Journal of Experimental Botany, Vol. 62, No. 6, pp. 2143–2154, 2011
doi:10.1093/jxb/erq408 Advance Access publication 14 February, 2011
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Characterization of JAZ-interacting bHLH transcription factors that regulate jasmonate responses in *Arabidopsis*

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Received 29 August 2010; Revised 19 November 2010; Accepted 23 November 2010

Abstract

The plant hormone jasmonate (JA) plays important roles in the regulation of plant defence and development. JASMONATE ZIM-DOMAIN (JAZ) proteins inhibit transcription factors that regulate early JA-responsive genes, and JA-induced degradation of JAZ proteins thus allows expression of these response genes. To date, MYC2 is the only transcription factor known to interact directly with JAZ proteins and regulate early JA responses, but the phenotype of *myc2* mutants suggests that other transcription factors also activate JA responses. To identify JAZ1-interacting proteins, a yeast two-hybrid screen of an *Arabidopsis* cDNA library was performed. Two basic helix-loop-helix (bHLH) proteins, MYC3 and MYC4, were identified. MYC3 and MYC4 share high sequence similarity with MYC2, suggesting they may have similar biological functions. MYC3 and MYC4 interact not only with JAZ1 but also with other JAZ proteins (JAZ3 and JAZ9) in both yeast two-hybrid and pull-down assays. MYC2, MYC3, and MYC4 were all capable of inducing expression of *JAZ::GUS* reporter constructs following transfection of carrot protoplasts. Although *myc3* and *myc4* loss-of-function mutants showed no phenotype, transgenic plants overexpressing *MYC3* and *MYC4* had higher levels of anthocyanin compared to the wild-type plants. In addition, roots of *MYC3* overexpression plants were hypersensitive to JA. Quantitative real-time RT-PCR expression analysis of nine JA-responsive genes revealed that eight of them were induced in *MYC3* and *MYC4* overexpression plants, except for a pathogen-responsive gene, *PDF1.2*. Similar to MYC2, MYC4 negatively regulates expression of *PDF1.2*. Together, these results suggest that MYC3 and MYC4 are JAZ-interacting transcription factors that regulate JA responses.

Key words: *Arabidopsis*, bHLH transcription factor, jasmonate, JAZ protein, MYC4 protein.

Introduction

As sessile organisms, plants utilize small molecules, phytohormones, to regulate their growth in response to environmental changes. Among these molecules are several forms of jasmonate (JA), including the active form of the JA hormone, (3R, 7S) jasmonoyl-isoleucine (JA-Ile) (Fonseca *et al.*, 2009). JA, a stress hormone, is involved in defence against insects and pathogens (Kessler and Baldwin, 2002; Turner *et al.*, 2002; Weber, 2002; Browse and Howe, 2008), responses to ultraviolet radiation (Conconi *et al.*, 1996), ozone (Rao *et al.*, 2000), drought (Fujita *et al.*, 2004), and other abiotic stresses (Moons, 2005; Ma *et al.*, 2006). JA can also induce the production of secondary metabolites, including alkaloids, anthocyanins, and terpenoid compounds (Feys *et al.*, 1994; Devoto *et al.*, 2005). Moreover, JA is an important regulator of plant growth and development, affecting root growth (Staswick *et al.*, 1992; McConn and Browse, 1996; Stintzi and Browse, 2000; Xiao *et al.*, 2004; Yoshida *et al.*, 2009), senescence (Xiao *et al.*, 2004), trichome patterning (Yoshida *et al.*, 2009), and reproductive development (Feys *et al.*, 1994; McConn and Browse, 1996; Stintzi and Browse, 2000). *Arabidopsis* mutants defective in JA synthesis or perception are male sterile (Feys *et al.*, 1994; McConn and Browse, 1996; Thines *et al.*, 2007).

Despite the diverse roles of JA, all these JA-mediated responses are dependent on the F-box protein, COI1. The *coi1* mutants were identified by screening *Arabidopsis* seedlings for resistance to a phytotoxin, coronatine (Feys *et al.*, 1994), which resembles the active jasmonate, JA-Ile. In *Arabidopsis*, COI1 has been shown to associate physically with SKP1 and CULLIN to form the SCF^{COI1} complex, a class of E3 ubiquitin ligases in the ubiquitin/26S proteasome pathway (Xie *et al.*, 1998; Xu *et al.*, 2002; Xiao *et al.*, 2004). Although the severe JA-insensitive phenotype of *coi1* mutants indicates that SCF^{COI1}-mediated protein ubiquitination is pivotal for the activation of JA responses (Xie *et al.*, 1998), it is only recently that the substrates of SCF^{COI1}, the JASMONATE ZIM-DOMAIN (JAZ) repressor proteins, were discovered (Chini *et al.*, 2007; Thines *et al.*, 2007; Yan *et al.*, 2007). The emerging model for JA perception is that, in the presence of JA-Ile, SCF^{COI1} binds JAZ proteins and catalyses the attachment of ubiquitin moieties to them. The ubiquitinated JAZ proteins are then degraded by the 26S proteasome so that JAZ-interacting transcription factors are derepressed, thus allowing expression of early response genes including JA-responsive transcription factors, and the *JAZ* genes themselves. The negative feedback loop created by elevating the expression of the repressors then attenuates the JA signal (Browse, 2009; Chini *et al.*, 2009). Yeast two-hybrid and pull-down assays demonstrated that JA-Ile, but not most other JA derivatives, can promote the COI1–JAZ interaction and that the SCF^{COI1}–JAZ complex is the perception site for JA-Ile (Thines *et al.*, 2007; Katsir *et al.*, 2008; Melotto *et al.*, 2008; Yan *et al.*, 2009). This model has received additional confirmation from the crystal structure of a JAZ1 peptide (amino acids 200–220) bound to COI1 in the presence of JA-Ile (Sheard *et al.*, 2010).

Although our understanding of the molecular mechanism for JA perception has improved over recent years, some key components are still missing. To date, there are 12 JAZ repressor proteins, but MYC2 is the only transcription factor known to interact directly with JAZ proteins and activate transcription of the early JA-responsive genes that encode downstream transcription factors and several JAZ proteins (Lorenzo *et al.*, 2004; Mandaokar *et al.*, 2006; Chini *et al.*, 2007, 2009; Melotto *et al.*, 2008; Chung and Howe, 2009). MYC2 was identified as a key regulator of JA signalling through two independent screens for JA-insensitive mutants and corresponds to the mutant loci *methyl jasmonate-insensitive1* (*jin1*) and *jasmonate-insensitive1* (*jai1*) (Berger *et al.*, 1996; Boter *et al.*, 2004; Lorenzo *et al.*, 2004). Both mutants exhibited reduced sensitivity to JA-mediated root growth inhibition, a typical JA-resistant phenotype. Interestingly, MYC2 differentially regulates two branches of JA-mediated responses; it positively regulates wound-responsive genes, including *VSP2, LOX3*, and *TAT*, but represses the expression of pathogen-responsive genes such as *PR4*, *PR1*, and *PDF1.2* (Lorenzo *et al.*, 2004). These complex interactions are co-mediated by the ethylene-responsive transcription factor ERF1 (Lorenzo *et al.*, 2003). MYC2 has also been proposed to have a role in abscisic acid signalling (Abe *et al.*, 2003).

MYC2 contains a basic helix-loop-helix (bHLH) domain which is responsible for DNA binding and the formation of homodimers and/or heterodimers between bHLH proteins (Ferre-D'Amare *et al.*, 1994; Shimizu *et al.*, 1997; Toledo-Ortiz *et al.*, 2003). As a MYC-related protein, MYC2 has a partially conserved leucine zipper (ZIP) motif adjacent to the bHLH domain, which may stabilize protein dimers (Heim *et al.*, 2003). In *Arabidopsis*, there are 133 *bHLH* genes, constituting one of the largest families of transcription factors (Heim *et al.*, 2003). Based on the amino acid sequence similarity both in and outside of the bHLH domain, *Arabidopsis* bHLH proteins are divided into 12 major groups and a total of 25 subgroups (Heim *et al.*, 2003). MYC2 is a member of the subgroup IIIe, along with MYC3 (At5g46760), MYC4 (At4g17880), and At5g46830 (bHLH28), which we have designated MYC5 (Abe *et al.*, 2003; Heim *et al.*, 2003).

In contrast to severe JA-synthesis and JA-perception mutants, *myc2* plants are male-fertile, and this indicates that there are other JAZ-interacting transcription factors that activate the expression of primary JA-responsive genes following JA-mediated removal of JAZ repressors. A yeast two-hybrid screen of an *Arabidopsis* cDNA library that used JAZ1 as bait and that identified MYC3 and MYC4 as JAZ1-interacting proteins is described here. MYC3 and MYC4 interact not only with JAZ1, but also with JAZ3 and JAZ9 proteins in both pull-down and yeast two-hybrid assays. Although *myc3* and *myc4* loss-of-function mutants did not show an evident JA-related phenotype, overexpression of cDNAs encoding MYC3 and MYC4 proteins resulted in anthocyanin accumulation and higher transcript levels of JA-responsive genes compared to wild-type plants. In addition, similar to plants overexpressing *MYC2*, *MYC3* overexpression plants were hypersensitive to JA-mediated root growth inhibition. Based on these results, it is concluded that MYC3 and MYC4 are JAZ-interacting transcription factors that act together with MYC2 to activate JA-responses.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana plants were grown in soil under a 16 h light cycle, with a light intensity of 100 μE m^{-2} s^{-1}, at 22 °C. In this study, wild-type (WT) refers to Columbia (Col-0). In experiments where seedlings were used, surface-sterilized seeds were grown on agar plates containing half-strength Murashige–Skoog salts (Sigma Co., MO) with 1% (w/v) sucrose.

Yeast two-hybrid screen and assays

HybriZAP®-2.1 Two-Hybrid System (Stratagene, CA) was used in the study. The *JAZ1* coding sequence was cloned into the Y2H bait vector pBD-GAL4 Cam resulting in a Gal4 DBD-JAZ1 fusion protein. This gene construct was transformed into *Saccharomyces cerevisiae* strain YRG-2 using the one-step transformation method (Chen *et al.*, 1992). Transformants were selected on SD medium with –Trp dropout supplement (Clontech, CA).

The screening procedure for isolation of *Arabidopsis* proteins interacting with JAZ1 protein was performed according to the manufacturer's protocol (Stratagene, La Jolla, CA). Briefly, the yeast strain expressing JAZ1 bait protein was transformed with a pooled *Arabidopsis* cDNA library (Du and Poovaiah, 2004) by the lithium acetate method. To screen the cDNA library, positive clones were initially selected for tryptophan, leucine, and histidine prototrophy and then assayed for lacZ activity using a filter β-galactosidase assay with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Plasmids were isolated from positive yeast clones and transformed into *Escherichia coli* DH10B for analysis of insert and sequencing.

Yeast two-hybrid assays were performed with HybriZAP®-2.1 Two-Hybrid System (Stratagene, CA). The coding sequences of *JAZ1*, *JAZ3*, and *JAZ9* genes were cloned into the bait vector to generate fusions with a Gal4 DNA binding domain (BD) and cotransformed into the yeast strain YRG-2 in combination with the prey vector containing fusions between the coding sequences of *MYC2*, *MYC3*, *MYC4*, and *MYC5* and the Gal4 activation domain (AD). Interactions between these constructs were determined by growth of transformants on SD medium with –Trp/–Leu/–His dropout supplement and the LacZ filter-lift assay as described by the manufacturer (Stratagene, CA).

In vitro *transcription/translation and pull-down assays*

The coding sequences of *MYC2, MYC3, MYC4*, and *MYC5* were cloned into the pTNT™ vector (Promega, WI) and used as the template to generate [^{35}S]Met-labelled proteins, using TNT® Coupled Reticulocyte Lysate System (Promega, WI) according to the manufacturer's instructions. Each pull-down assay contained 20 µl of *in vitro* translation products and 10 µg purified MBP–JAZ–His fusion protein in a total volume of 300 µl. The fusion protein was first immobilized on Dynabeads® TALON™ superparamagnetic beads (Invitrogen, CA) in incubation buffer [50 mM TRIS-Cl, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 10% (v/v) glycerol, 0.05% (v/v) Tween-20, 25 mM imidazole, and the EDTA-free protease inhibitor cocktail (Sigma Aldrich, MO)] for 1 h at 4 °C. Following the addition of [^{35}S]MYC protein, the reaction was incubated for an additional 1 h at 4 °C. The beads were recovered by magnetic force with a Dynalmagnet and then washed four times at 4 C for 5 min each with 800 µl of incubation buffer. The beads were eluted with 30 µl of elution buffer containing 250 mM imidazole. Bound proteins were separated by SDS-PAGE and visualized by PhosphorImager 445 SI system (Molecular Dynamics, UK).

Transient expression and localization in onion epidermal cells

The coding sequences of *MYC3* and *MYC4* were cloned into the pENTR-D/TOPO vector (Invitrogen, CA). Transient expression vectors producing GFP-MYC3 and GFP–MYC4 fusion protein were created by combining pENTR clones and the destination vector p2FGW7 (Karimi *et al.*, 2002), in which the GFP–MYC fusions were expressed under the control of the cauliflower mosaic virus 35S promoter. Tungsten particles of 1.1 µm in diameter were soaked in 70% ethanol and washed in water. The particles were then suspended in 50% glycerol with a concentration of 60 mg ml^{-1}. After the addition of 10 µl of 2.5 M $CaCl_2$ and 4 µl of 0.1 M spermidine, 1 µg of DNA was precipitated on 0.5 mg tungsten particles at room temperature for 3 min with continuous vortexing. The pellet was washed in 70% ethanol and then in 100% ethanol before being resuspended in 10 µl of 100% ethanol. Aliquots of tungsten particles coated with DNA were loaded on to macrocarriers and used to transform onion epidermal cells. Bombardments were performed using the Biolistic PDS-1000/He Particle Delivery System (Bio-Rad, CA) at a helium pressure of 1300 psi. Each sample was bombarded three times, then samples were incubated at room temperature for 16–18 h. A single layer of epidermal cells was peeled from the onion scale leaves and examined with an Olympus IX70 microscope (Olympus, PA). Nuclei were stained by adding 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining solution (Molecular Probes, Eugene, OR) to the onion cells. Images were taken using a Coolpix 990 digital camera (Nikon, Japan).

Reporter and effector constructs

DNA fragments of 3576 bp, 2122 bp, 1663 bp, 3501 bp, 2474 bp, and 1760 bp containing *JAZ1*, *JAZ2*, *JAZ5*, *JAZ6*, *JAZ7*, and *JAZ9* promoters, respectively, their 5′-UTR region and encoding for the first three amino acids of JAZ were cloned via Gateway reactions into a plant binary plasmid pMDC162 (Curtis and Grossniklaus, 2003) generating the reporter construct *JAZ::GUS*. The reporter plasmid was isolated from the *E. coli* strain ER2925 which is Dam^{-}/Dcm^{-} (New England Biolabs, MA). The *35S::LUC* reporter construct has been described previously (Liu *et al.*, 1994). The effector constructs *35S::bHLH* were generated by cloning *bHLH* coding region via Gateway reactions into a plant transient expression plasmid p2GWF7 (Karimi *et al.*, 2002).

Carrot protoplasts isolation and transfection

Isolation of protoplasts from carrot (*Daucus carota*) suspension culture cells, transfections, and GUS assays have been described previously (Liu *et al.*, 1994; Tiwari *et al.*, 2006). Reporter and effector plasmids used for protoplast transfection were prepared using Wizard Plasmid Midi kit (Promega, WI). Ten micrograms of each effector and reporter plasmids were used in each transfection assay. β-Glucuronidase (GUS) activities were standardized by cotransfections with a cauliflower mosaic virus (CaMV) *35S::LUC* reporter gene as described by Liu *et al.* (1994). GUS and LUC activities were measured using a luminescence spectrometer (Perkin Elmer LS-50B, MA). For measuring GUS activity, an excitation wavelength of 365 nm and an emission wavelength of 455 nm were used. For measuring LUC activity, a Luciferase Assay System (Promega, WI) and a luminometer with an emission wavelength of 550 nm and a photomultiplier gain of 775 V was used. Measured LUC activities were used to correct for variation in transfection efficiency as described by Liu *et al.* (1994). Each transfection assay was performed in triplicate, and two independent transfection assays were performed for each experiment, as has been described by Tiwari *et al.* (2004).

Genotyping of T-DNA mutants

The T-DNA insertion lines were obtained through the *Arabidopsis* Biological Resource Center (Alonso *et al.*, 2003) and GABI-Kat (Rosso *et al.*, 2003). PCR was performed by using the T-DNA left-border primer (LBa1 for SALK lines, 5′-TGGTTCACG-TAGTGGGCCATCG-3′ and GABI_08409 for Gabi_Kat lines, ATATTGACCATCATACTCATTGC) and genomic sequences that correspond to the flanking DNA to identify the mutant allele or both of the genomic primers to identify the wild-type allele. Primers specific for the *MYC3* gene were 048028-LP, 5′-AAAAATTGAACGGAAGTTGCTATG-3′; 048028-RP, 5′-AGAGAGATGAGTGGTGGTTTGTTC-3′; 012763-LP, 5′-AAAGATGATTGGAGAAAGAAAACAC-3′; 012763-RP, 5′-CGAGAGTTTAAGAAAGATTCTCCG-3′; 445B11-LP, 5′-CCCATTTACAACCACTTATTTTCC-3′; and 445B11-RP, 5′-GTTGAATCATGTTGAAGCAGAGAG-3′. Primers specific for the *MYC4* gene were 491E10-LP, 5′-AACTTTGATGTAA-AAGGCTCCTTG-3′ and 491E10-RP, 5′-TTGTAACCCA-TAAATCTGACCTTG-3′. Homozygous mutant plants were then used in RT-PCR assays to test the transcript levels of the corresponding gene. Primers used for RT-PCR were *MYC3* RT-For, 5′-ATGAACGGCACAACATCATCAAT-3′; *MYC3* RT-Rev 5′-TCAATAGTTTTCTCCGACTTTCG-3′; *MYC4* RT-For, 5′-ATGTCTCCGACGAATGTTCAAGT-3′; *MYC4* RT-Rev, 5′-GCTGACTTCAATTCATGGACATTC-3′.

Generation of overexpression plants

The coding sequences of *MYC2, MYC3*, and *MYC4* genes were amplified by RT-PCR with the following primers, which incorporated *Xma*I or *Cla*I restriction sites, MYC2 cDNA-Xma-F, 5′-TCCCCCCGGGACTACGAAGACTTTCTCCTATCTC-3′; MYC2 cDNA-Cla-R, 5′-CCATCGATCAGTAACTAACTCATATTACTCAT-3′; MYC3 CDS-Xma-F, 5′-TCCCCCCGGGATGAACGGCACAACATCATCAATC-3′; MYC3 CDS-Cla-R, 5′-CCATCGATTCAATAGTTTTCTCCGACTTTCGT-3′; MYC4 cDNA-Xma-F, 5′-TCCCCCCGGGCCCGAAACAATCAAACCAAACACA-3′ and MYC4 cDNA-Cla-R, 5′-CCATCGATAGTCCCATTTGTCTTATTTCTAAC-3′. The resulting PCR products were cleaved with *Cla*I and *Xma*I and cloned into pART7 vector, followed by digesting with *Not*I. The cassette containing the *CaMV 35S* promoter, the coding sequence of the *MYC* gene, and the *ocs* terminator was then inserted into a binary vector pBART (Stintzi and Browse, 2000). The resulting plasmids were introduced into *Agrobacterium tumefaciens* (GV3101). Wild-type plants were transformed using the floral dip method (Clough and Bent, 1998). Seeds from these plants were selected on soil containing diluted Finale BASTA (120 mg l^{-1}).

qRT-PCR analysis

Polymerase chain reactions were performed with an Mx3005P Real-Time PCR System (Stratagene, CA), using SYBR® Green to monitor dsDNA synthesis. Reactions contained 10 µl 2× SYBR® Green Master Mix reagent (Invitrogen, CA), 1 µl of 1:4 diluted reverse transcription reaction, and 150 nM of each gene-specific primer in a final volume of 20 µl. The following standard thermal profile was used for all PCRs: 50 °C for 2 min; 95 °C for 2 min; 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. Data were analysed using Mx3005P system software (Stratagene, CA). All amplification plots were analysed with an R_n threshold of 0.2 to obtain C_T (threshold cycle) values. In order to compare data from different PCR runs or cDNA samples, C_T values for genes were normalized to the C_T value of *TUB2*, which was a housekeeping gene included in each PCR run. The sequences of the primer pairs used were JAZ1 qPCR-For, 5′-AGCTTCACTTCACCGGTTCTTGGA-3′; JAZ1 qPCR-Rev, 5′-TCTTGTCTTGAAGCAACGTCGTCA-3′; JAZ3 qPCR-For, 5′-TGTAATGGCTCCAACAGTGGCATTAC-3′; JAZ3 qPCR-Rev, 5′-ATTCAGACATTGATCTGCGACAATCTGT-3′; JAZ6 qPCR-For, 5′-TCATCTTCCTCCCAAGCCAGAGAT-3′; JAZ6 qPCR-Rev, 5′-ACTAGAAACGTGAACTCGATCGTGCAT-3′; JAZ7 qPCR-For, 5′-TTCGGATCCTCCAACAATCCCA-3′; JAZ7 qPCR-Rev, 5′-TCAAGACAATTGGATTATTATGTTACAGT-3′; JAZ10 qPCR-For, 5′-TCGCAAGGAGAAAGTCACTGCAAC-3′; JAZ10 qPCR-Rev, 5′-CGATTTAGCAACGACGAAGAAGGC-3′; PDF1.2 qPCR-For, 5′-TGTTCTCTTTGCTGCTTTCGACGC-3′; PDF1.2 qPCR-Rev, 5′-TGTGTGCTGGGAAGACATAGTTGC-3′; TAT3 qPCR-For, 5′-AAGCTGAAGGCCGAGGATGTGTAT-3′; TAT3 qPCR-Rev, 5′-TCCCGGCCTTGGAAGTAGAATGTT-3′; VSP2 qPCR-For, 5′-CAAAATATGGATACGGGACA-3′; VSP2 qPCR-Rev, 5′- ATTGCCAACGATGTTGTATC-3′; LOX3 qPCR-For, 5′-CGGATAGAGAAAGAGATTGAGAAAAGGAAC-3′; LOX3 qPCR-Rev, 5′-AGGTACACCTCTACACGTAACACCAGGC-3′; TUB2 qPCR-For, 5′-ACTGTCTCCAAGGGTTCCAGGTTT-3′; TUB2 qPCR-Rev, 5′-ACCGAGAAGGTAAGCATCATGCGA-3′.

Anthocyanin quantification

Extraction of anthocyanins from 10-d-old *Arabidopsis* seedlings was performed following the protocols of Mehrtens *et al.* (2005) with minor modifications. One millilitre of acidic methanol (1% HCl, w/v) was added to about 200 mg of fresh plant material. Samples were incubated for 18 h at room temperature under moderate shaking. Plant material was sedimented by centrifugation at 14 000 rpm for 2 min at room temperature and 400 µl of the supernatant was added to 600 µl of acidic methanol. Absorption of the extracts at 530 nm and 657 nm wavelength was determined spectrophotometrically. Quantification of anthocyanins was performed using the following equation: $Q_{\text{Anthocyanins}}=(A_{530}-0.25\times A_{657})\times M^{-1}$, where $Q_{\text{Anthocyanins}}$ is the amount of anthocyanins, A_{530} and A_{657} is the absorption at the indicated wavelengths, and M is the fresh weight, in grams, of the plant material used for extraction. All samples were measured as replicates in two independent biological replicates.

Root growth assay

After incubation at 4 °C for 2–3 d, surface-sterilized seeds were sown on agar plates containing 1% (w/v) sucrose and different concentrations of JA (as the methyl ester) (Bedoukian, CT) as indicated. Plates were then placed vertically in a culture chamber and grown for 10 d at 23 °C under 16/8 h fluorescent light/dark cycles. Root lengths were measured, and JA treatments were expressed as percentages compared to the untreated control for each genotype.

Results

A yeast two-hybrid screen identifies MYC3 and MYC4 as JAZ1-interacting proteins

Our yeast two-hybrid screen was conducted using the Hybri ZAP 2.1 System. The full-length coding sequence of JAZ1 was inserted into the pBD-GAL4 Cam vector to construct a bait plasmid encoding a fusion protein with the DNA-binding domain of Gal4p. This bait plasmid was transformed into the yeast strain YRG-2 containing both *lacZ* and *HIS3* reporter genes. After confirming that JAZ1 was incapable of inducing expression of the reporters in the absence of an interacting protein, we introduced a library of *Arabidopsis* cDNAs encoding C-terminal fusion proteins with the Gal4p activation domain into the yeast strain containing the JAZ1 bait plasmid.

A screen of approximately 1.6×10^5 yeast transformants resulted in the isolation of 44 positive colonies as determined by *lacZ* staining. Sequencing of the plasmids in these clones indicated that 20 corresponded to At4g28910 encoding the JAZ corepressor, NINJA (Pauwels *et al.*, 2010), and nine to At3g02540, a member of the ubiquitin gene family (see Supplementary Table S1 at *JXB* online). Two clones encoded MYC3 (At5g46760) and two encoded MYC4 (At4g17880). MYC3 and MYC4 belong to the bHLH subgroup IIIe which also consists of MYC2 and MYC5/bHLH28 (Heim *et al.*, 2003). These four bHLH proteins exhibit high sequence similarity (56%), especially in the N-terminal conserved regions and the bHLH domains that are nearly identical (Fig. 1A). Although MYC2 is known to interact with JAZ proteins (Chini *et al.*, 2007), it was not identified in our screen, possibly because it was poorly represented in the particular cDNA library that was used.

In order to verify the specificity of interactions of MYC3 and MYC4 with JAZ1 identified from the Y2H screen, the full-length MYC3 and MYC4 proteins (prey) were coexpressed with JAZ1 (bait) in yeast and the interaction

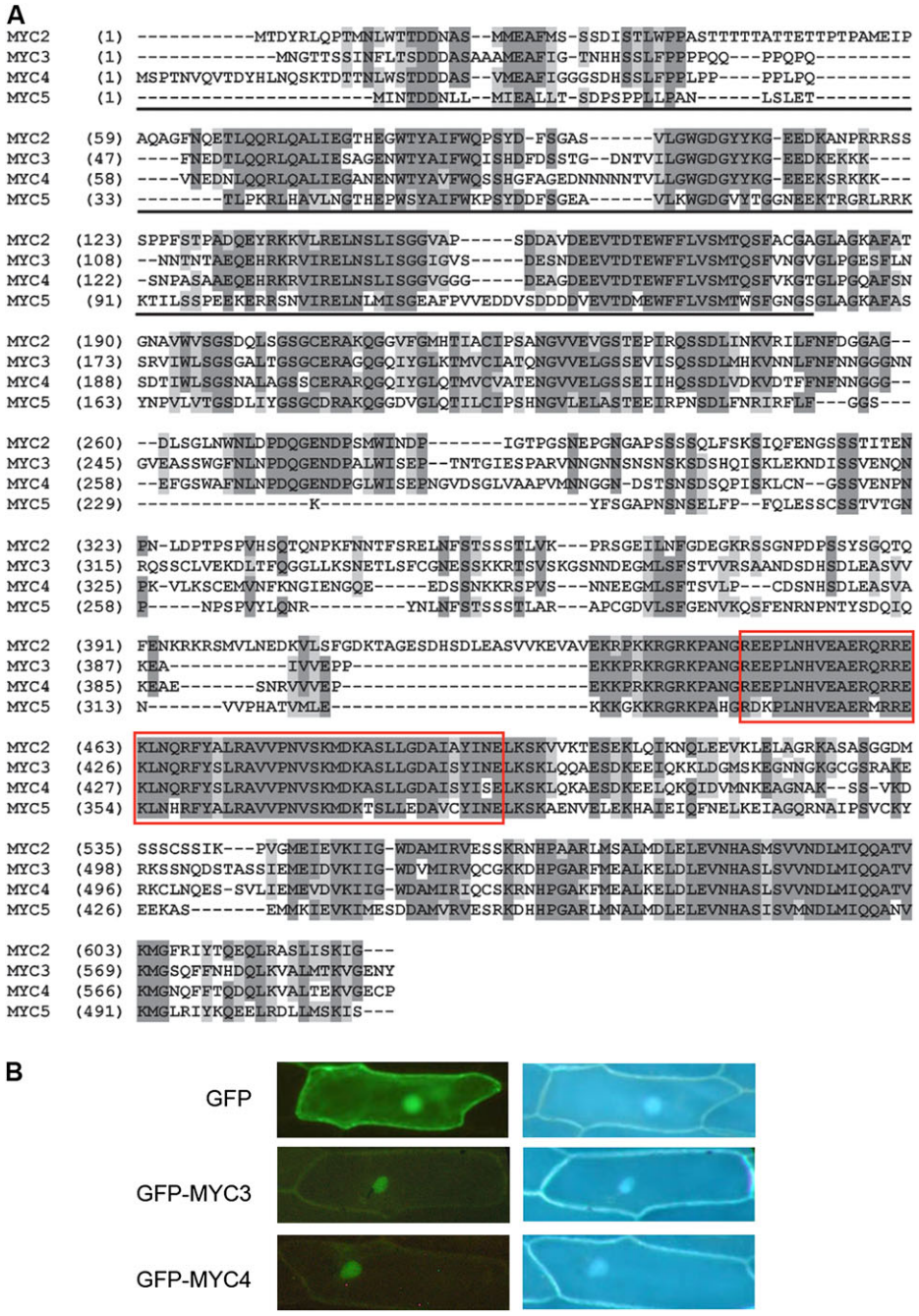

Fig. 1. Sequence comparisons and subcellular localization of JAZ1-interacting proteins, MYC3 and MYC4. (A) Sequence alignment of members in bHLH subgroup IIIe. Shaded letters indicate the conserved amino acid residues. The N-terminal region in MYC2 required for the interaction with JAZ3 is identified by lines under the sequences (Chini *et al.*, 2007). Their conserved bHLH domain is highlighted in the red box. (B) Nuclear localization of GFP–MYC3 and GFP–MYC4 fusion proteins in onion epidermal cells. Nuclei were stained with DAPI (4,6-diamidino-2-phenylindole dihydrochloride).

MYC3/JAZ1 and MYC4/JAZ1 were assayed using the reporter systems described above. These strains coexpressing AD-MYC (AD: activation domain) and BD-JAZ1 (BD: GAL4 DNA binding domain) were able to grow on medium lacking histidine and exhibited β-galactosidase activity, whereas clones expressing AD only and the bait BD-JAZ1 only grew on medium supplemented with histidine (Fig. 2A). These data confirmed our screening results.

MYC3 and MYC4 interact with multiple JAZ proteins besides JAZ1

MYC2 has been shown to interact with most JAZ proteins (Chini *et al.*, 2007, 2009; Melotto *et al.*, 2008; Chung and Howe, 2009). To explore the possible interactions between other members of the bHLH IIIe subfamily and JAZ proteins, combinations of the bait (JAZ1, JAZ3, JAZ9) and prey (MYC3, MYC4, MYC5) were tested in the yeast two-hybrid

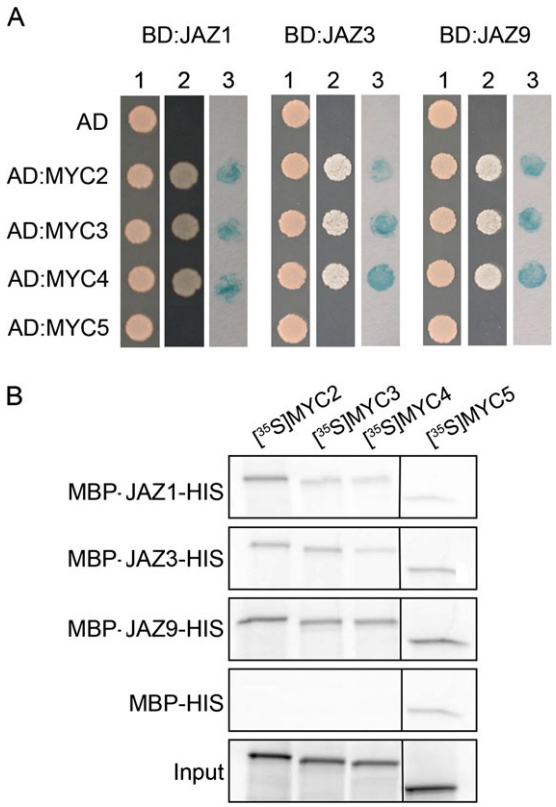

Fig. 2. MYC3 and MYC4 interact with JAZ repressors. (A) MYC3 and MYC4 interact with JAZ proteins in Y2H. (1) YRG2 yeast cells expressing both bait and prey fusions were grown on yeast synthetic minimal SD medium with the omission of leucine and tryptophan, and (2) on yeast SD medium with the omission of leucine, histidine, and tryptophan for examination of *HIS3* reporter gene (3) assayed for lacZ activity. (B) Pull-down of [^{35}S]bHLHs (MYC2, MYC3, MYC4, and MYC5) produced by *in vitro* transcription/translation, using Dynabeads® TALON™ superparamagnetic beads containing MBP–His or MBP–JAZ–His fusion proteins. The input lane shows 10% of [^{35}S]-Met labelled products used in each pull-down assay.

system with the AD and AD-MYC2 as controls. Consistent with previous results, MYC2 interacts not only with JAZ1 but also with JAZ3 and JAZ9 (Fig. 2A). Like MYC2, MYC3 and MYC4 also associate with each of these JAZ proteins, as demonstrated by transcriptional activation of reporter genes, *HIS3* and *lacZ*, in the two-hybrid system (Fig. 2A). MYC5 did not show interaction with any of the three JAZ proteins (Fig. 2A) even though its amino acid sequence shares high homology with those of MYC2, MYC3, and MYC4.

To test further for possible interactions, pull-down assays were performed with purified recombinant JAZ proteins containing an N-terminal maltose-binding protein (MBP) tag and C-terminal 6× His tag. [^{35}S]-Methionine labelled MYC proteins were synthesized by *in vitro* coupled transcription/translation, and then incubated with MBP–JAZ–His fusion proteins that were bound to superparamagnetic beads. After extensive washing, bound, radiolabelled proteins were detected by SDS-PAGE and phosphorimaging. In accordance with the yeast two-hybrid results, MYC2, MYC3, and MYC4 were found to bind to MBP–JAZ1–His, MBP–JAZ3–His, and MBP–JAZ9–His, but not to the control MBP–His (Fig. 2B). In these pull-down assays, MYC5 also showed interactions with all of the MBP–JAZ–His proteins, but a comparable interaction was observed between MYC5 and the MBP–His control (Fig. 2A, B). The choice was therefore made to conduct additional experiments with MYC3 and MYC4.

MYC3 and MYC4 are localized to the nucleus

As MYC3 and MYC4 contain the conserved bHLH domain, they are considered to be putative transcription factors. Furthermore, in order to interact with JAZ proteins *in vivo*, MYC3 and MYC4 are required to colocalize to the same subcellular compartment as JAZ proteins which have been determined to be localized to the nucleus (Chini *et al.*, 2007; Thines *et al.*, 2007).

To determine their subcellular localizations, the full-length *MYC3* and *MYC4* coding sequences were fused in-frame to the C-terminus of green fluorescent protein (GFP). Transient expression of these constructs in onion epidermal cells indicated that, in contrast to the GFP control, which was distributed extensively within the cells, GFP–MYC3 and GFP–MYC4 fusion proteins were localized to the nucleus (Fig. 1B).

MYC2, MYC3, and MYC4 all transactivate JAZ promoters

A *JAZ2::GUS* reporter construct expressed in carrot protoplasts (Liu *et al.*, 1994) was initially used to test the ability of bHLH transcription factors to activate the JA-responsive *JAZ2* promoter. This carrot protoplast system has previously been used to investigate auxin signalling, which is similar to JA signalling (Tiwari *et al.*, 2006). Expression of the *JAZ2::GUS* reporter alone resulted in a low level of GUS activity upon assay. Co-expression of MYC2, MYC3 or MYC4 resulted in 3–4-fold higher GUS activity demonstrating the ability of these transcription factors to activate the *JAZ2* promoter (Fig. 3). By contrast, co-expression of bHLH proteins from subfamily IIId (bHLH3, bHLH13 or bHLH17) did not result in any increase in GUS activity above that of the reporter alone.

To extend this investigation of the three MYC transcription factors, promoter–GUS reporter constructs were generated for *JAZ1*, *JAZ5*, *JAZ6*, *JAZ7*, and *JAZ9*, all of which are strongly induced by JA (Thines *et al.*, 2007). When compared to the basal GUS activity (protoplasts expressing the *JAZ::GUS* reporter alone), co-expression of MYC2, MYC3 or MYC4 resulted in induction of the reporter and increased GUS activity (Table 1). These results indicate that MYC3 and MYC4 act like MYC2 (Chini *et al.*, 2007) in directly activating the promoters of multiple *JAZ* genes.

Loss-of-function mutants of MYC3 and MYC4 show no JA-related phenotype

Since MYC3 and MYC4 share high homology with MYC2 and, more importantly, they physically associate with JAZ

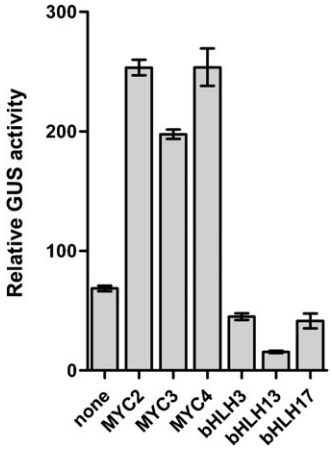

Fig. 3. MYC2, MYC3, and MYC4 induce expression of a *JAZ2::GUS* reporter. Carrot protoplasts were transfected with the *JAZ2::GUS* reporter alone (none) or together with one of six *35S::bHLH* constructs as indicated. GUS activities were standardized using a cotransfected *35S::LUC* reporter. Data are mean ±SE from three separate transfections.

Table 1. MYC transcription factors induce *JAZ::GUS* reporters

Reporter and effector constructs were expressed in carrot protoplast for 18 h and GUS activity was assayed following lysis of the protoplasts. The results show reporter induction relative to an empty vector control. Data are mean ±SE from two independent experiments.

	Promoter driving GUS					
Effector	**JAZ1**	**JAZ2**	**JAZ5**	**JAZ6**	**JAZ7**	**JAZ9**
MYC2	3.0±0.1	3.4±0.2	2.0±0.2	8.2±0.4	20.5±2.4	9.8±1.7
MYC3	1.9±0.1	2.0±0.4	1.8±0.3	4.5±1.2	5.5±1.1	4.4±0.1
MYC4	3.0±0.1	2.9±0.4	1.6±0.2	16.1±0.4	17.1±0.6	8.4±1.6

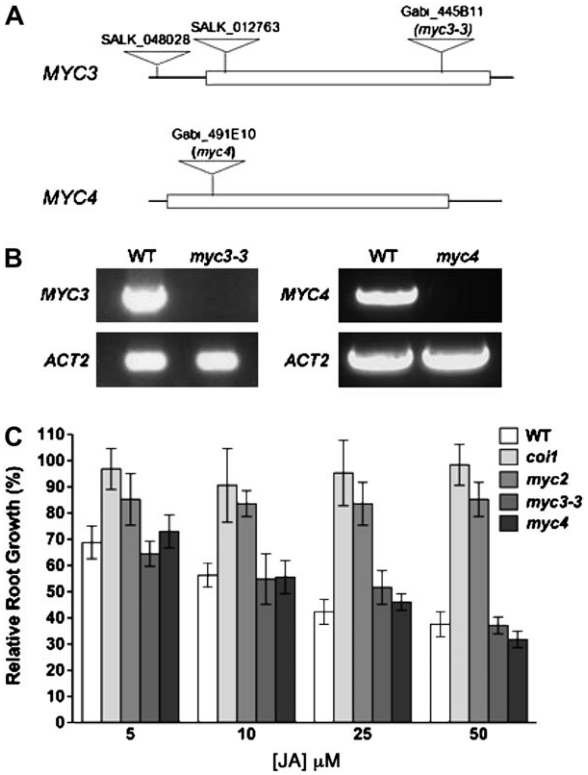

Fig. 4. T-DNA insertion lines for *myc3* and *myc4* genes. (A) Diagram of the genomic sequences of *MYC3* and *MYC4* showing the T-DNA insertion sites of the mutant lines. Open boxes indicate exons. (B) RT-PCR analysis confirms *myc3-3* (Gabi_445B11) and *myc4* (Gabi_491E10) are null. The gene *ACT2* (*Actin2*) was used as the internal control. (C) Relative root growth of seedlings on JA-containing medium compared to root growth on unsupplemented medium (100%) (n=15 seedlings). *myc2* represents *jin1-7* (SALK_040500) (Lorenzo *et al.*, 2004). Error bars indicate the standard deviation for experiments performed in triplicate.

repressors, it raises the possibility that MYC3 and MYC4 are involved in the regulation of JA responses. To investigate the functions of MYC3 and MYC4, a reverse-genetic approach was first employed by identifying T-DNA insertion lines. Four available lines (three lines for *MYC3* and one line for *MYC4*) were examined, but only two of them are null based on the lack of the full-length transcripts of the corresponding genes (Fig. 4A, B). The two null mutants, Gabi_445B11 and Gabi_491E10, are designated as *myc3-3* and *myc4*, respectively. These *myc3-3* and *myc4* null mutants showed no obvious phenotypic differences from the wild-type under normal growth conditions.

To examine whether the mutants have JA-related phenotypes, JA-mediated root growth inhibition in *myc3-3* and *myc4* mutant seedlings was compared with that of *myc2* (*jin1-7*; SALK_040500), *coi1-1*, and wild-type seedlings. When grown on MS media containing various concentrations of JA (5, 10, 25, and 50 μM), the *myc2* mutant showed a degree of insensitivity to JA-inhibited root growth that was slightly less than that observed in the *coi1* mutant. However, root growth of *myc3-3* and *myc4* seedlings was inhibited by JA and the root lengths of these mutants were comparable with that of the wild-type plants (Fig. 4C). The lack of an obvious JA-related phenotype in *myc3-3* and *myc4* mutants may be due to functional redundancy among these, and possibly other, bHLH transcription factors.

Constitutive expression of MYC *genes induces anthocyanin accumulation*

Transgenic plants overexpressing the *MYC3* or *MYC4* cDNA under the control of the cauliflower mosaic virus *CaMV 35S* promoter (*35S::MYC3* and *35S::MYC4*) were then generated. *MYC2*-overexpression plants (*35S::MYC2*) were also generated using the same vector, and these were included as controls in our experiments. Approximately 20 independent T_1 plants for each construct were analysed by reverse transcription-PCR (RT-PCR) and ~80% showed increased transcript accumulation of the corresponding gene compared with wild-type plants. A single homozygous line for each construct that provided >40-fold increase in expression was selected for further characterization.

Compared with wild-type controls, seedlings of lines overexpressing *MYC2*, *MYC3*, or *MYC4* all accumulated

more purple pigmentation, when grown on agar medium, suggesting the accumulation of anthocyanin. To determine the anthocyanin levels, wild-type and overexpression transgenic seedlings were germinated on agar plates and then harvested 10 d after germination. Figure 5A shows that in *35S::MYC2*, *35S::MYC3*, and *35S::MYC4* seedlings, the anthocyanin contents were 3.4-, 15.4-, and 3.6-fold higher, respectively, than that of the wild-type seedlings. JA signalling is known to regulate anthocyanin accumulation in *Arabidopsis* (Feys *et al.*, 1994). Significantly, anthocyanin biosynthetic genes, such as *DIHYDROFLAVONOL REDUCTASE* (*DFR*), are up-regulated by JA (Devoto *et al.*, 2005; Chen *et al.*, 2007). In accordance with the anthocyanin levels, the expression level of *DFR* was higher in overexpression seedlings than in wild-type when quantified by qRT-PCR (Fig. 5B). These results indicate that overexpression of *MYC3* and *MYC4* produced a JA-related phenotype, anthocyanin accumulation, and this accumulation was correlated with increased expression of a gene involved in the production of anthocyanin.

Overexpression of MYC3 *but not* MYC4 *confers hypersensitivity to JA*

Overexpression plants were next tested for another JA-related phenotype, root growth inhibition induced by JA. As shown in Fig. 5C, consistent with the results previously reported (Lorenzo *et al.*, 2004), *35S::MYC2* seedlings were more sensitive to root growth inhibition by JA than wild-type seedlings. In contrast to *35S::MYC4* seedlings that exhibited a similar level of root length reduction as wild-type seedlings, *35S::MYC3* transgenic seedlings showed enhanced inhibition in root growth when grown on MS medium containing JA, but to a lesser extent compared with *35S::MYC2* seedlings. These results indicate that in addition to MYC2, MYC3 plays a role in JA-mediated inhibition of root growth.

Altered transcription of JA-responsive genes in 35S::MYC *transgenic plants*

To explore the effects of overexpression of *MYC3* and *MYC4* on JA signalling further, qRT-PCR was conducted to determine the expression levels of JA-responsive genes in *35S::MYC2*, *35S::MYC3*, and *35S::MYC4* transgenic plants. Two independent biologically replicated experiments were set up with 10-d-old seedlings of the transgenic lines and wild-type controls. Figure 6 depicts the results of the qRT-PCR analysis. Some of the *JAZ* genes are among the earliest genes induced by JA signalling (Thines *et al.*, 2007; Chung *et al.*, 2008). Five of these were chosen for analysis, and each of these was expressed at higher levels in all three

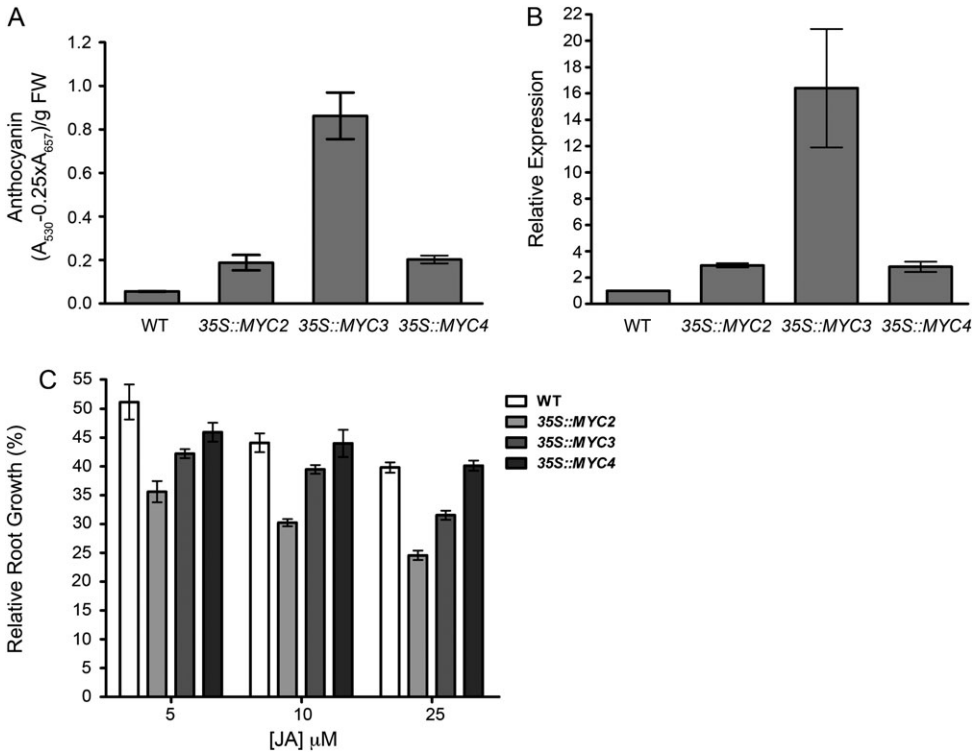

Fig. 5. *MYC* overexpression plants accumulate anthocyanin and are hypersensitive to JA. (A) Photometric determination of anthocyanin content in methanolic extracts of 10-d-old seedlings. All samples were measured as replicates in two independent biological replicates. Data are mean ±SE. (B) Quantitative real-time RT-PCR expression analysis of *DFR* gene in 10-d-old seedlings. Relative expression was determined in replicate measurements in two independent biological replicates. Data are mean ±SE. (C) Relative root growth of wild-type and overexpression transgenic seedlings. Seedlings were grown on normal MS plates for 4 d and transferred to new MS plates containing 0, 5, 10, and 25 μM JA. Root length was measured 6 d after transfer. Root growth in the absence of JA was set to 100%. Data are mean ±SE for n=20 seedlings.

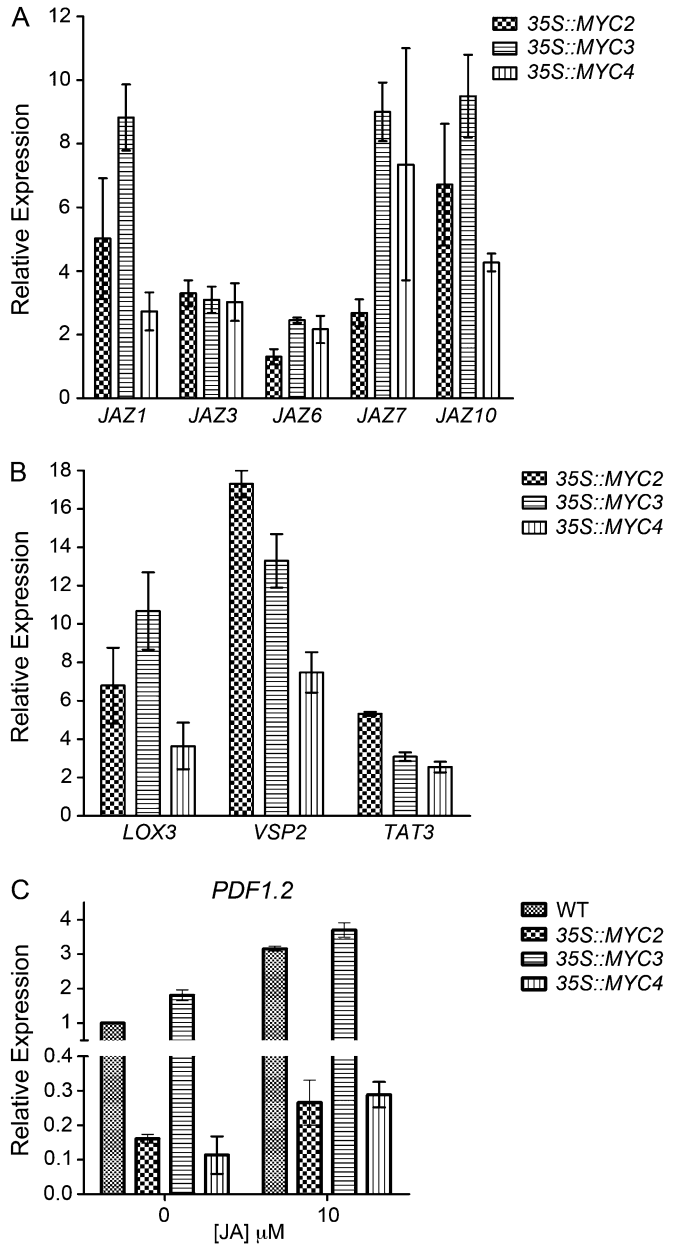

Fig. 6. MYC3 and MYC4 regulate expression of JA-responsive genes. (A, B) qRT-PCR expression analysis of *JAZ* genes and wound-responsive genes in 10-d-old wild-type and *MYC* overexpression seedlings without JA treatment. Wild-type samples served as a calibrator for the calculation of relative expression levels (arbitrarily set to one). (C) Relative transcript levels of the *PDF1.2* gene in 10-d-old wild-type and overexpression transgenic seedlings with or without 10 μM JA treatment (incubated for 6 h). Wild-type samples without JA treatment served as a calibrator, and relative expression was determined from replicate measurements in two independent biological replicates. Data are mean ±SE.

35S::MYC transgenic lines than in the wild type (Fig. 6A), suggesting that, besides MYC2 (Chini *et al.*, 2007), MYC3 and MYC4 also act as transcriptional activators during early JA signalling. In addition, overexpression of these bHLH transcription factors caused increased transcript levels of JA-mediated wound-response genes, *LIPOXYGENASE 3* (*LOX3*), *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*), and *TYROSINE AMINOTRANSFERASE 3* (*TAT3*) (Fig. 6B).

Interestingly, the transcription of the pathogen-responsive gene *PLANT DEFENSIN1.2* (*PDF1.2*) was repressed in *35S::MYC2* and *35S::MYC4* plants compared to the wild-type plants (6-fold and 9-fold repression relative to the wild-type, respectively), whereas in *35S::MYC3* plants the *PDF1.2* transcript level was not substantially changed (less than 2-fold induction) (Fig. 6C). Furthermore, although treatment with 10 μM JA induced expression of *PDF1.2* in all the plants tested, the repression of *PDF1.2* expression was not released by JA treatment in the *35S::MYC2* and *35S::MYC4* transgenic plants. Following JA treatment, *PDF1.2* transcript levels in *35S::MYC2* and *35S::MYC4* plants were still less than 30% of those in untreated wild-type controls (Fig. 6C). These results suggest that MYC4 acts like MYC2 in positively regulating wound-response genes, while repressing the expression of pathogen-responsive genes.

Discussion

Jasmonates are oxylipin signalling molecules that contribute to the regulation of many processes, including growth, defence against pathogens and insects, responses to abiotic stresses, and reproductive development. The recent discovery of the JAZ repressors has greatly improved our understanding of the mechanism of JA signalling, which is similar in several respects, to the model for auxin signalling (Santner and Estelle, 2009). In both systems, repressor proteins bind to specific transcription factors and recruit corepressor proteins such as TOPLESS (which for JA signalling occurs indirectly through NINJA) to prevent transcription of early-response genes (Szemenyei *et al.*, 2008; Pauwels *et al.*, 2010). Increased concentrations of the hormone (auxin or JA-Ile) enhances interaction of the repressor proteins with an SCF ubiquitin ligase, and results in their degradation via the ubiquitin/26S-proteasome pathway (Chini *et al.*, 2007; Tan *et al.*, 2007; Thines *et al.*, 2007). In auxin signalling, 23 ARF transcription factors are known or proposed targets of 29 Aux/IAA repressors, in *Arabidopsis* (Santner and Estelle, 2009). For JA signalling, there are 12 recognized JAZ repressors in *Arabidopsis* but, so far, MYC2 is the only characterized JAZ-interacting transcription factor that has been shown to activate transcription of early JA-responsive genes. However, genetic evidence indicates that additional transcription factors are also involved in JA signalling (Browse, 2009; Chini *et al.*, 2007).

Using JAZ1 as the bait in a yeast two-hybrid screen, two bHLH proteins, MYC3 and MYC4, that share high sequence similarity with MYC2, were identified. In addition to JAZ1, MYC3 and MYC4 directly interact with JAZ3 and JAZ9, in both yeast two-hybrid and pull-down assays. Transient expression of GFP fusions with MYC3 and

MYC4 indicated that they are localized to the nucleus. Furthermore, in carrot suspension cell protoplasts, both MYC3 and MYC4 activated transcription of *GUS* reporter genes under the control of the native *JAZ* promoters. These data suggest that MYC3 and MYC4 are candidates as transcriptional activators whose activities are controlled by JAZ proteins in JA signalling.

In order to search for a JA-related phenotype, T-DNA knockout mutants in *MYC3* and *MYC4* were identified. However, they showed no observable differences from wild-type plants, indicating that there may be functional redundancy among these, and other, MYC2-related bHLH transcription factors. It is considered likely that additional bHLH proteins may also be involved in mediating JA responses. Besides MYC3 and MYC4, three other bHLH proteins are also candidates for JAZ-interacting transcription factors, including MYC5 (bHLH28), which is in the same bHLH subgroup IIIe as MYC2, MYC3, and MYC4. Although MYC5 did not interact with JAZ proteins in yeast two-hybrid assays, it was pulled-down by MBP–JAZ–His proteins (Fig. 2). Additional techniques (e.g. bimolecular fluorescence complementation) could be useful to test for the interaction between MYC5 and JAZ proteins *in vivo*. In addition, bHLH13 and bHLH17 in subgroup IIId also interacted with JAZ1 in our yeast two-hybrid assays (data not shown). It is noteworthy that the N-terminal region of MYC2 (Fig. 1A) that is required for the interaction with JAZs (Chini *et al.*, 2007) is conserved among these bHLH proteins, supporting their possible roles as JAZ-interacting transcription factors. Additional characterization of these five bHLH proteins, including the production of multiple mutant lines, should help to refine and develop the model of JA signalling.

As an alternative approach to investigate the biological roles of these transcription factors, transgenic plants constitutively expressing *MYC3* and *MYC4* were generated. Although the *35S::MYC3* and *35S::MYC4* overexpression plants grew normally, they accumulated higher levels of anthocyanin than did wild-type plants. These results are consistent with MYC3 and MYC4 activation of JA signalling because JA is known to have a role in the production of secondary metabolites, such as anthocyanin (Feys *et al.*, 1994; Gundlach *et al.*, 1992). Previous studies also showed that JA induces the expression of some anthocyanin-related genes (Devoto *et al.*, 2005), and MYC2 is known to function as a positive regulator of JA-mediated anthocyanin biosysthesis (Dombrecht *et al.*, 2007). Consistent with these previous studies, *MYC2* overexpression plants generated in this study also have higher anthocyanin content compared with the wild-type plants. The results of anthocyanin quantification assays showed that the anthocyanin content in *35S::MYC3* transgenic seedlings was more than 4-fold higher than in *35S::MYC2* and *35S::MYC4* seedlings. In addition, the relative expression level of an anthocyanin biosynthesis gene, *DFR*, was also higher (more than 5-fold) in *35S::MYC3* plants compared with *35S::MYC2* and *35S::MYC4* plants. These results demonstrate that, besides MYC2, MYC3 and MYC4 positively regulate anthocyanin biosynthesis and MYC3 may have a dominant role in this process.

In addition to anthocyanin accumulation, *35S::MYC3* plants and also *35S::MYC2* plants showed hypersensitivity to JA in terms of root growth inhibition. However, the level of root length reduction of *35S::MYC4* plants was similar to that of wild-type plants in the presence of JA. These results suggest that MYC3, but not MYC4, is involved in regulating JA-mediated inhibition of root growth.

To understand the function of MYC3 and MYC4 further, the expression of JA-regulated genes in *35S::MYC3* and *35S::MYC4* overexpression plants was analysed. As expected, early JA-responsive genes, such as *JAZ* genes, were induced in these overexpression plants and the levels of induction were comparable with those in *35S::MYC2* plants. These results suggest that *JAZ* genes are targets of MYC3 and MYC4. In addition, the expression of genes involved in two branches of JA responses, including wound-responsive genes, *VSP2*, *LOX3*, and *TAT3*, and a pathogen-responsive gene, *PDF1.2*, were examined. MYC2 differentially regulates expression of these genes (Lorenzo *et al.*, 2004). MYC4 induces expression of three genes involved in wounding (Fig. 6B) and represses transcription of a pathogen defence gene, *PDF1.2* (Fig. 6C), indicating that similar to MYC2, MYC4 is likely to have differential effects on different JA responses.

In contrast, the expression levels of tested JA-responsive genes suggest that MYC3 may only participate in the activation of the wound response, but not regulate expression of pathogen-responsive genes. A previous study (Smolen *et al.*, 2002) also found that *MYC3* overexpression had no effect on transcript levels of *PDF1.2*. This finding was reported as part of an investigation of a dominant, altered-function allele of *MYC3* named *altered tryptophan regulation 2* (*atr2D*), which does show increased *PDF1.2* expression relative to the wild type. The *atr2D* mutation results in an Asp94Asn change in the MYC3 protein and is associated with a pleiotropic phenotype, particularly in transgenic lines expressing a *35S::atr2D* construct. Although a mechanistic explanation of the *atr2D* phenotype is lacking, the results of Smolen *et al.* (2002) and those reported here point to a complex relationship among the transcription factors that mediate JA signalling and response.

Taken together, these results on *MYC3* and *MYC4* overexpression plants suggest that these two bHLH proteins are transcription factors involved in JA signalling and that they have overlapping functions with other bHLH proteins, including MYC2.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Table S1. Summary data on clones testing positive for interaction with JAZ1 in a yeast-two-hybrid screen.

Acknowledgements

We thank Tom Guilfoyle and Gretchen Hagen (University of Missouri) for carrot cell cultures, constructs and assistance, and Shiv Tiwari (Mendel Biotechnology Inc) for assistance with protoplasts isolation and transfection. This research was supported by the U.S. Department of Energy grant DE-FG02-99ER20323 and the Agricultural Research Center at Washington State University.

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