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The MIXTA-like Transcription factor MYB16 is a major regulator of cuticle formation in vegetative organs

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Cuticle secreted on the surface of the epidermis of aerial organs protects plants from the external environment. We recently found that *Arabidopsis* MIXTA-like R2R3-MYB family members MYB16 and MYB106 regulate cuticle formation in reproductive organs and trichomes. However, the artificial miRNA (amiRNA)-mediated knockdown plants showed no clear phenotypic abnormality in vegetative tissues. In this study, we used RNA interference (RNAi) targeting MYB16 to produce plants with reduced expression of both *MYB16* and *MYB106*. The rosette leaves of RNAi plants showed more severe permeable cuticle phenotypes than the *myb106* mutants expressing the *MYB16* amiRNA in the previous study. The RNAi plants also showed reduced expression of cuticle biosynthesis genes *LACERATA* and *ECERIFERUM1*. By contrast, expression of a gain-of-function MYB16 construct induced over-accumulation of waxy substances on leaves. These results suggest that MYB16 functions as a major regulator of cuticle formation in vegetative organs, in addition to its effect in reproductive organs and trichomes.

In plants, the outmost cell layer, or epidermis, differentiates into many types of cells, including leaf pavement cells, stomatal guard cells, trichomes, and petal conical cells. The epidermal cells of the above-ground parts of the plant secrete a cuticle of cutin and wax outside of the cell wall; this cuticle protects plants from biotic and abiotic stresses, confers mechanical strength, regulates water and gas exchange (together with stomata), and prevents organ fusion.[1]

MIXTA homologs belonging to the R2R3-MYB family subgroup 9 regulate epidermal cell outgrowth of petal conical cells and trichomes. For example, in *Antirrhinum majus*, the *mixta* mutant shows alterations in petal color intensity, in which conical cells become flat.[2] Other MIXTA homologs in *A. majus* and petunia promote anticlinal expansion of epidermal cells, as shown by analysis of mutants and overexpression lines.[3-5] Overexpression of MYB16, an *Arabidopsis* MIXTA homolog, induced similar ectopic outgrowths in petals of *Arabidopsis* and tobacco, suggesting it has similar functions to petunia MYB1 and *A. majus* MYBML2.[4] Another *Arabidopsis* homolog, MYB106/NOK, regulates trichome maturation, including promotion and limitation of trichome branching.[6,7]

We recently described additional functions of MIXTA-like proteins in cuticle development.[8] Expression of MYB106 and MYB16 chimeric repressor fusions (MYB106-SRDX, MYB16-SRDX) under the control of the CaMV 35S promoter induced cuticle deficiencies such as permeable surfaces, reduced epicuticular wax crystals, and loss of nanoridges, in whole plants.[8] By contrast, expression of the dominant active form of MYB106 (MYB106-VP16), which has a virus-derived strong transcriptional activation domain VP16,[9] induced ectopic formation of nanoridges, which are usually only formed in floral organs, and plate-like wax crystals on rosette leaves.[8] These data, together with the results of transcriptome analysis of these transgenic plants and effector reporter assays, revealed that MYB106 positively regulates cuticle formation through activation of the expression of cutin and wax biosynthetic genes and an APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) transcription factor, *WAX INDUCER1/SHINE1,* which also positively regulates cutin biosynthetic genes.[8,10-13] We also analyzed *myb106–2* mutants and *MYB16* artificial miRNA (*MYB16-amiRNA*) plants and demonstrated that MYB106 and MYB16 have redundant functions in cuticle nanoridge formation in petal and stamen, and in morphogenesis of petal conical cell and trichome. However, the *MYB16-amiRNA myb106–2* double knockdown/out plants did not show a cuticle deficiency in leaves. A leaf phenotype was expected because the *MYB16* promoter has activity in leaves and MYB16-SRDX driven by the *MYB16* promoter produces an organ fusion phenotype in leaves.[8] These data suggest that the amiRNA-mediated suppression of *MYB16* was not sufficient, or there is another functionally redundant gene in addition to *MYB16* and *MYB106*. To answer this question, in this study, we produced *MYB16* silenced plants by introducing a *35S:MYB16-RNAi* construct into wild type and *myb106–2* mutants. To construct *35S:MYB16-RNAi,* the coding sequence of *MYB16* was transferred by Gateway LR reaction, into pHG8-based vector.[8,14] qRT-PCR procedure

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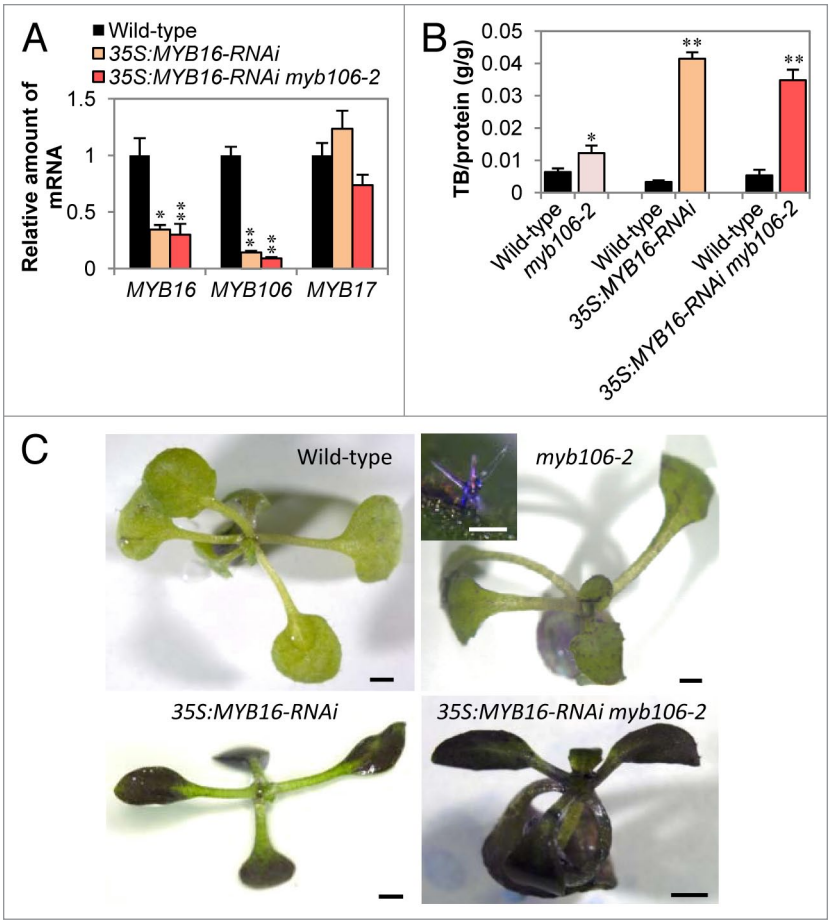

Figure 1. Surface permeability of *MYB16* and *MYB106* silenced plants. (**A**) Quantitative RT-PCR analysis of 5′ or 3′ untranslated region of *MYB16* and *MYB106*, respectively, and the coding region of *MYB17* in 2-week-old plants (n = 5). Error bars represent standard error. Single and double asterisks indicate $P < 0.05$ and $P < 0.01$ in the Welch *t*-test, respectively. (**B**) TB uptake per gram protein (n = 8) after staining by 0.05% TB for 2 min. (**C**) Seedling stained with TB. Bars = 1 mm. The inset panel shows a stained trichome of *myb106–2*. Bar = 100 μm.

and analysis method were described before.[8] 3′ untranslated region of MYB106 was amplified by using the specific primers MYB106–3′f 5′-ACCGTCCGATTCCGCGACGA-3′ and MYB106–3′r 5′-ACCGTCCGATTCCGCGACGA-3′.[8] 5′ untranslated region of *MYB16* was amplified by using the specific primers AT5G15310pRB 5′-CGCGGATCCTGTTTTGAGAGCAAAGAAATAAGA-3′[8] and AT5G15310pS 5′-AACATTTCCACAACTGTAGCCAAACT-3′. *MYB17* was amplified by using the primers 3G61250Rf1 5′-AAAGGTCCTTGGACGCCTGAA-3′ and 3G61250Rr1 5′-TCTTCCCACAGCGAAGTAAACCA-3′. In *35S:MYB16-RNAi* and *35S:MYB16-RNAi myb106–2* plants, expression of both *MYB16* and *MYB106* mRNAs was reduced, but expression of *MYB17*, which belongs to R2R3-MYB family subgroup 9 and is closest to *MYB16* and *MYB106*, was not affected (**Fig. 1A**). The high nucleotide sequence similarity between *MYB16* and *MYB106* likely causes the silencing effect of the *35S:MYB16-RNAi* construct on *MYB106*.

To investigate cuticle permeability in the RNAi plants, we performed toluidine blue (TB) staining.[15] Broad regions of leaves in *35S:MYB16-RNAi* and *35S:MYB16-RNAi myb106–2* plants were stained by TB, but only the trichome cells in *myb106–2* were stained (**Fig. 1B** and **1C**). *MYB106* is expressed in trichomes of leaves and the single mutant of *MYB106* exhibited abnormalities only in the trichome cells;[6,8] therefore, the cuticle deficiency of the leaves in *MYB16-RNAi* plants is likely to be mainly caused by the suppression of *MYB16*. We also found that the cuticle related genes *LACERATA* (LCR) and *ECERIFERUM1* (*CER1*) were also suppressed in the *MYB16-RNAi* plants. Expression of *LCR* and *CER1* was reduced to approximately half and less than one fifth of wild type, respectively (**Fig. 2**). *LCR* encodes a cytochrome P450 monooxygenase CYP86A8 which catalyzes ω-hydration of fatty acids, and is suggested to be involved in the biosynthesis of cutin; indeed, *lcr* mutants show typical cutin-deficient phenotypes like postgenital organ fusion and hydration of wild type pollen on mutant leaves, which indicates permeable leaf cuticle.[16] CER1 protein is a core component of the very-long-chain (VLC) alkane synthesis complex.[17] The mutants of *CER1* showed reduced wax VLC alkane accumulation and increased cuticle permeability, and *CER1* overexpression increased VLC alkane derivatives and reduced permeability, resulting in drought tolerance and increased susceptibility to pathogens.[18,19] *MYB16-RNAi* expression affected the expression of these cutin and wax synthesis genes in leaves, suggesting that MYB16 functions through the regulation of these enzymatic genes in cuticle formation.

To examine the ability of MYB16 to induce cuticle synthesis, we generated plants expressing the dominant active form of MYB16, in which MYB16 was fused with the VP16 activation domain from herpes simplex virus (*35S:MYB16-VP16*). The *35S:MYB16-VP16* plants had slightly shiny leaves (**Fig. 3A**) and scanning electron microscopy revealed over-accumulation of epicuticular wax-like substances (**Fig. 3B** and **3C**), as observed in *35S:MYB106-VP16*, *35S:MYB106* and *35S:WIN1* plants.[8] MYB16 activated expression from the promoters of *WIN1/SHN1* and the cutin biosynthesis gene *CYP86A4 in vivo*,[8] indicating that MYB16 functions as a positive regulator of cuticle formation. Taking these results together, we conclude that MYB16 is a major regulator of cuticle formation in vegetative tissues.

SHN3, a paralog of WIN1/SHN1, has been suggested to regulate cuticle formation in leaves, based on its expression pattern and ability to induce accumulation of cuticle.[10] However, *SHN1/2/3* triple amiRNA plants did not show cuticle deficiency in vegetative organs.[13] HD-Zip IV family transcription factors specifying epidermal cell identity during various stages in epidermal cell differentiation also regulate some cuticle related genes via the L1-box in their promoters.[1,20-22] Mutants of these HD-Zip IV transcription factors showed protodermal and epidermal

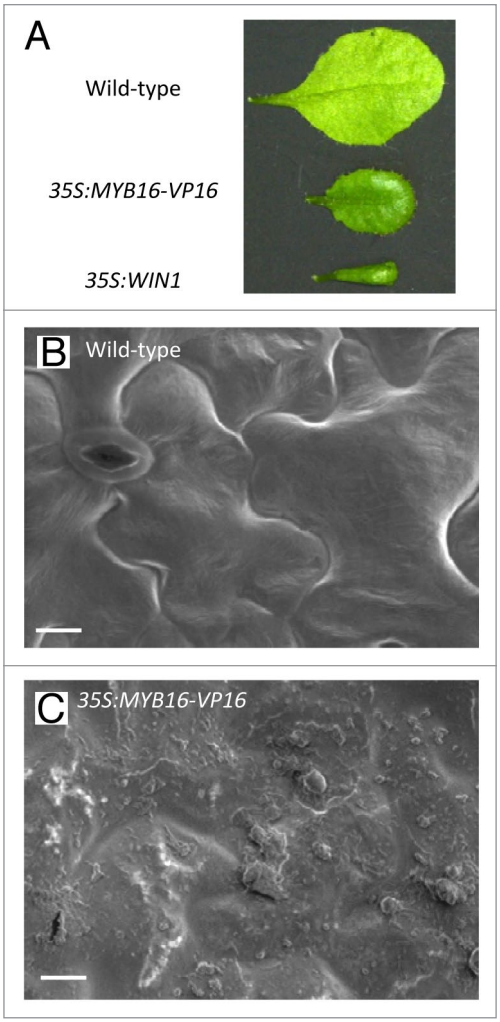

Figure 2. Ectopic accumulation of cuticular substances in *35S:MYB16-VP16* plants. (**A**) Rosette leaves excised from wild-type, *35S:MYB16-VP16*, and *35S:WIN1* plants. (**B**) and (**C**) Adaxial surface of rosette leaves of wild-type (**B**) and *35S:MYB16-VP16* (**C**) plants observed by scanning electron microscopy. Bars = 10μm.

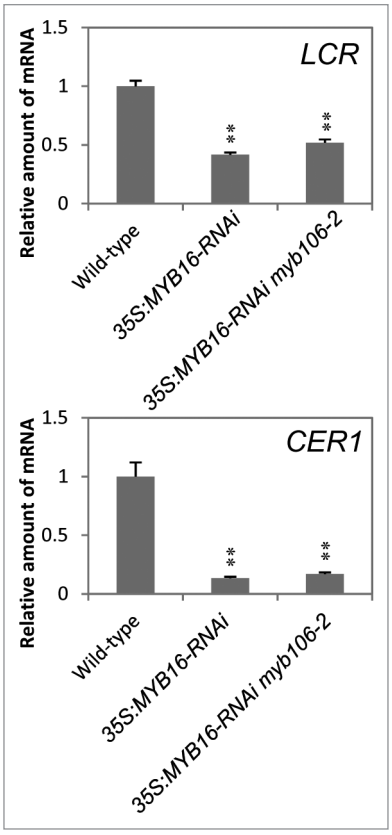

Figure 3. Expression of cuticle-related genes in transgenic plants. qRT-PCR analysis of expression of *LCR* and *CER1* in 2-week-old wild type, *35S:MYB16-RNAi* and *35S:MYB16-RNAi myb106–2* plants. Expression level in the wild type is set as 1. Error bars represent SE (n = 5). Double asterisks indicate $P < 0.01$ in the Welch *t*-test.

defects in early developmental stages, or no phenotype due to redundancy.[20,21] Cuticle development in leaves after differentiation of epidermal cells requires *LCR* and *CER1*, and other cutin and wax synthesizing enzyme and transporter genes (with or without the L1-box in their promoters).[23-26] Assessing the involvement of MIXTA-like MYB transcription factors in the regulation of these genes may provide valuable insights into the regulatory mechanism of cuticle development in vegetative organs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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