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The bHLH Transcription Factor MYC3 Interacts with the Jasmonate ZIM-Domain Proteins to Mediate Jasmonate Response in *Arabidopsis*

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ABSTRACT The *Arabidopsis* Jasmonate ZIM-domain proteins (JAZs) act as substrates of SCF^{COI1} complex to repress their downstream targets, which are essential for JA-regulated plant development and defense. The bHLH transcription factor MYC2 was found to interact with JAZs and mediate JA responses including JA-inhibitory root growth. Here, we identified another bHLH transcription factor MYC3 which directly interacted with JAZs by virtue of its N-terminal region to regulate JA responses. The transgenic plants with overexpression of *MYC3* exhibited hypersensitivity in JA-inhibitory root elongation and seedling development. The JAZ-interacting pattern and the JA-induced expression pattern of MYC3 were distinguishable from those of MYC2. We speculate that MYC3 and MYC2 may have redundant but also distinguishable functions in regulation of JA responses.

Key Words: MYC3; JAZ; COI1; Jasmonate.

INTRODUCTION

The phytohormone jasmonates (JAs) are fatty acid-derived signaling molecules, which regulate development of many plant tissues, including root growth, tuberization, fruit ripening, tendril coiling, and pollen development. JAs are also involved in plant response to diverse environmental stresses, such as wounding, water deficit, ozone exposure, pest attack, and pathogen infection (Penninckx et al., 1996; Creelman and Mullet, 1997; McConn et al., 1997; Pieterse et al., 1998; Overmyer et al., 2000; Farmer, 2001; Rao et al., 2002; Turner et al., 2002; Bu et al., 2008; Browse, 2009; Sun et al., 2009).

The *Arabidopsis* Jasmonate-ZIM-domain proteins (JAZs) consist of 12 members (Chini et al., 2007; Thines et al., 2007), serve as repressors to suppress JA responses including JA-inhibitory root growth (Chini et al., 2007; Thines et al., 2007), plant fertility (Chini et al., 2007; Thines et al., 2007), and resistance against pathogen *Pseudomonas syringae* (Thines et al., 2007). Upon perception of Jasmonate-Isoleucine, a biological-active form of JA (Thines et al., 2007), the JA receptor Coronatine Insensitive 1 (COI1) (Yan et al., 2009) recruits JAZs for ubiquitination and degradation by 26S proteasome (Chini et al., 2007; Thines et al., 2007). As a result of JAZs degradation, the JAZs-interacting transcription factors, such as MYC2, will be released to activate various JA responses (Chini et al., 2007; Browse, 2009; Chini et al., 2009).

The essential role for MYC2 in regulation of JA responses was revealed through characterization of a *jasmonate insensitive1* (*jin1*) mutant with a mutation in the at1g32640 gene encoding a basic/helix-loop-helix (bHLH) family member named MYC2 (Lorenzo et al., 2004). The *myc2/jin1* mutant exhibits insensitivity to JA-inhibitory root growth (Lorenzo et al., 2004) and shows increased resistance to *Necrotrophic* pathogens (Anderson et al., 2004; Lorenzo et al., 2004) and bacterial pathogen *Pseudomonas syringae* (Nickstadt et al., 2004; Laurie-Berry et al., 2006). MYC2 acts as a negative regulator to control pathogen defense genes like *PDF1.2*, *CHIB/PR3*, and *HEL/PR4* (Anderson et al., 2004; Lorenzo et al., 2004), as a positive regulator to mediate tolerance to oxidative stress and resistance to insects such as *Helicoverpa armigera* (Dombrecht et al., 2007). Transgenic plants with overexpression of *MYC2* are hypersensitive to JA inhibition on root growth (Lorenzo et al., 2004). To date, the bHLH transcription factor MYC2 is the only well characterized direct target for JAZ proteins in jasmonate signal pathway. In this study, we identified and characterized another bHLH transcription factor MYC3 as

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a direct target of JAZs to mediate JA-inhibitory seedling growth. We also observed a discrepancy in JAZ-interacting pattern and JA-induced expression pattern between MYC3 and MYC2.

RESULTS

MYC3 Interacts with JAZ Proteins

To identify JAZ1-interating proteins in the yeast two-hybrid system, we used JAZ1 as bait to screen *Arabidopsis* cDNA library. Putative JAZ1-interating colonies were sequenced. The *Arabidopsis* gene At5g46760, which encodes a bHLH transcription factor MYC3, was identified to interact with JAZ1.

MYC3 shows similarity to a bHLH transcription factor MYC2 (Figure 1A) (Bailey et al., 2003; Abe et al., 2003). Among the bHLH protein family, MYC2 is a well known transcription factor in the jasmonate pathway (Abe et al., 2003; Boter et al., 2004; Lorenzo et al., 2004). MYC2 is able to interact with all the 12 JAZ proteins (Browse, 2009) (Figure 1B). However, MYC3

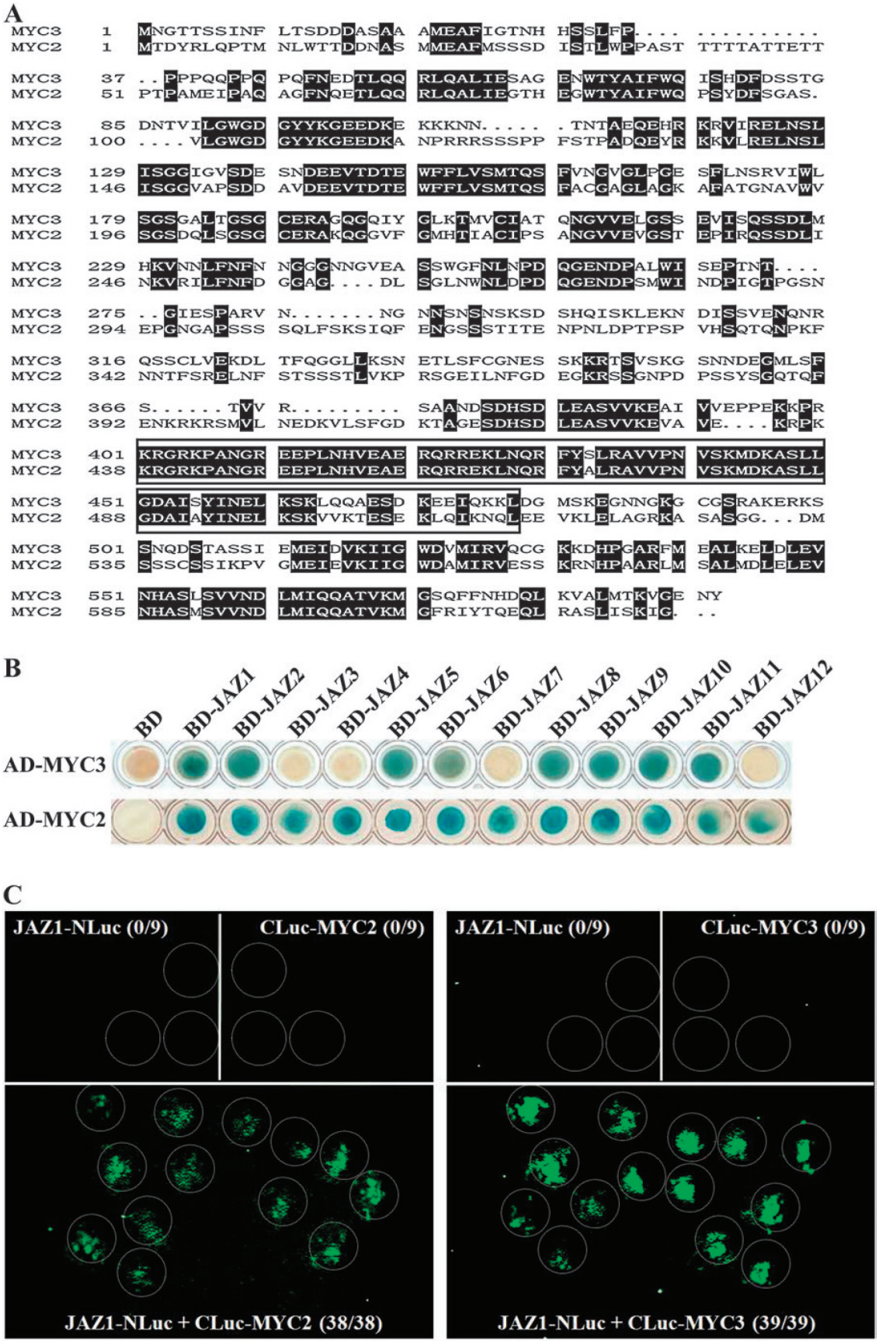

Figure 1. The Protein–Protein Interactions between MYC3 and JAZ Proteins.
(A) Amino acid sequence alignment between MYC3 and MYC2. The highlighted sequences with black color show the conservative regions. The area in the black frame is the conservative function domain of bHLH family proteins. The alignment was performed using Vector NTI software.
(B) JAZs-interacting patterns of MYC3 and MYC2 in yeast two-hybrid system (Y2H). JAZ proteins were fused with LexA DNA binding domain (BD-JAZs); MYC3 and MYC2 were fused with activating domain (AD-MYC3 and AD-MYC2), respectively. BD represents empty pLexA vector containing DNA binding domain only. Photographs were taken after 2 d of growth of yeast transformants on a 96-well plate-containing Gal/raffinose/SD/-His-Trp-Leu-Ura/X-β-Gal medium at 30°C.
(C) Luciferase Complementation Imaging Assay (LCI) shows *in vivo* interactions between JAZ1 and MYC3 or MYC2. JAZ1 was fused with the N-terminal fragment of firefly luciferase (JAZ1-NLuc) while MYC3 and MYC2 were fused with CLuc (CLuc-MYC3 and CLuc-MYC2), respectively. The indicated construct pairs were co-expressed in *Nicotiana benthamiana* leave the area (white circles) via *Agrobacterium*-mediated infiltration, whereas the single construct (JAZ-NLuc, CLuc-MYC2, or CLuc-MYC3) was infiltrated as negative control. Images were taken 60 h after infiltration. Numbers in brackets represent the number of infiltration sites in each experiment (infiltration sites with Luc complementation/total infiltrations).

showed strong interactions with eight JAZ proteins (JAZ1, JAZ2, JAZ5, JAZ6, JAZ8, JAZ9, JAZ10, and JAZ11) in yeast (Figure 1B). It would be interesting to verify the interaction affinity between MYC3 and various JAZ proteins.

We further used the firefly luciferase (Luc) complementation imaging (LCI) assay (Chen et al., 2008) to verify the interaction between MYC3 and JAZs *in planta*. MYC3 fused with C-terminal fragment of Luc (35S:CLuc-MYC3) and JAZ1 with N-terminal fragment of Luc (35S:JAZ1-NLuc) were co-expressed in *Nicotiana benthamiana* leaf via *Agrobacterium*-mediated infiltration assay. As shown in Figure 1C, similar to the positive control MYC2, MYC3 exhibited a strong interaction with JAZ1 in *N. benthamiana* leaves.

We further divided MYC3 into N-terminal region, MYC3△CT (from the first amino acid (aa) to 312 aa), and C-terminal region, MYC3△NT (164–593 aa), to test which domain in MYC3 is required for JAZs interaction. As shown in Figure 2B, JAZs showed strong interaction with MYC3△CT but not with MYC3△NT in the yeast two-hybrid system. Similar interactions of JAZs with MYC3△CT were also observed in LCI assay (data not shown). These data demonstrate that the N-terminus of MYC3 (1–163 aa) is essential for its protein–protein interaction with JAZs.

Transgenic Plants Overexpressing *MYC3* Are Hypersensitive to JA Treatment

To determine a possible role for MYC3 in regulation of JA responses, we generated transgenic plants with overexpression of *MYC3* (*MYC3–OE*). We found that *MYC3–OE1* and *MYC3–OE2*, two individual homozygous lines with increased expression level of *MYC3* by approximate 64- and seven-fold, respectively (Figure 3A), were more sensitive to JA-inhibitory root growth compared with wild-type (Col-0) (Figure 3B and 3C). As shown in Figure 3B, relative root length of wild-type seedlings, treated with 10 μM methyl jasmonate (MeJA) for 7 d, was about 39.5% of its own root length on Murashige and Skoog (MS) plate without MeJA. However, relative root lengths of *MYC3–OE1* and *MYC3–OE2* were about 27.6% and 30% of their own root length without JA treatment. As expected, *coi1-2* was insensitive to JA-inhibitory root growth.

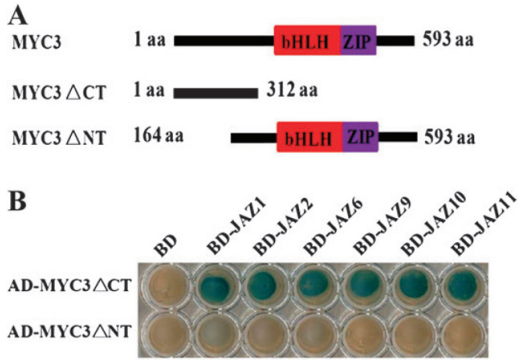

Figure 2. The N-Terminal Region of MYC3 Is Required for Its Interaction with JAZs. **(A)** Schematic diagram shows different truncations of MYC3. bHLH (red) represents the conservative basic/helix-loop-helix domain and ZIP (purple) shows the leucine-zipper motif. Numbers represent the terminal amino acid residues. **(B)** MYC3ΔCT, but not MYC3ΔNT, interacts with JAZs in the yeast two-hybrid system.

Except for the hypersensitivity in JA-inhibitory root growth, the aerial parts of *MYC3–OE1* and *MYC3–OE2* seedlings were severely inhibited by JA. When grown on MS containing MeJA, growth of *coi1-2* seedling was not affected, while wild-type seedling was obviously inhibited (Figure 3D). *MYC3–OE1* and *MYC3–OE2* were severely attenuated compared with wild-type seedlings (Figure 3D). When grown on medium containing 10 μM MeJA, cotyledons of *MYC3–OE1* and *MYC3–OE2* were unable to expand, the seedling sizes of *MYC3–OE1* and *MYC3–OE2* were much smaller than wild-type (Figure 3D). Consistent with severe inhibition on the aerial part growth, the fresh weights of *MYC3–OE1* and *MYC3–OE2* were also significantly reduced, compared with that of wild-type seedlings, in response to MeJA treatment (data not shown).

Taken together, these results demonstrate that transgenic plants with overexpression of *MYC3* are more sensitive to exogenous JA treatment.

We generated transgenic plants with down-regulation of *MYC3* using the RNAi approach. The transgenic line *MYC3–RNAi1* contained about 70% of wild-type *MYC3* (Figure 3A). We found that the root elongation of *MYC3–RNAi1* seedlings (data not shown) and the aerial part growth of *MYC3–RNAi1* seedlings (Figure 3D) were inhibited by MeJA, which were comparable to that of wild-type seedlings (Figure 3D). Consistently, the T-DNA insertion mutant of *MYC3* also did not show clear defects in JA response (Lorenzo et al., 2004). It is possible that other transcription factors, such as MYC2, might have a redundant function with MYC3. Compared with *MYC3–RNAi1* seedlings and wild-type (Col-0), *MYC3–OE1* and *MYC3–OE2* were severely inhibited by MeJA, demonstrating that the transgenic plants with overexpression of *MYC3* are undoubtedly hypersensitive to JA treatment.

Overexpression of *MYC3* Causes Change in Gene Expression Profile

To thoroughly investigate the MYC3-regulated gene expression profile, we sampled wild-type (Col-0) and *MYC3–OE1* seedlings for microarray analysis using the GeneChip *Arabidopsis* ATH1 genome array. Compared with Col-0, 46 genes in *MYC3–OE1* were significantly up-regulated (log ratio ⩾ 2) (Table 1). *MYC3* was increased (log ratio = 4.4) as expected, indicating that the microarray data possess sufficient validity for analyzing the transcriptional regulation of MYC3.

Among these up-regulated genes categorized with Expression Angler (Toufighi et al., 2005), At5g05340 is responsive to oxidative stress; At3g25760 is related to jasmonic acid biosynthetic process; At1g56650 and At4g23600 respond to salt stress; At5g13220, At4g24350, and At5g43580 are involved in response to wounding; and At3g28740, At4g23600, At5g06870, and At3g50970 are related to defense response against microbe or insect infection. These data indicate that

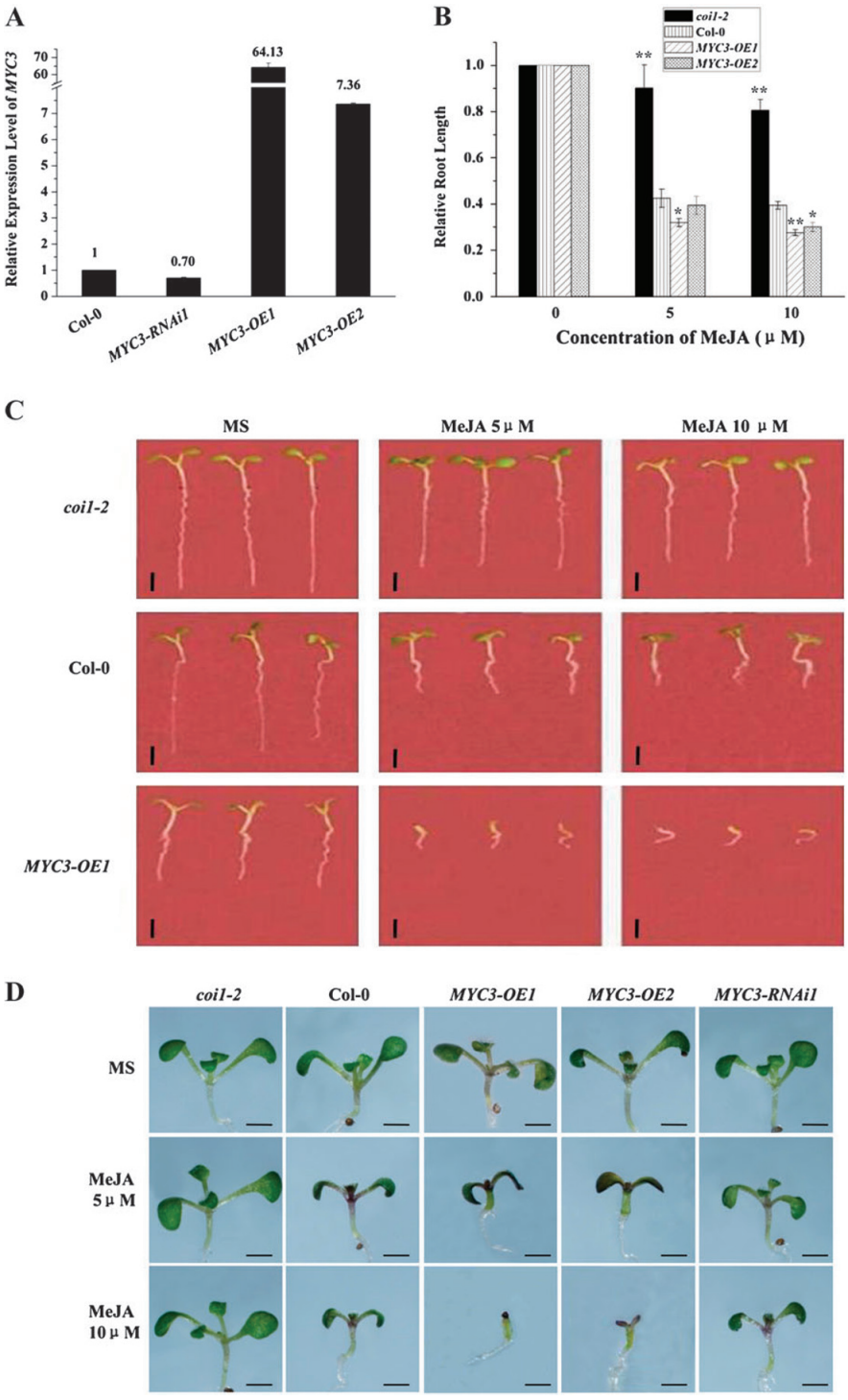

Figure 3. Transgenic Plants Overexpressing *MYC3* (*MYC3–OE*) Show Hypersensitivity in JA-Inhibitory Root Elongation and Aerial Part Growth. **(A)** Real-time PCR assay of the relative expression levels of *MYC3* in 3-week-old *MYC3–RNAi1*, *MYC3–OE1*, and *MYC3–OE2* seedlings. Numbers above the columns represent the relative *MYC3* levels in indicated genotypes. *ACTIN2* and *ACTIN7* served as normalizing controls; wild-type (Col-0) served as reference sample. **(B)** Relative root lengths of 7-day-old *coi1-2*, wild-type, *MYC3–OE1*, and *MYC3–OE2* seedlings treated with various concentrations of MeJA. Relative root lengths were shown in the percentage of their own root length on MS medium. Error bars represent SE ($n \geqslant 30$). Asterisks denote Student's *t*-test significance compared with wild-type (* $P < 0.05$, ** $P < 0.01$). **(C)** Phenotypes of 7-day-old *coi1-2*, wild-type (Col-0), and *MYC3–OE1* seedlings grown on MS plates containing indicated concentration of MeJA. Root elongation of *MYC3–OE1* seedlings were significantly inhibited by MeJA. Bars represent 5 mm. **(D)** The enlarged aerial parts of 7-day-old *coi1-2*, wild-type (Col-0), *MYC3–OE1*, *MYC3–OE2*, and *MYC3–RNAi1* seedlings grown on MS plates with indicated concentration of MeJA. Bars represent 2 mm.

MYC3 may be involved in transcriptional regulation of various stress-induced genes.

MYC3 Is a Nuclear-Localized Protein

Both MYC2 and MYC3 are bHLH transcription factors interacting with JAZs to regulate JA responses (Chini et al., 2007; Pauwels et al., 2010) (Figure 1B). We further compared their expression patterns in response to MeJA treatment. As shown in Figure 4, both *MYC3* and *MYC2* were induced by MeJA. However, *MYC2* was rapidly induced by MeJA: at the point of 0.5 h of treatment with MeJA, *MYC2* exhibited a strong increase to about 16-fold, while no induction of *MYC3* was

Table 1. Significantly Up-Regulated Transcripts in *MYC3–OE1.*

Probe set ID[a]	Gene ID	Ratio[b]	Annotation
248864_at	At5g46760	4.4	Basic helix-loop-helix (bHLH) family protein (MYC3)
262661_s_at	At1g14250	4.1	Nucleoside phosphatase family protein/GDA1/CD39 family protein
245422_at	At4g17470	3.8	Palmitoyl protein thioesterase family protein
251770_at	At3g55970	3.7	Oxidoreductase, 2OG-Fe(II) oxygenase family protein (JRG21)
249101_at	At5g43580	3.3	Predicted to encode a PR (pathogenesis-related) peptide that belongs to the PR-6 proteinase inhibitor family
254314_at	At4g22470	3.3	Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
262616_at	At1g06620	3.1	Similar to a 2-oxoglutarate-dependent dioxygenase
254150_at	At4g24350	3	Phosphorylase family protein
249971_at	At5g19110	2.8	Extracellular dermal glycoprotein-related/EDGP-related
250292_at	At5g13220	2.8	Jasmonate ZIM-domain protein 10 (JAZ10)
254163_s_at	At4g24340	2.8	Phosphorylase family protein
245244_at	At1g44350	2.7	Similar to IAA amino acid conjugate hydrolase
254232_at	At4g23600	2.7	Cystine lyase, involved in amino acid metabolism (JR2)
260408_at	At1g69880	2.7	Thioredoxin H-type 8 (ATH8)
250669_at	At5g06870	2.6	Polygalacturonase inhibiting protein (PGIP2)
257641_s_at	At3g25760	2.6	Allene oxide cyclase (AOC1)
250793_at	At5g05600	2.5	Oxidoreductase, 2OG-Fe(II) oxygenase family protein
261443_at	At1g28480	2.5	GRX480, glutaredoxin family protein, regulates protein redox state
250083_at	At5g17220	2.4	Glutathione transferase belonging to the phi class of GSTs
259975_at	At1g76470	2.4	3-beta-hydroxy-delta5-steroid dehydrogenase
245628_at	At1g56650	2.3	Putative MYB domain containing transcription factor involved in anthocyanin metabolism and radical scavenging
248432_at	At5g51390	2.3	Unknown protein
267147_at	At2g38240	2.3	Oxidoreductase, 2OG-Fe(II) oxygenase family protein
249215_at	At5g42800	2.2	Dihydroflavonol reductase (DFR)
250500_at	At5g09530	2.2	Hydroxyproline-rich glycoprotein family protein
256017_at	At1g19180	2.2	Jasmonate ZIM-domain protein 1 (JAZ1)
256589_at	At3g28740	2.2	A member of the cytochrome p450 family (CYP81D1)
263032_at	At1g23850	2.2	Unknown protein
265620_at	At2g27310	2.2	F-box family protein
246238_at	At4g36670	2.1	Putative mannitol transporter (AP22)
251438_s_at	At3g59930	2.1	A defensin-like (DEFL) family protein
252102_at	At3g50970	2.1	Belongs to the dehydrin protein family
256382_at	At1g66860	2.1	Hydrolase
258507_at	At3g06500	2.1	Beta-fructofuranosidase
262309_at	At1g70820	2.1	Putative phosphoglucomutase, glucose phosphomutase
263475_at	At2g31945	2.1	Unknown protein
266977_at	At2g39420	2.1	Esterase/lipase/thioesterase family protein
250798_at	At5g05340	2	Peroxidase, putative
252114_at	At3g51450	2	Strictosidine synthase family protein
256324_at	At1g66760	2	MATE efflux family protein
256461_s_at	At4g18440	2	Putative adenylosuccinate lyase, adenylosuccinase
259725_at	At1g61065	2	Unknown protein
261033_at	At1g17380	2	Jasmonate ZIM-domain protein 5 (JAZ5)
263680_at	At1g26930	2	Kelch repeat-containing F-box family protein
263963_at	At2g36080	2	A plant-specific B3 DNA-binding domain transcription factor
267524_at	At2g30600	2	BTB/POZ domain-containing protein; protein binding

a Representation of Affymetrix probe set on GeneChip.
b Signal ratio represents the changed fold, when compared with the normalized data of Col-0.

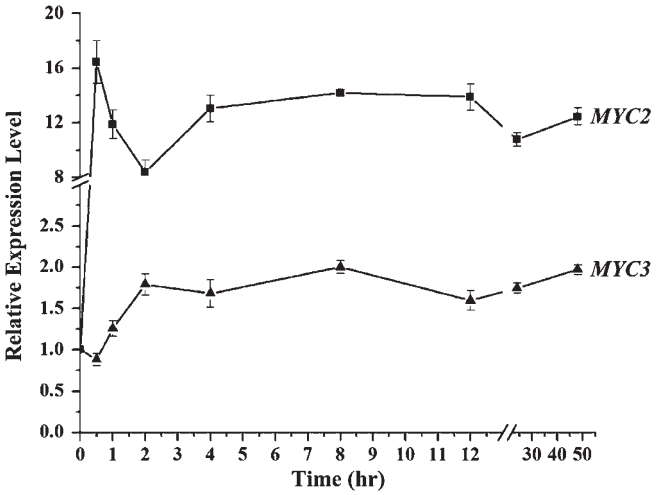

Figure 4. JA-Induced Expression Patterns of *MYC3* and *MYC2*. Real -time PCR analysis of relative expression levels of *MYC3* and *MYC2* in 3-week-old wild-type seedlings treated with 100 μM MeJA for indicated time. *EEF1* and *CDKA* served as internal controls.

detected. At the point of 2 h of treatment with MeJA, *MYC3* was induced to about two-fold, while the level of *MYC2* decreased to about eight-fold. During 2–12 h of JA treatment, the expression of *MYC3* fluctuated between 1.5 and two-fold, while *MYC2* was 8–14-fold. *MYC3* may act as a late-response factor in JA signaling as the slow increase in its expression induced by MeJA, while *MYC2* acts much earlier. In terms of JA-induced expression level, *MYC3* responds to exogenous MeJA in a mild pattern, while *MYC2* is undoubtedly a strong responsor.

To investigate the sub-cellular localization of MYC3, we made a transient expression of MYC3–GFP fusion protein in *Nicotiana benthamiana* leaves. DAPI staining indicated the nuclei in blue color at an excitation wavelength of 405 nm, while GFP was shown in green at 488 nm when inspected under a Laser Scanning Confocal Microscope. As shown in Figure 5, MYC3–GFP fusion protein was observed and co-localized with nucleus in *N. benthamiana* leaves. As a control, free GFP (35S:GFP) lacked certain localization signal and therefore gave off green signal all over the epidermal cells including nuclei (Figure 5). These data suggest that MYC3 is a nuclear-localized protein, which is very consistent with its function as a transcription factor.

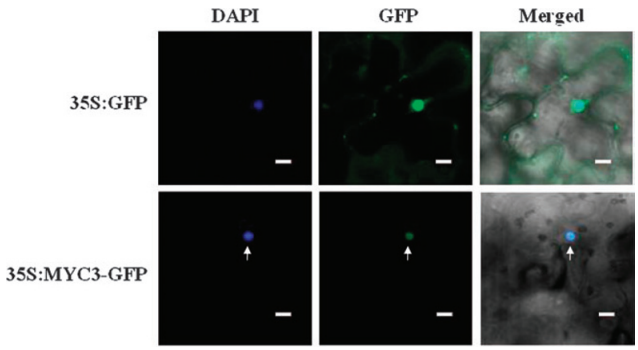

Figure 5. MYC3 Is a Nuclear-Localized Protein. DAPI (5 μg ml^{-1}) were used for nuclei staining and shown in blue color, and green for GFP signal; the excitation wavelength used for DAPI is 405 nm, while 488 nm for GFP. The free GFP (35S:GFP) expressing all over the epidermal cells served as control (top panel). MYC3 was fused with GFP (35S:MYC3–GFP) and transiently expressed in *Nicotiana benthamiana* leaves (bottom panel). Arrows indicate the localization of 35S:MYC3–GFP fusion protein in nucleus. White bars represent 10 μm.

DISCUSSION

JAZ proteins (Chini et al., 2007; Thines et al., 2007) function as substrates of SCF^{COI1} (Xie et al., 1998; Xu et al., 2002; Liu et al., 2004; Xiao et al., 2004; Ren et al., 2005) to interact with their downstream transcription factors and exert negative regulation on JA responses including plant fertility, root growth, anthocyanin accumulation, and plant defense (Franceschi and Grimes, 1991; Feys et al., 1994; Howe et al., 1996; McConn and Browse, 1996; Sanders et al., 2000; Stintzi and Browse, 2000; Ishiguro et al., 2001; Park et al., 2002; Shan et al., 2009). Here, we identified a bHLH transcription factor, named MYC3 (Bailey et al., 2003), which interacted with JAZs to mediate JA responses. MYC3 showed high similarity with the bHLH member MYC2 (Abe et al., 2003), which was previously identified as a direct target of JAZs to mediate JA responses (Chini et al., 2007). Similarly to MYC2 (Abe et al., 2003; Boter et al., 2004), the N-terminal region of MYC3 was required for its interaction with JAZs; overexpression of *MYC3* caused hypersensitivity to JA-inhibitory root elongation and aerial part growth. Therefore, *MYC3* and *MYC2* may exhibit functional redundancy in regulation of JA-regulated responses.

However, we noticed that the JAZ interaction and JA induction of *MYC3* were distinguishable from those of MYC2. MYC3 interacted with eight JAZ proteins (JAZ1, JAZ2, JAZ5, JAZ6, JAZ8, JAZ9, JAZ10, and JAZ11) (Figure 1B) whereas MYC2 interacted with all

12 JAZ proteins in yeast (Figure 1B) (Browse, 2009). *MYC2* was rapidly and significantly induced by JA (16-fold increment in response to 0.5-h JA treatment), while *MYC3* responded more slowly and mildly (two-fold increment after 2 h of JA inducement). MYC3 and MYC2 may have overlapping but also distinguishable functions in JA-regulated biological processes.

Indeed, we noticed that *dihydroflavonol reductase* (*DFR*), a 'late' anthocyanin biosynthetic gene, was obviously up-regulated in *MYC3–OE1* (Table 1) and that more anthocyanin was accumulated in *MYC3–OE1* seedlings (Supplemental Figure 1); we observed that some of the *MYC3–OE1* lines exhibited reduced fertility (Supplemental Figure 2). MYC2 was not considered to play a role in anthocyanin accumulation (Ramsay et al., 2003; Shan et al., 2009) and in regulation of plant fertility (Lorenzo et al., 2004). More genetic and physiological evidences will be required to further verify the possible role for MYC3 in regulation of plant fertility and anthocyanin accumulation.

METHODS

Plant Materials and Growth Conditions

The *Arabidopsis* mutant *coronatine insensitive1-2* (*coi1-2*) was previously described (Xu et al., 2002).

Arabidopsis seeds were surface-sterilized with 20% bleach, germinated on MS (Sigma, St Louis, MO, USA) plates (supplemented with 2% sucrose and 0.7% agar powder, adjust pH to 6.0), chilled in the dark at 4°C for 3 d, then grown in a greenhouse under a 16-h (22–24°C)/8-h (16–19°C) light/dark photoperiod.

Nicotiana benthamiana seeds were directly sown into soil and grown under the same conditions.

Identification of JAZ1-Interacting Proteins by Yeast Two-Hybrid (Y2H)

JAZ1 was fused to the DNA binding domain (BD) in pLexA vector at *Eco*RI and *Xho*I sites for screening the pB42AD (containing activating domain, AD)-based *Arabidopsis* cDNA library according to the MATCHMAKER LexA Libraries User Manual and Yeast Protocols Handbook (Clontech, Palo Alto, CA) and published methods (Xu et al., 2002); a total of 1×10^7 yeast transformants were screened on Gal/raffinose/SD/-His-Trp-Leu-Ura/X-β-Gal plates (Clontech, Palo Alto, CA).

For protein–protein interaction assay, MYC3 and MYC2 were fused with AD domain in pB42AD vector, and other JAZ proteins were fused to BD domain in pLexA vector, respectively. pB42AD-MYC3△CT and pB42AD-MYC3△NT constructs were generated by respectively fusing MYC3△CT (1–312 aa) and MYC3△NT (164–593 aa) with AD domain. Indicated construct pairs were co-transformed into yeast. Yeast transformants were independently re-suspended in 10 μl SD/-His-Trp-Ura liquid medium (Clontech, Palo Alto, CA) and 5 μl of each suspension was dropped into one well of a 96-well plate containing Gal/raffinose/SD/-His-Trp-Leu-Ura/X-β-Gal agar medium (Clontech, Palo Alto, CA). Photographs were taken after 2-d incubation in the 96-well plates at 30°C. Interaction verification was repeated at least three times.

Luciferase Complementation Imaging Assay (LCI)

JAZ1 was fused with the N-terminal fragment of firefly luciferase (Luc) in pCAMBIA-Nluc vector (35S:JAZ1-NLuc); MYC3 and MYC2 were fused with the C-terminal fragment of Luc in the pCAMBIA–CLuc vector (35S:CLuc-MYC3 and 35S:CLuc-MYC2) according to the published method (Chen et al., 2008).

Agrobacterium tumefaciens strain *GV3101* containing indicated constructs were cultured, pelleted, and re-suspended in infiltration buffer (10 mM $MgCl_2$, 10 mM MES, 200 μM Acetosyringone). For interaction detection, indicated suspensions were mixed with equal volume and co-infiltrated into fully expanded *Nicotiana benthamiana* leaves (about 3–5 weeks old) with a needleless syringe. Sixty hours after infiltration, *N. benthamiana* leaves were sprayed with luciferin solution (100 μM luciferin, 0.1% Triton X-100, 1 mM NaOH) and kept in the dark for 6 min to quench the fluorescence. Images were captured with low-light cooled CCD imaging apparatus. The CCD camera was cooled to –70°C; an exposure time of 10 min was used for all images. Andor iXon software was used for further analyzing the LCI images. This experiment was repeated at least three times.

Real-Time PCR

Total RNAs were isolated using the Trizol method. Reverse transcription was performed according to the manufacturer's protocol (TAKARA Chemicals, Shiga, Japan). Real-time PCR was performed on an ABi7500 real-time PCR system using RealMasterMix (SYBR Green I) (TAKARA Chemicals, Shiga, Japan). Reaction system and amplification protocol refer to the manufacturer's introduction. *ACTIN2* and *ACTIN7* were used as normalizing controls. Each real-time PCR reaction was repeated at least three times.

For real-time PCR analysis on JA-treated materials (Figure 4), 3-week-old *Arabidopsis* seedlings grown on MS medium were drenched respectively in 100 μM MeJA (Aldrich, Milwaukee, WI, USA) solution or water for the indicated time then collected for RNA isolation. The *CDKA* and *EEF1* were used as normalizing controls. Each treatment was repeated at least three times.

The primer pairs used in real-time PCR analysis are as follows:

MYC2: 5′-tccgagtccggttcattct-3′ and 5′-tctcgggagaaagtgttattgaa-3′;
MYC3: 5′-aggttgggatgtgatgatacg-3′ and 5′-aacctagcaccgggatgat-3′;
CDKA: 5′-attgcgtattgccactctcatagg-3′ and 5′-tcctgacagggataccgaatgc-3′;
EEF1: 5′-ctggaggttttgaggctggtat-3′ and 5′-ccaagggtgaaagcaagaaga-3′;
ACTIN2: 5′-ccgctctttctttccaagc-3′ and 5′-ccggtaccattgtcacacac-3′;

ACTIN7: 5′-cgctgcttctcgaatcttct-3′ and 5′-ccattccagttccattgtca-3′.

Generation of Transgenic Plants with Overexpression (*MYC3–OE*) or Down-Regulation (*MYC3–RNAi*) of *MYC3*

Full-length cDNA of *Arabidopsis* gene *MYC3* was cloned into pCXSN vector (Chen et al., 2009) to generate the overexpression construct (*MYC3–OE*) driven by the cauliflower mosaic virus (CaMV) 35S promoter. Primers used for cDNA amplification were 5′-cgacatgaacggcacaacatcatc-3′ and 5′-tagtatagttttctccgactttcg-3′.

The down-regulation construct (*MYC3–RNAi*) was generated by overlapping PCR. The target fragment of *MYC3* was amplified with primers 5′-atgaacggcacaacatcatc-3′ and 5′-ctggagcggcaaacacctctcctttgtagtaaccatc-3′, and the stuffer sequence fragment, a section of *beta-glucuronidase* (*GUS*), was amplified with primers 5′-gtgtttgccgctccagatctacccgcttc-3′ and 5′-gtgtttgccgctccagtaatcgcctgtaag-3′. The two fragments were fused together as an inverted-repeat cassette through amplification with the forward primer of the target fragment; the product fragment was cloned into the pCXSN vector (Chen et al., 2009) under the control of the 35S promoter.

The *MYC3–RNAi* and *MYC3–OE* constructs were transformed into wild-type (Col-0) plants according to the *Agrobacterium*-mediated flower dip method (Clough and Bent, 1998; Zhang et al., 2006). T2 seeds from each of the selected transgenic plants were sown on MS medium containing hygromycin (30 μg l^{-1}), and the homozygous lines were selected. Homozygous T3 plants were examined for *MYC3* level by real-time PCR. Four homozygous lines with elevated expression of *MYC3* (*MYC3–OE1*, *2*, *3*, and *4*) were identified and used for further analysis, and similar phenotypes were observed in these four homozygous lines.

For *MYC3–RNAi* constructs, more than 30 transgenic lines were examined; only a slight reduction in *MYC3* was observed. Three lines (*MYC3–RNAi1*, *2*, and *3*), with about 30, 20, and 10% reduction in *MYC3*, respectively, were used for further analysis, and no obvious phenotype was observed in these lines, compared with wild-type (Col-0).

The data from *MYC3–OE1*, *MYC3–OE2*, and *MYC3–RNAi1* were representatively shown in figures.

Root Length Measurement

Seven-day-old seedlings grown on MS plates with various concentrations of MeJA (0, 5, and 10 μM) were stretched out on plates containing 1% agarose for photographing. Root lengths were determined using Digimizer software ($n \geqslant 30$). The measurements were repeated at least three times.

Affymetrix Microarray Assay

Three-week-old plants grown in soil were sampled for RNA isolation. For each sample, 8 μg total RNA was used to produce biotin-labeled cRNA targets. cRNA fragmentation, hybridization, washing, staining, and scanning were performed according to Affymetrix ATH1 Gene Chip Protocol (Eukaryotic Target Preparation).

Data normalization and comparative analysis were performed with Affymetrix GCOS software (Liu et al., 2010). Changes in the gene transcription level detected by each probe were calculated as *P*-values, which presented the differences between target samples and wild-type (Col-0) (Wang et al., 2008).

Microsoft Excel was used to extract and manage the microarray data. The Affymetrix company web site www.affymetrix.com/estore/analysis/index was visited for inquiring gene information on each chip.

Laser Scanning Confocal Microscopy

MYC3 was fused with GFP in the pCXSN–DG vector driven by the 35S promoter; the resulting construct 35S:MYC3–GFP was introduced into *Agrobacterium tumefaciens* for *Nicotiana benthamiana* infiltration. Sixty hours after infiltration, *N. benthamiana* leaves were cut off into small squares and immersed into PBS buffer (1.4 M NaCl, 100 mM Na_2HPO_4, 30 mM KCl, 20 mM KH_2PO_4) containing 5 μg ml^{-1} DAPI for staining of the nuclei. Slides carrying the drenched leaf squares were observed under a Laser Scanning Confocal Microscope (Carl Zeiss, LSM710). The excitation wavelength used for GFP was 488 nm and 405 nm for DAPI. Images were merged using ZEN software (Carl Zeiss, 2009 light edition). This experiment was repeated at least three times.

Accession Numbers

The Arabidopsis Genome Initiative numbers for genes mentioned in this article are as follows: JAZ1 (At1g19180), JAZ2 (At1g74950), JAZ3 (At3g17860), JAZ4 (At1g48500), JAZ5 (At1G17380), JAZ6 (At1g72450), JAZ7 (At2g34600), JAZ8 (At1g30135), JAZ9 (At1g70700), JAZ10 (At5g13220), JAZ11 (At3g43440), JAZ12 (At5g20900), MYC2 (At1g32640), MYC3 (At5g46760), COI1 (At2g39940), EEF1 (At5G60390), CDKA (At3G48750), ACTIN2 (AtG18780), ACTIN7 (AtG09810).

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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