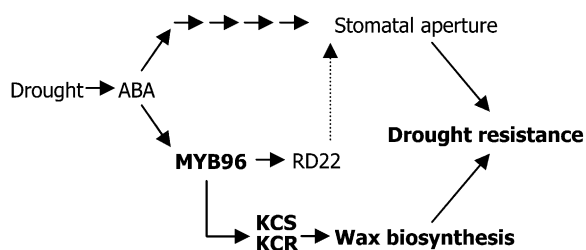


Figure 10. Schematic Working Model of MYB96 Function in Cuticular Wax Biosynthesis under Drought Conditions.

MYB96 transcription is upregulated in response to ABA-mediated drought signals. The MYB96 protein activates the cuticular wax biosynthetic genes by binding directly to the gene promoters, resulting in accumulation of cuticular waxes. MYB96 may also contribute to drought resistance via the *RD22* pathway by regulating stomatal aperture (Seo et al., 2009).

permeability by altering cuticle biosynthesis (Cominelli et al., 2008). However, the gene regulatory networks and underlying molecular mechanisms governing cuticular wax biosynthesis have been unexplored at the molecular level in most cases.

Here, we found that the MYB96 transcription factor, which is a part of an ABA signaling cascade in the drought resistance response (Seo et al., 2009), modulates cuticular wax biosynthesis in response to drought by regulating cuticular wax biosynthetic genes. The MYB96 transcription factor binds directly to conserved sequences existing in the promoters of the cuticular wax biosynthetic genes and transcriptionally activates the target genes. These observations are consistent with altered cuticular wax loads in the *MYB96*-overexpressing and *myb96-1* mutant plants. It has been observed that ABA induces accumulation of cuticular waxes, although it is possible that cuticular wax biosynthesis is also induced by abiotic stresses in an ABA-independent manner (Kosma et al., 2009). We found that the cuticular wax biosynthetic genes regulated by MYB96 are also induced by ABA and drought stress. Furthermore, our results demonstrate that the inductive effects of ABA and drought stress are, at least in part, dependent on MYB96, confirming that MYB96 regulates cuticular wax biosynthesis via ABA-mediated drought stress signaling.

It has been shown that changes in cuticular wax accumulation and composition influence epidermal properties (Gray et al., 2000; Aharoni et al., 2004). In addition, the effects of cuticular transpiration on drought resistance are relatively smaller than those of stomatal transpiration (Kerstiens, 1996). Therefore, another possibility is that the drought-resistant and -susceptible phenotypes of the *myb96-1D* and *myb96* mutants, respectively, would be caused by alterations in epidermal properties rather than by changes in cuticular transpiration. We previously observed that stomatal density and index and trichome morphology and distribution are not altered in the *myb96-1D* and *myb96-1* mutants (Seo et al., 2009). We found that cuticular transpiration is detectably reduced in the drought-resistant *myb96-1D* leaves but increased in the drought-susceptible *myb96-1* leaves. The previous and our own data support that MYB96 does not affect leaf epidermal properties. We therefore believe that cuticular wax accumulation

is associated intimately with drought resistance, in which MYB96 plays a role by modulating cuticular wax biosynthesis. However, it is still possible that certain aspects of stomatal properties, such as microstructures and assembly, would be altered due to changes in cuticular wax accumulation and composition in the mutants. This uncertainty could be clarified by measuring stomatal conductance in the leaves of Columbia-0 (*Col-0*) and mutant plants grown under normal and drought conditions.

It is interesting that MYB96 also plays a role in pathogen resistance (Seo and Park, 2010). Cuticular waxes modulate plant-microbe interactions by providing mechanical strength and viscoelastic properties (Baker et al., 1982; Lee et al., 2009a). Therefore, it is likely that MYB96 promotes pathogen resistance by inducing cuticular wax accumulation. Notably, it has been observed that the salicylic acid (SA) content is elevated and transcription of the SA biosynthetic *SA INDUCTION DEFICIENT2* gene is upregulated in the *myb96-1D* mutant (Seo and Park, 2010). It is yet unknown whether SA biosynthesis and cuticular wax accumulation are interrelated or not. SA biosynthesis may be induced indirectly because the disease resistance response is triggered due to cuticular wax accumulation in the mutant. Alternatively, the two processes may be independently regulated by MYB96.

Cutin biosynthesis and deposition are also influenced by drought stress. Endogenous contents of cutin monomers are elevated in *Arabidopsis* leaves that are exposed to drought conditions (Kosma et al., 2009). However, contents of cutin monomers were not discernibly changed in the *MYB96*-overexpressing and deficient mutant plants, and cutin monomer biosynthetic genes were not regulated by MYB96. It is notable that the levels of cutin monomers are largely unaffected by ABA treatments (Kosma et al., 2009). Previous and our own data support that MYB96 is not directly involved in cutin biosynthesis.

We found that MYB96 regulates the *KCS* genes by directly binding to the gene promoters. Transcription of the *KCS* genes was upregulated in the *myb96-1D* mutant but downregulated in the *myb96-1* mutant. Roles of several *KCS* enzymes have been reported in fatty acid chain elongation. Among the 22 *KCS* isoforms identified in *Arabidopsis* (Beisson et al., 2003; Li-Beisson et al., 2010), *KCS1*, *KCS2/DAISY*, *KCS6/CER6/CUT1*, *KCS5/CER60*, and *KCS20* have been shown to be involved in cuticular wax biosynthesis (Millar and Kunst, 1997; Millar et al., 1999; Todd et al., 1999; Fiebig et al., 2000; Clemens and Kunst, 2001; Hooker et al., 2002; Lee et al., 2009b), although their biochemical activities are not well understood. Of particular interest is *KCS2*. It is induced by osmotic stress and ABA (Lee et al., 2009b). In addition, the *KCS2* enzyme is involved in the VLCFA biosynthesis required for suberin accumulation in the root endodermis and seed chalazal-micropyle (Franke et al., 2009; Lee et al., 2009b). It is supposed that the *KCS2*-mediated regulation of cuticular wax biosynthesis via MYB96 may be related with the role of MYB96 in lateral root development and drought resistance response (Seo et al., 2009).

Genes encoding four major components of the fatty acid elongation complex, including *KCS*, *KCR*, *ECR*, and 3-hydroxyacyl-CoA dehydratase (*PAS2*), are transcriptionally upregulated in the *myb96-1D* mutant. Those enzymes involved in the subsequent reactions, such as *CER3* and *MAH1* in the decarbonylation pathway and *CER4* and *WSD1* in the acyl reduction pathway

(Rowland et al., 2006, 2007; Greer et al., 2007; Li et al., 2008), are also induced in the mutant. In addition, *WBC11*, which encodes a plasma membrane-localized ABC transporter functioning as the exporter of cutin monomers and waxes (Bird et al., 2007; Luo et al., 2007; Panikashvili et al., 2007; Ukitsu et al., 2007), is also regulated in a similar manner by MYB96. Furthermore, additional genes encoding LTP and ABC transporters are also upregulated in the *myb96-1D* mutant. We therefore propose that the MYB96 transcription factor may act as a master transcriptional regulator for cuticular wax biosynthesis and accumulation in response to drought.

Alkane biosynthesis is one of the key responses of plants under osmotic stress conditions (Kosma et al., 2009). Alkanes, primary alcohols, and aldehydes are known to confer resistance to water movement through artificial membranes more efficiently than do fatty acids. It has been shown that alkanes constitute the most impermeable crystalline barrier against water movement (Grncarevic and Radler, 1967; Kosma et al., 2009). Consistent with the role of alkanes serving as a barrier against water movement, alkane contents were dramatically elevated in the drought-resistant *myb96-1D* mutant but reduced in the drought-susceptible *myb96-1* mutant. It is particularly noteworthy that endogenous contents of alkanes, a major constituent of gasoline and diesel, were increased >8-fold in the *myb96-1D* mutant, suggesting that our findings would be applicable to engineering of alkane mass production in terrestrial plants (Jetter and Kunst, 2008; Schirmer et al., 2010).

METHODS

Plant Materials and Growth Conditions

All *Arabidopsis thaliana* lines used were in the Col-0 background. Plants were grown in a controlled culture room or on half-strength Murashige and Skoog (MS)-agar plates (hereafter referred to as MS-agar plates) at 22°C under long-day conditions (16 h light and 8 h dark). White light illumination (120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) was provided by fluorescent FLR40D/A tubes (Osram). The activation-tagged *myb96-1D* and T-DNA insertional *myb96-1* mutants have been described previously (Seo et al., 2009).

The primers used for subcloning of the *MYB96* gene under the control of a β -estradiol-inducible promoter were MYB96 (pER8)-F and MYB96 (pER8)-R (see Supplemental Table 1 online). The PCR product was subcloned into the *Xho*I and *Pac*I sites of the pER8 vector (Zuo et al., 2000). Two-week-old transformants grown on MS-agar plates were used for induction of the *MYB96* gene by 10 μM β -estradiol.

To examine the effects of growth hormones and stress conditions on gene expression, 2-week-old plants grown on MS-agar plates were transferred to MS liquid cultures supplemented with 20 μM ABA or 100 μM SA and incubated for 6 h. To examine the effects of drought, plants grown in soil for 20 d were subject to drought treatment by halting watering. To examine the effects of high salinity, plants were soaked in MS liquid cultures containing 150 mM NaCl and incubated for 6 h. For cold treatments, plants were exposed to 4°C for 24 h. For flagellin22 (flg22) treatments, plants were soaked in MS liquid cultures containing 5 μM flg22 and incubated for 24 h.

Microarray Assays

Two-week-old whole plants grown on MS-agar plates at 22°C under long-day conditions (16 h light and 8 h dark) were used for extracting total

RNAs using the RNeasy plant mini kit (Qiagen). The total RNA samples were pretreated with RNase-free DNase I and cleaned up using the Plant Total RNA isolation kit (Qiagen). Three independent RNA samples were assayed and statistically treated. Probe preparation, hybridization to the GeneChip *Arabidopsis* ATH1 Genome Arrays (Affymetrix), and subsequent processing steps were performed according to the manufacturer's procedure. Global normalization and expression estimates were analyzed by GC-robust multiarray implemented in the bioconductor (<http://www.bioconductor.org>) and R software (Wu et al., 2004). Wild-type (Col-0) plant values were used as the baseline to calculate the intensity ratio/fold changes of the mutant plants. FDRs were calculated by significance analysis of microarrays algorithm (Tusher et al., 2001; Gentleman et al., 2004), and genes with fold change of at least 2 and a FDR-corrected P value lower than 0.05 were identified. The microarray data set is deposited into ArrayExpress with accession number E-MEXP-2965 at <http://www.ebi.ac.uk/at-miamexpress>.

Analysis of Transcript Levels

qRT-PCR reactions were performed in 96-well blocks with an Applied Biosystems 7500 Real-Time PCR system using the SYBR Green I master mix in a volume of 25 μL . The reactions were performed in biological triplicates using RNA samples extracted from three independent plant materials and gene-specific primers listed in Supplemental Table 1 online. Data processing and determination of the reaction specificities were performed as described previously (Seo et al., 2009).

Scanning Electron Microscopy and Nile Red Staining

For epicuticular wax crystal observation, the fourth rosette leaves or inflorescence stem segments from tip to 2 cm of 4- to 5-week-old plants grown in soil were examined by scanning electron microscopy as described previously (Lee et al., 2009b).

For Nile red staining, plant materials were incubated for 20 min in a 5 $\mu\text{g mL}^{-1}$ Nile red solution. Stained samples were washed with deionized water and analyzed using a TCS SP5 AOBs/Tandem confocal laser scanning microscope (Leica). Nile red was excited with a 488-nm laser and collected with a 560- to 615-nm filter as described previously (Pighin et al., 2004).

Analysis of Cuticular Wax Composition and Loads

Cuticular waxes were extracted from the leaves (200 to 1000 mg) and stems (200 mg) of 4-week-old plants in chloroform for 30 s at room temperature. *n*-Octacosane, docosanoic acid, and 1-tricosanol were added to the extracted chloroform solvent as internal standards. The solvent was subsequently evaporated under a gentle stream of nitrogen and redissolved in a mixture of 100 μL of pyridine and 100 μL of bis-*N,N*-(trimethylsilyl)trifluoroacetamide. The wax mixtures were heated at 90°C for 30 min to convert waxes into trimethylsilyl derivatives. Qualitative and quantitative composition analyses were conducted as described previously (Lee et al., 2009a, 2009b). The P values from each comparison were corrected for multiple tests using FDR control (Benjamini and Hochberg, 1995).

Analysis of Cutin Polyester Monomers

Rosette leaves of 4-week-old plants grown in soil were used to quantify cutin polyester monomers. Methyl heptadecanoate and ω -pentadecalactone were added as internal standards into the delipidated and dried leaves and then depolymerized by hydrogenolysis with LiAlH_4 or by methanolysis with NaOCH_3 . Cutin polyesters were analyzed by gas chromatography-mass spectrometry (GCMS-QP2010; Shimadzu) with a

HP-5 column (60 m, 0.32 mm inner diameter, film thickness 0.1 mm; Agilent). The analysis system was maintained at 110°C. The temperature was increased to 300°C at a rate of 2.5°C min⁻¹ and maintained at 300°C for 3 min.

Electrophoretic Mobility Shift Assays

The *MYB96* gene was subcloned into the pMAL-c2X *Escherichia coli* expression vector (NEB), which has an MBP coding sequence, using MYB96 (MBP)-F and MYB96 (MBP)-R primers (see Supplemental Table 1 online). The MBP-MYB96 fusion protein was purified according to the manufacturer's procedure using the pMAL Protein Fusion and Purification System (#E8000S). DNA fragments were end-labeled with [γ -³²P]dATP using T4 polynucleotide kinase. Labeled probes were then incubated with ~0.5 μ g of the purified MBP-MYB96 protein for 30 min at 25°C in a binding buffer (10 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA, 5 mM DTT, and 5% glycerol) with or without competitor DNA fragments. The reaction mixtures were electrophoresed on 6% native PAGE gels. The gels were dried on Whatman 3MM paper and exposed to x-ray films.

ChIP Assays

A MYC-coding sequence was fused in frame to the 3' end of the *MYB96* gene, and the gene fusion was subcloned under the CaMV 35S promoter (Kim et al., 2006). The expression construct was transformed into Col-0 plants. Two-week-old 35S:96-MYC transgenic plants grown on MS-agar plates were used for extraction of total proteins. The processing of plant materials and qRT-PCR were performed as described previously (Lawrence et al., 2004). The qRT-PCR primers used are listed in Supplemental Table 2 online.

Transcriptional Activation Activity Assays

The *MYB96* gene sequence was fused in frame to the 3' end of the GAL4 DNA binding domain-coding sequence in the effector plasmid. The activity of the MYB96 transcription factor was examined by a GAL4 transient expression system using *Arabidopsis* protoplasts as described previously (Miura et al., 2007).

Chlorophyll Leaching Assays

Rosette leaves of 4-week-old plants grown in soil were used. The leaves were weighed, and ~2 g of each leaf sample was incubated on ice for 30 min and immersed in 30 mL of 80% ethanol in 50 mL conical tubes at room temperature. Aliquots of 100 μ L were removed from the solution at every 10 min after initial immersion. The amount of extracted chlorophylls was quantified by measuring absorbance at 647 and 664 nm using a diode array spectrophotometer (WPA Biowave) as described previously (Lolle et al., 1998).

Accession Numbers

Sequence data from this article can be obtained from the Arabidopsis Genome Initiative database under the following accession numbers: *MYB96* (At5g62470), *KCS1* (At1g01120), *KCS2* (At1g04220), *KCS6* (At1g68530), *KCR1* (At1g67730), *CER1* (At1g02205), and *CER3* (At5g57800). The microarray data set is deposited into ArrayExpress with accession number E-MEXP-2965 at <http://www.ebi.ac.uk/at-miamexpress>.

Supplemental Data

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

Supplemental Figure 1. Expression of *MYB96* in Epidermal Cells of Stems.

Supplemental Figure 2. Epicuticular Wax Deposition in *myb96-1* Stems.

Supplemental Figure 3. Cuticular Wax Composition in *myb96-1* Leaves.

Supplemental Figure 4. Cuticular Wax Composition in *myb96-1* Stems.

Supplemental Figure 5. Cutin Biosynthesis in *myb96-1D* Leaves.

Supplemental Figure 6. Expression of Cutin Biosynthetic Genes in *myb96-1D* and *myb96-1*.

Supplemental Figure 7. Rate of Chlorophyll Leaching in *myb96-1D* and *myb96-1* Leaves.

Supplemental Figure 8. MYB96 Regulation of Wax Biosynthetic Genes in Response to ABA.

Supplemental Figure 9. Effects of *MYB96* Induction on Wax Biosynthetic Gene Expression.

Supplemental Figure 10. Binding of MYB96 to the Consensus Sequences in the Promoters of Wax Biosynthetic Genes in Vitro.

Supplemental Figure 11. In Vitro Binding of MBP Alone to the Consensus Sequences.

Supplemental Figure 12. Direct Binding of MYB96 to the Promoters of Wax Biosynthetic Genes in Vivo.

Supplemental Table 1. Primers Used in qRT-PCR and Subcloning.

Supplemental Table 2. Primers Used in ChIP Assays.

Supplemental Data Set 1A. Upregulated Genes in the *myb96-1D* Mutant.

Supplemental Data Set 1B. Ontologies of Upregulated Genes in the *myb96-1D* Mutant.

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