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Transcription Factor AsMYC2 Controls the Jasmonate-Responsive Expression of *ASS1* Regulating Sesquiterpene Biosynthesis in *Aquilaria sinensis* (Lour.) Gilg

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Sesquiterpenes are one of the most important defensive secondary metabolite components of agarwood. Agarwood, which is a product of the *Aquilaria sinensis* response to external damage, is a fragrant and resinous wood that is widely used in traditional medicines, incense and perfume. We previously reported that jasmonic acid (JA) plays an important role in promoting agarwood sesquiterpene biosynthesis and induces expression of the sesquiterpene synthase ASS1, which is a key enzyme that is responsible for the biosynthesis of agarwood sesquiterpenes in *A. sinensis*. However, little is known about this molecular regulation mechanism. Here, we characterized a basic helix–loop–helix transcription factor, AsMYC2, from *A. sinensis* as an activator of *ASS1* expression. *AsMYC2* is an immediate-early jasmonate-responsive gene and is co-induced with *ASS1*. Using a combination of yeast one-hybrid assays and chromatin immunoprecipitation analyses, we showed that AsMYC2 bound the promoter of *ASS1* containing a G-box motif. AsMYC2 activated expression of *ASS1* in tobacco epidermis cells and up-regulated expression of sesquiterpene synthase genes (*TPS21* and *TPS11*) in Arabidopsis, which was also promoted by methyl jasmonate. Our results suggest that AsMYC2 participates in the regulation of agarwood sesquiterpene biosynthesis in *A. sinensis* by controlling the expression of *ASS1* through the JA signaling pathway.

Keywords: Agarwood • Jasmonate • MYC2 • Sesquiterpene synthase • Transcription factor.

Abbreviations: bHLH, basic helix–loop–helix; ChIP, chromatin immunoprecipitation; GFP, green fluorescent protein; GST, glutathione *S*-transferase; JA, jasmonic acid; JAZ, jasmonate-ZIM domain protein; MeJA, methyl jasmonate; MS, Murashige and Skoog; ORF, open reading frame; RACE, rapid amplification of cDNA ends; RT-PCR, real-time PCR.

Introduction

Agarwood is a non-timber dark resinous wood that is formed in the stems, branches and roots of *Aquilaria* and *Gyrinops* trees after they have been wounded (Ng 1997, Itoh et al. 2002, Pojanagaroon and Kaewrak 2005, Persoon, 2008). It is widely used in traditional medicines such as digestive, sedative and antiemetic drugs (China Pharmacopoeia Committee 2015), and is popular as incense and in perfumes in the Middle East, South Asia, Japan and China (Kumeta and Ito, 2010, Y.Y. Liu et al. 2013, Xu et al. 2013). In the international market, high-quality agarwood commands a higher price than gold. *Aquilaria sinensis* (Lour.) Gilg is one of the most important plant resources for producing agarwood in China, and it is also the only certified source of agarwood listed in the China Pharmacopoeia (China Pharmacopoeia Committee 2015). Sesquiterpenes are one of the major components in agarwood (Hashimoto et al. 1985, Chen et al. 2011, Chen et al. 2012), which is formed only when healthy *Aquilaria* trees are wounded. Like other sesquiterpene phytoalexins, agarwood sesquiterpenes are defensive compounds with antimicrobial and antidisease activity (Kumeta and Ito 2010, Chen et al. 2011) and only accumulate in response to elicitation or wound signals (Ito et al. 2005, Okudera and Ito 2009, Kumeta and Ito 2010, Chen et al. 2011, Xu et al. 2013).

Jasmonic acid (JA) and its derivates, collectively known as jasmonates (JAs), are essential signaling molecules that coordinate the plant defense response to biotic and abiotic challenges, in addition to multiple developmental processes (Wasternack 2007, Balbi and Devoto 2008, Browse and Howe 2008, Chico et al. 2008). Recent investigations hypothesized that, in the absence of JAs, jasmonate-ZIM domain proteins (JAZs) may repress expression of JA-responsive genes via their interactions with a series of transcription factors; when JA

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signaling is initiated, degradation of JAZs would disrupt these interactions, leading to activation of these transcription factors, which mediate various JA-regulated biological processes (Chini et al. 2007, Thines et al. 2007, Chico et al. 2008, Kazan and Manners 2008, Browse 2009, Pauwel et al, 2010). MYC2, a basic helix–loop–helix (bHLH)-type transcription factor, is a direct target of JAZs (Chini et al. 2007). As a master regulator in the JA signaling pathway, MYC2 both positively and negatively regulates diverse aspects of JA responses, including JA-mediated biosynthesis of secondary metabolites (for a review, see Kazan and Manners 2008, Kazan and Manners 2013).

Studies have shown that JA-induced biosynthesis of nicotine in tobacco (Todd et al. 2010, Shoji and Hashimoto 2011), sesquiterpenes in Arabidopsis (Hong et al. 2012, Ran et al. 2014), alkaloids in *Catharanthus roseus* (Zhang et al. 2011) and sakuranetin, a flavonoid antifungal phytoalexin in rice (Ogawa et al. 2017), are all mediated by the transcription factor MYC2. In tobacco, NtMYC2 binds to the G-box sequence in the promoter of the putrescine *N*-methyltransferase (*PMT*) gene, regulating the expression of nicotine biosynthesis genes (Todd et al. 2010). Meanwhile, NtMYC2 also activates an AP2/ERF transcription factor gene, *ERF189*, that directly regulates several JA-inducible nicotine biosynthesis genes (Shoji and Hashimoto 2011). In *C. roseus*, methyl jasmonate (MeJA)-responsive expression of alkaloid biosynthesis genes is controlled by a transcription factor cascade; CrMYC2 binds to the promoter and activates expression of the AP2/ERF-domain transcription factors ORCA2 and ORCA3, which in turn regulate a subset of alkaloid biosynthesis genes (Zhang et al. 2011). In rice, OsMYC2 drastically enhances the activity of the *OsNOMT* promoter and is reinforced by OsMYL1 and OsMYL2 by interacting with each other, resulting in the inductive production of sakuranetin in the JA signaling pathway (Ogawa et al. 2017). MYC2 also positively regulates terpene synthase genes, such as *AtTPS10* and *NbTPS1*, two whitefly defense-related genes (Ran et al. 2014) and two sesquiterpene synthase genes, *TPS21* and *TPS11*, in Arabidopsis inflorescence (Hong et al. 2012) by directly binding to their promoters. In *Aquilaria*, the induction role played by JA has also been widely demonstrated in recent years. Exogenously applied MeJA in *Aquilaria* cell suspension cultures or calluses induced biosynthesis and accumulation of sesquiterpene compounds, especially δ-guaiene (Ito et al. 2005, Okudera and Ito 2009, Kumeta and Ito 2010, Xu et al. 2013, Xu et al. 2016). According to our latest results, JA is the most crucial endogenous signal molecule in the accumulation of biosynthesized sesquiterpenes in *A. sinensis* (Xu et al. 2016). However, the molecular mechanism by which JA regulates sesquiterpene biosynthesis is largely unknown.

The first committed step of sesquiterpene biosynthesis is the cyclization of farnesyl diphosphate, which is catalyzed by sesquiterpene synthases. The genes for two of these enzymes, AcC1 and ASS1, have been independently identified in *Aquilaria* by two groups (Kumeta and Ito 2010, Xu et al. 2013). Both genes encode enzymes that convert farnesyl diphosphate into δ-guaiene in addition to a few minor products such as α-guaiene and α-humulene. In healthy *Aquilaria* calluses or cell cultures, expression of the δ-guaiene synthase *ASS1* was barely detectable, whereas in wounded tissues or MeJA-treated calluses, its expression increased significantly, and the sesquiterpene compound content correspondingly increased (Xu et al. 2013). These results demonstrate that ASS1 is the key enzyme that is responsible for the biosynthesis of agarwood sesquiterpene, whose expression is regulated at the transcriptional level by JA and wound signals.

In this study, to reveal the mechanism by which JA induces expression of *ASS1* and agarwood sesquiterpene biosynthesis, we focused on the transcription factor AsMYC2, the master regulator in the JA signaling pathway in *A. sinensis*. Overexpression of AsMYC2 in Arabidopsis up-regulates expression of sesquiterpene synthase genes (*TPS21* and *TPS11*) and partly rescues their expression and the MeJA responsiveness of the *myc2-2* mutant. AsMYC2 binds the promoter of *ASS1*, which contains a G-box motif, and activates its expression in response to MeJA. These data suggest that AsMYC2 participates in the regulation of agarwood sesquiterpene biosynthesis by positively regulating *ASS1* expression at the transcriptional level through a JA signaling pathway.

Results

Isolation of AsMYC2 from *A. sinensis*

MYC2, a key downstream component of the JA signaling pathway, has been reported as a master that regulates most JA-responsive genes (Kazan and Manners 2008, Kazan and Manners 2013). The importance of JA signaling in regulating sesquiterpene biosynthesis and *ASS1* expression in *Aquilaria* spp. (Ito et al. 2005, Okudera and Ito 2009, Kumeta and Ito 2010, Xu et al. 2013, Xu et al. 2016) prompted us to investigate the function of MYC2 in *A. sinensis*. Based on the partial sequences of unigenes in *A. sinensis* transcriptome data (Xu et al. 2013), the full-length cDNA of *AsMYC2* was obtained using the 5′/3′-RACE (rapid amplification of cDNA ends) method (accession No. KP677282). It was found to encode a 673 amino acid protein with a predicted molecular mass of 73.5 kDa and a pI of 5.53 (**Fig. 1A**). The search for conserved domains in the AsMYC2 protein against the NCBI Conserved Domain Database showed that AsMYC2 contains an HLH domain in its carboxyl region and a bHLH_MYC_N domain (**Fig. 1A**). By aligning multiple amino acid sequences, an unrooted Neighbor–Joining tree was constructed, showing that AsMYC2 is most homologous (73%) with the *Theobroma cacao* bHLH protein (**Fig. 1B**).

Analysis of AsMYC2 characteristics

A putative nuclear localization signal (NLS) in AsMYC2 is indicated in **Fig. 1A**. It was previously reported that C-terminal NLSs are responsible for targeting to the nucleus (Hedhili et al. 2010). To confirm that AsMYC2 is a nuclear protein in *A. sinensis*, the open reading frame (ORF) of the *AsMYC2* gene was fused to the N-terminus of a green fluorescent protein (GFP) reporter gene under the control of the *Cauliflower mosaic virus* (CaMV) 35 S promoter. The recombinant constructs of the AsMYC2–GFP fusion and GFP alone were

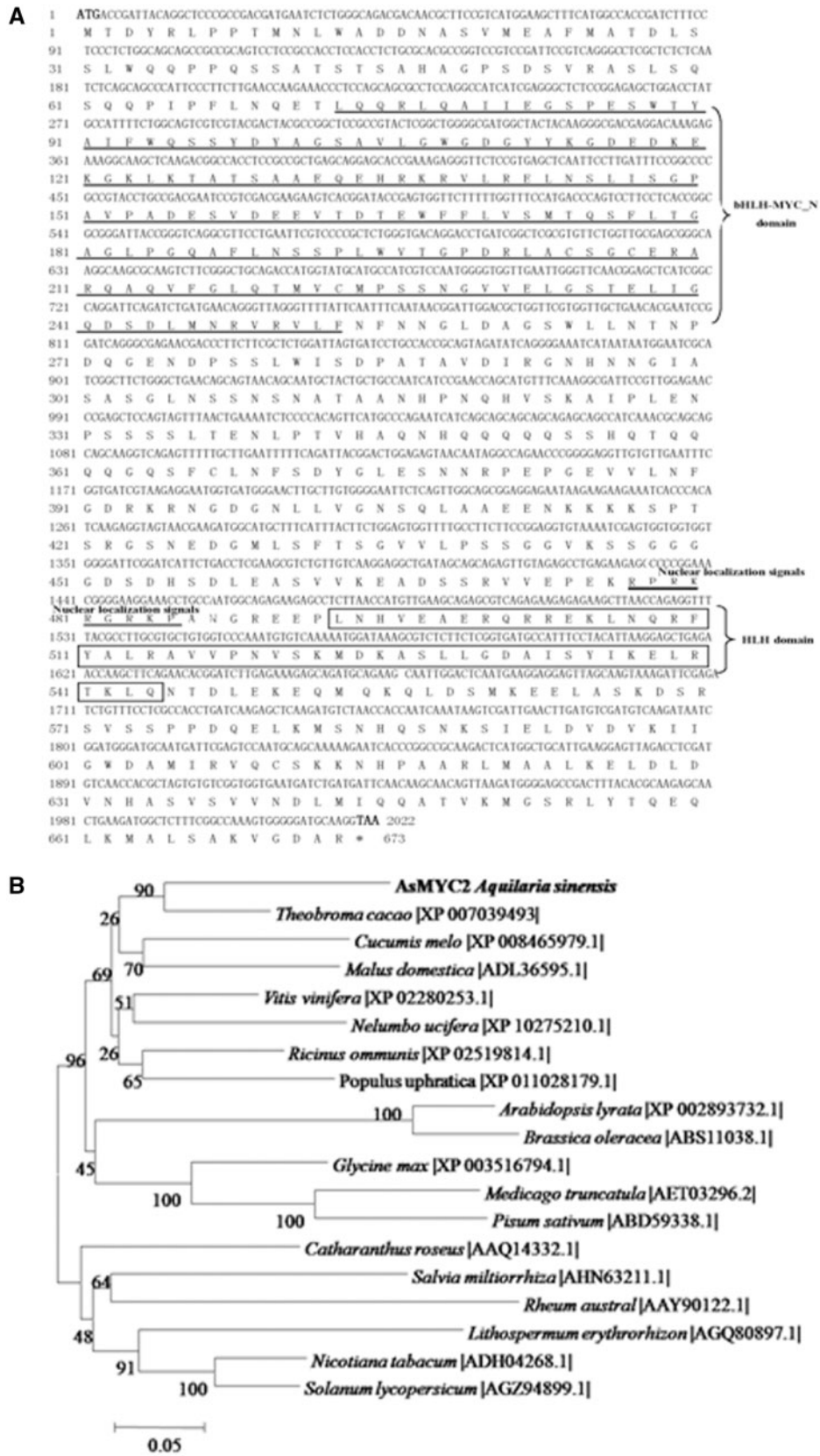

Fig. 1 Analysis of the protein sequence of AsMYC2. (A)The CDS sequence and the deduced amino acid sequence of *AsMYC2*. (B) Phylogenetic tree based on the amino acid sequence of AsMYC2 and other homologous sequences.

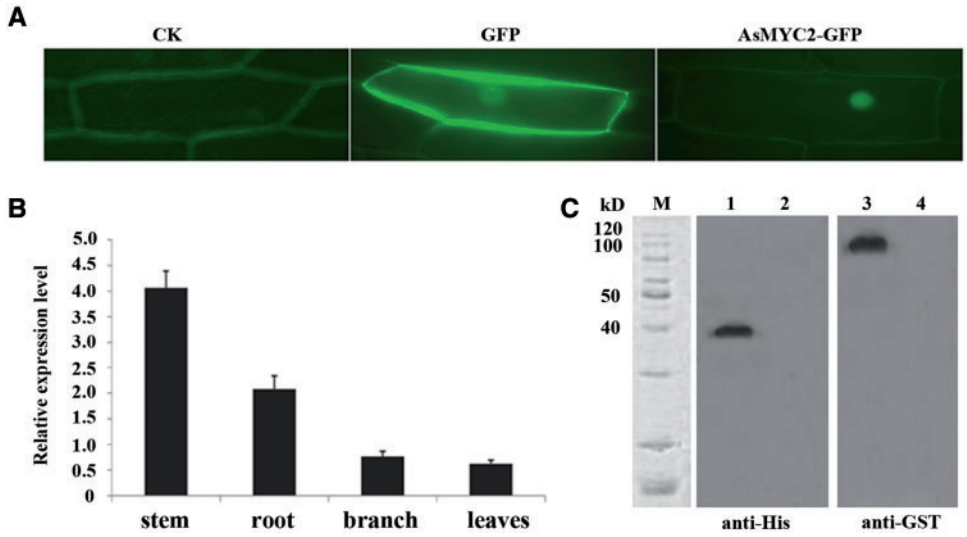

Fig. 2 Analysis of AsMYC2 characteristics. (A) Transient expression in onion epidermis shows that AsMYC2 localized in nuclei. Ck, GFP and AsMYC2-GFP indicate the bright field, the fluorescence of the empty GFP vector and fluorescence of the AsMYC2–GFP fusion protein, respectively. (B) The tissue expression pattern of *AsMYC2* in *A. sinensis*. (C) AsMYC2 interacts with the AsJAZ1 repressor in a pull-down experiment. (1) effluent of AsMYC2–GST + AsJAZ1-His protein from the GST resin, (2) effluent of pGEX-4 T-1 + AsJAZ1-His protein from the GST resin, (3) effluent of AsMYC2–GST + AsJAZ1-His protein from Ni-NTA His Bind resin and (4) effluent of pET-28a + AsMYC2–GST protein from Ni-NTA His Bind resin.

individually introduced into onion epidermal cells by gold particle bombardment. As shown in **Fig. 2A**, the AsMYC2–GFP fusion protein was specifically localized in the nucleus, whereas GFP alone was ubiquitously distributed in both the nucleus and the cytoplasm.

Next, we preliminarily elucidated the function of AsMYC2 by analyzing its expression patterns in the roots, stems, leaves and branches of 4-year-old *A. sinensis* trees. Quantitatiuve real-time PCR (RT-PCR) experiments showed that *AsMYC2* was expressed in all the analyzed tissues (**Fig. 2B**), with the highest transcript level, approximately eight times higher than that in leaves, occurring in stems; roots expressed the second highest amount, four times higher than that in leaves (**Fig. 2B**). These data indicated that AsMYC2 is mainly accumulated and functions in stems and roots during the development of *Aquilaria* trees. Coincidentally or not, agarwood is mainly formed in stems and roots of wounded *Aquilaria* trees, implying that there may be a positive correlation in different tissues between AsMYC2 expression and the active principle content of agarwood.

JA signaling is regulated by some protein complexes. MYC2, as a master regulator in JA signaling, is recruited by JAZ repressors to form COI1–JAZ–MYC2 complexes and inhibit MYC2-targeted gene transcripts (Thines et al. 2007, Chico et al. 2008, Kazan et al. 2008). To demonstrate whether there is an interaction between AsJAZ1 and AsMYC2 proteins in *A. sinensis*, we performed pull-down experiments using recombinant purified glutathione *S*-transferase (GST)–AsMYC2 and His-AsJAZ1 fusion proteins. As shown in **Fig. 2C**, AsJAZ1 could be pulled down by the GST–AsMYC2 fusion protein (lane 1), and AsMYC2 could also be pulled down by His-AsJAZ1 fusion protein (lane 3), demonstrating that there is an interaction between AsJAZ1 and AsMYC2 proteins.

AsMYC2 is co-induced with ASS1 by MeJA

Because *ASS1* transcription could be activated by mechanical wounding and MeJA (Xu et al. 2013), we would expect the inducible expression of *AsMYC2* if it regulates *ASS1*. Therefore, we analyzed its expression in response to MeJA exposure. Examination by RT-PCR revealed that *AsMYC2* was significantly up-regulated by MeJA treatment (**Fig. 3**), showing two peaks within a 24 h window, with the first peak at 0.5 h and the second peak at 4 h; this second peak was followed by a slow decline and finally returned to normal at 24 h. Comparatively, the sesquiterpene biosynthesis gene *ASS1* showed a slower rate of induction, its transcriptional level was not significantly elevated until approximately 1 h post treatment and it reached its peak expression at 6 h (**Fig. 3**). Thus, the transcript level of *AsMYC2* increased rapidly and transiently upon elicitation, preceding *ASS1* induction, which suggested that AsMYC2 is an immediate-early jasmonate-responsive gene in *A. sinensis* and is co-induced with *ASS1*.

Overexpression of AsMYC2 up-regulates expression of sesquiterpene synthase genes *TPS21* and *TPS11* in Arabidopsis

To determine further whether AsMYC2 is a regulator of sesquiterpene biosynthesis genes, we transformed the *AsMYC2* gene into wild-type Arabidopsis (marked as *35 S-AsMYC2*) and AtMYC2 knockout mutant *myc2-2* Arabidopsis (marked as *35 S-AsMYC2-myc2-2*) and then analyzed the expression of the sesquiterpene synthase genes *TPS21* and *TPS11*, which are responsible for the formation of virtually all Arabidopsis floral volatile sesquiterpenes (Tholl et al. 2006, Hong et al. 2012). As shown in **Fig. 4**, in the *myc2-2* mutant, the knocking out of AtMYC2 down-regulates the expression levels of *TPS11* and

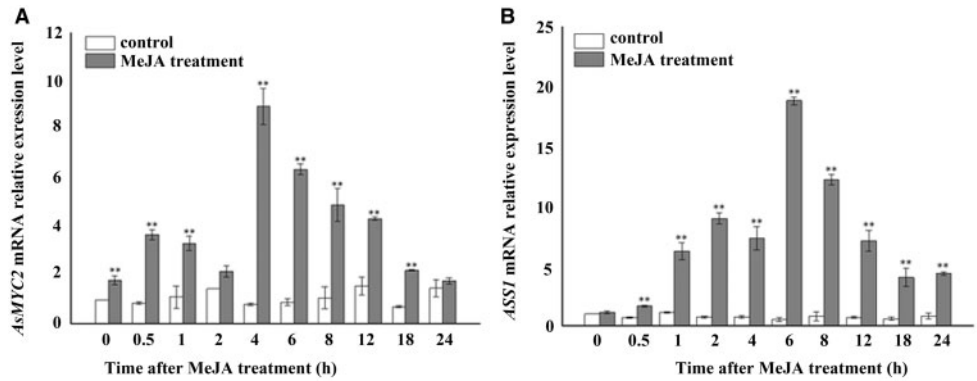

Fig. 3 Expression of *AsMYC2* and *ASS1* is co-induced by MeJA. The transcript level of *AsMYC2* and *ASS1* was examined in *A. sinensis* cell suspension with MeJA treatment. MeJA at 100 μM was added to the *A. sinensis* cultured cell suspension and samples at the appointed times (0, 0.5, 2, 4, 6, 12 and 24 h). Healthy cells in suspension cultures that lacked MeJA were used as controls. Expression levels of *AsMYC2* and *ASS1* were assayed using real-time PCR analysis, and the *Tubulin* gene was used as the internal control. Asterisks (*) indicate a significant difference from control samples at $^{*}P < 0.05$ or $^{**}P < 0.01$ (Student's *t*-test). Data are presented as the means ± SD of three independent experiments.

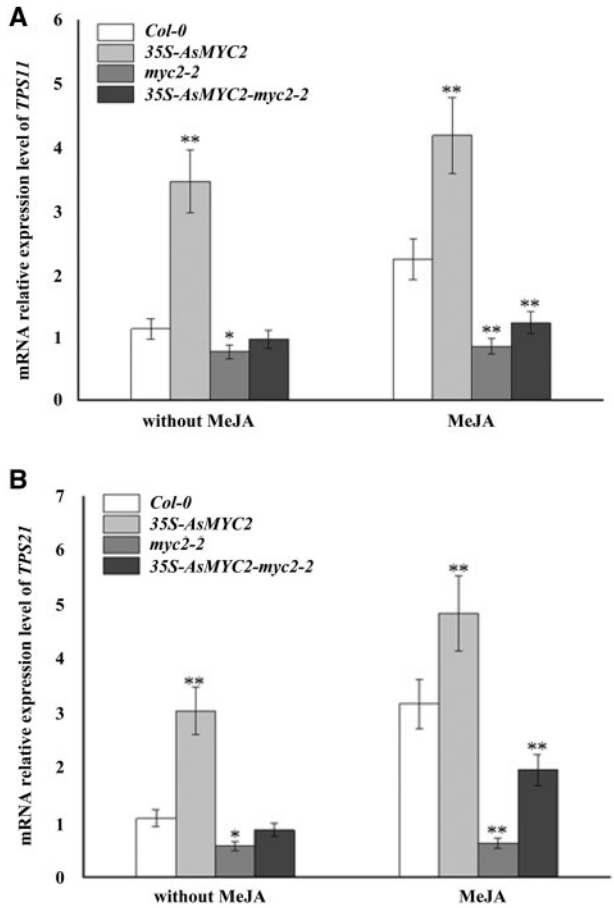

Fig. 4 *Aquilaria sinensis AsMYC2* up-regulates the expression of sesquiterpene synthase genes *TPS11* and *TPS21* in Arabidopsis in the JA signaling pathway. *AsMYC2* up-regulates the expression of *TPS11* and *TPS21* in wild-type Arabidopsis and partially rescues the expression of *TPS11* and *TPS21* in the *myc2-2* mutant, and MeJA promotes this effect. (A, B) Real-time PCR analysis of *TPS11* and *TPS21* transcription levels in Col-0, *35 S-AsMYC2*, *myc2-2* and *35 S-AsMYC2-myc2-2* using actin2/8 as internal controls. Asterisks (*) indicate a significant difference from control samples at $^{*}P < 0.05$ or $^{**}P < 0.01$ (Student's *t*-test).

TPS21, and the mutant showed a MeJA-insensitive phenotype in the MeJA-induced promotion of *TPS11* and *TPS21* expression; induction by MeJA was almost lost compared with the response in the wild type, demonstrating that AtMYC2 is essential for MeJA-responsive expression of *TPS11* and *TPS21*. Interestingly, overexpression of *AsMYC2* partially rescued the expression of *TPS11* and *TPS21* to wild-type levels and restored their inducibility by MeJA. Meanwhile, in the *35 S-AsMYC2* transgenic lines, *AsMYC2* increased the transcription levels of *TPS11* and *TPS21* 2-fold, and MeJA treatment significantly induced their expression (**Fig. 4**). These data provided genetic evidence that the function of AsMYC2 is similar to that of AtMYC2 in Arabidopsis: specifically, it regulates the expression of sesquiterpene synthase genes through a JA signaling pathway (Hong et al. 2012).

AsMYC2 binds the promoter of *ASS1* containing a G-box motif and activates its expression

To test whether AsMYC2 binds to the *ASS1* promoter in vitro, a yeast one-hybrid assay was performed. It was shown that AsMYC2 interacts with the promoter of *ASS1*, as the cells grow well on SD medium lacking tryptophan, leucine and histidine (**Fig. 5B**). Previous in vitro and in vivo binding assays showed that MYC2 binds directly to the G-box *cis*-element (CACGTG) and G-box-like motifs (AACGTG or CATGTG or CACATG) (Boter et al. 2004, Dombrecht et al. 2007, Hou et al. 2010, Hong et al. 2012, Li et al. 2014). In a broader sense, E-box elements (CANNTG) serve as binding sites of bHLH transcription factors (Chaudhary and Skinner 1999). Coincidentally, six E-box (CANNTG) motifs were found in the promoter of *ASS1* (P_{ASS1}) and thus may be the binding site of AsMYC2. To investigate the binding sites of AsMYC2 in the *ASS1* promoter, a chromatin immunoprecipitation (ChIP) assay was performed, followed by RT-PCR. In the ChIP assay assessing AsMYC2–P_{ASS1} interactions, three domains in the *ASS1* promoter were isolated (**Supplementary Table S1**): domain I (−973 to −731) covers two E-boxes (CACCTG and CAAGTG), domain II (−707 to −436) covers one E-box (CATTTG) and domain

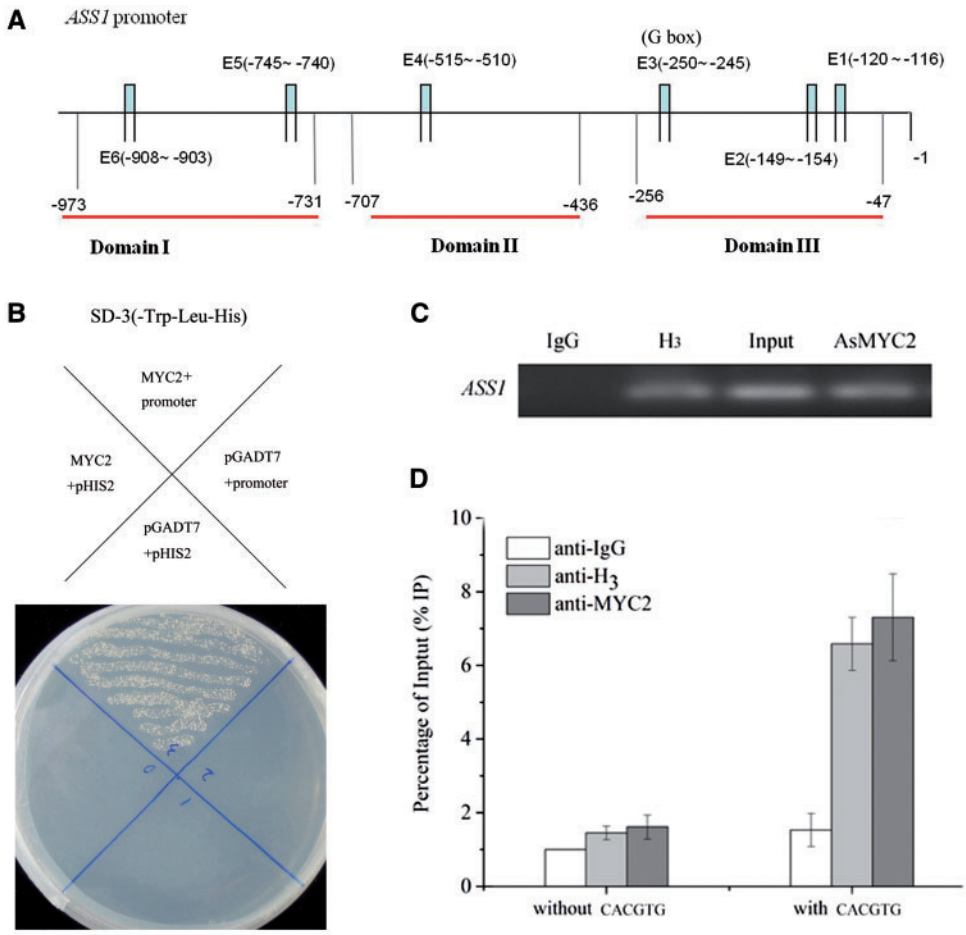

Fig. 5 AsMYC2 binds to the *ASS1* promoter containing a G-box sequence, and activates its expression. (A) The promoter structure of the *ASS1* genes. E*n* (E1–E6) indicates E-boxes that are numbered from right to left, with their sequence sites relative to the translation start codon (ATG). Red lines indicate the sequences detected by ChIP assays. (B) AsMYC2 interacts with the promoters of the *ASS1* genes in a yeast one-hybrid assay. The prey vector, harboring *AsMYC2* (pGADT7-*AsMYC2*, indicated by MYC2), and the bait vector pHIS2, harboring the *ASS1* promoter, were transformed into yeast cells. Transformations with empty vectors pGADT7 and pHIS2 were used as negative controls. The experiments were repeated three times and yielded the same results. (C, D) ChIP assay showing that AsMYC2 interacts with the *ASS1* promoter. (C) PCR data from ChIP assays with the AsMYC2-specific antibody. Lanes: IgG, PCR product from ChIP with pre-immune serum (as a negative control); H3, PCR product from ChIP with anti-acetyl-histone 3 antibody (as a positive control); input, PCR product from the chromatin DNA; AsMYC2, PCR product from ChIP with the antibody against AsMYC2. (D) Real-time PCR data from the ChIP assay with the antibody against AsMYC2; *Actin* was used as a negative control.

III (−256 to −47) covers three E-boxes (CACGTG, CAAGTG and CAGGTG). Our results demonstrated that AsMYC2 binds domain III (**Fig. 5C, D**), as the experiment showed that ChIP with either anti-acetyl-histone 3 or anti-AsMYC2 antibody detected the *ASS1* promoter amplification products (**Fig. 5C**, lanes 2 and 4). However, almost no products were observed in ChIP assays with anti-rabbit IgG (**Fig. 5C**, lane 1). Samples of DNA that was not immunoprecipitated displayed a bright lane (**Fig. 5C**, lane 3) and thus served as a control to determine ChIP efficiency. ChIP-qPCR analysis also illustrated that AsMYC2 strongly binds to the *ASS1* promoter that encompasses the G-box motifs; in contrast, no interaction was observed between AsMYC2 and the *ASS1* promoter without the G-box motifs (**Fig. 5D**). These results suggested that AsMYC2 binds to *ASS1* promoter that contains the G-box motifs.

We tested whether AsMYC2 regulates the promoter activity of the *ASS1* gene using an in vivo system that involved co-transforming tobacco leaves with AsMYC2 and the *ASS1* promoter linked to GFP (P_{ASS1}–GFP). The result showed that AsMYC2 also interacts with the *ASS1* promoter in vivo and activates its expression, as reported by the GFP fluorescence. GFP fluorescence from tobacco leaves that had been infiltrated by both *35 S:AsMYC2* and P_{ASS1}-*GFP* recombinant plasmids was stronger than the fluorescence of leaves with P_{ASS1}–GFP alone (**Fig. 6**). In addition, applications of exogenous MeJA specifically promoted their interaction, as epidermal cells of tobacco leaves with these applications showed the strongest fluorescence (**Fig. 6**).

Discussion

Agarwood, a type of precious and rare traditional Chinese medicinal material and a highly valued worldwide resource, is a

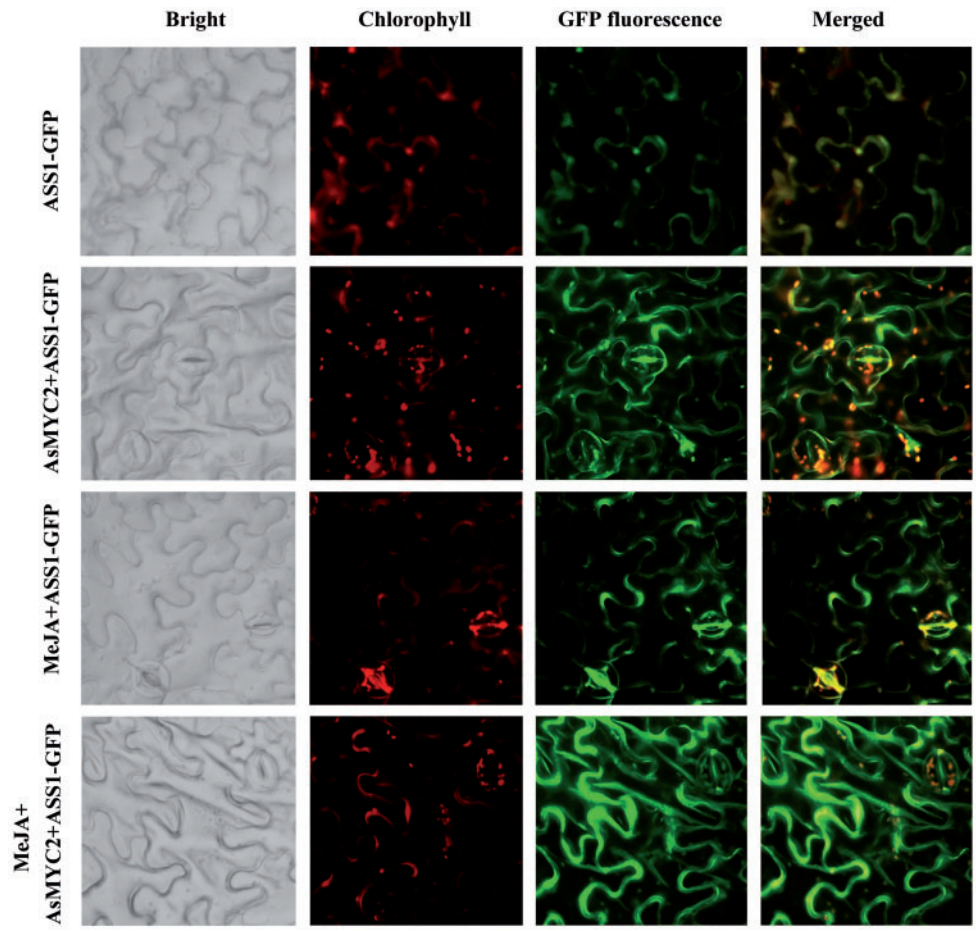

Fig. 6 AsMYC2 activates *ASS1* expression in vivo. AsMYC2 activates the promoter activity of ASS1, and MeJA promotes this process. Tobacco leaves were transformed with the constructs P_{ASS1}–GFP alone and P_{ASS1}–GFP plus *AsMYC2*. Note that co-transformation of *AsMYC2* and P_{ASS1}–GFP promotes P_{ASS1}–GFP expression. '+ MeJA' indicates that the transgenic tobacco leaves were sprayed with 0.1 mM MeJA 2 h before observation. The experiments were repeated three times and yielded the same results. Bright, Chlorophyll, GFP, and Merged indicate bright-field, Chl autofluorescence, the P_{ASS1}–GFP fusion protein and the merged image of the GFP and the autofluorescence with the bright field, respectively. Images were taken with identical parameters to allow fluorescence intensities to be compared.

resinous heartwood produced by wounded *Aquilaria* spp. trees, and sesquiterpenes are one of its major components (Hashimoto et al. 1985, Chen et al. 2011, Chen et al. 2012). It has been widely demonstrated that JA plays important roles in agarwood sesquiterpene biosynthesis in *A. sinensis* (Okudera and Ito 2009, Kumeta and Ito 2010, Xu et al. 2013), but whether there is a direct relationship between JA and this process is unknown, as is the exact regulatory mechanism of the JA signaling pathway that is involved in agarwood sesquiterpene biosynthesis.

As extensively reported, primary signal transduction processes following JA perception converge on related bHLH transcription factors. Of these, the best characterized and most multifunctional is MYC2. Considering the important role of MYC2 in JA signaling and plant secondary metabolism (for a review, see Kazan and Manners 2013), as well as the positive role of JA in agarwood production (Ito et al. 2005, Okudera and Ito 2009, Kumeta and Ito 2010, Xu et al. 2013, Xu et al. 2016), it is interesting to ask whether the transcription factor MYC2 regulates the expression of sesquiterpene synthase in *A. sinensis*.

In this study, we first isolated and characterized the transcription factor AsMYC2 from *A. sinensis*. We provided several lines of evidence that AsMYC2 is a positive regulator of *ASS1* in the JA signaling pathway. First, AsMYC2 is an immediate JA-responsive gene that is co-induced with *ASS1* and is mainly expressed in the stems and roots of *A. sinensis*, where agarwood is usually formed (**Figs. 2B**, **3**), demonstrating that it is likely to function mostly in these parts and might be a regulator of *ASS1*. Secondly, the expression of sesquiterpene synthase genes *TPS11* and *TPS21* in Arabidopsis is up-regulated by overexpression of AsMYC2 in the *myc2-2* mutant and partly restores their response to MeJA (**Fig. 4**), which provides genetic evidence that AsMYC2 has a function similar to that of AtMYC2 in regulating sesquiterpene synthase genes through the JA signaling pathway. Thirdly, yeast one-hybrid and ChIP assays showed that AsMYC2 directly binds the *ASS1* promoter, which contains a G-box motif (**Fig. 5**). Lastly, in tobacco leaves co-transformed with both 35 S-*AsMYC2* and *ASS1* native promoter–GFP constructs, we observed that AsMYC2 specifically activates expression of *ASS1* in vivo and MeJA promoted this process (**Fig. 6**).

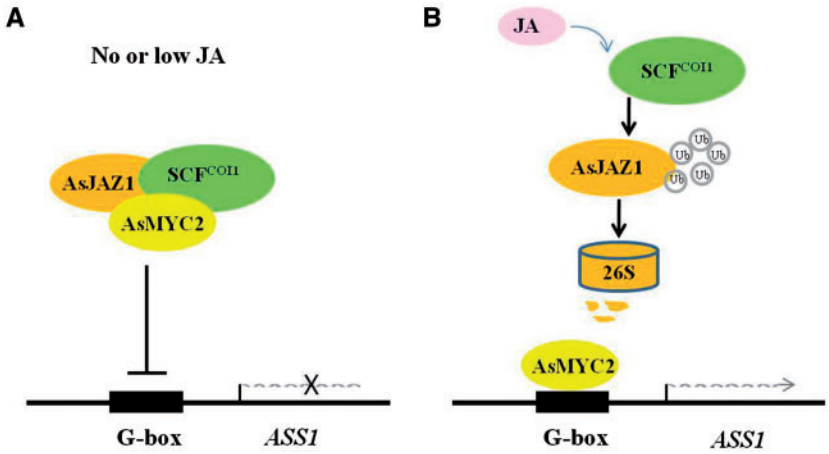

Fig. 7 A simple model for the molecular mechanism of AsMYC2 regulation of *ASS1* expression in *A. sinensis*.

Thus, the present experiments allow us to identify AsMYC2 as a positive regulator that targets *ASS1* and promotes its expression in *A. sinensis*.

Additionally, pull-down assays indicated that there exists an interaction between AsJAZ1 and AsMYC2 in vitro (**Fig. 2C**), which is consistent with the previously described working model in which in the absence of JA, MYC2 is recruited by JAZ repressors to form COI1–JAZ–MYC2 complexes and inhibit MYC2-targeted gene transcripts (Thines et al. 2007, Chico et al. 2008, Kazan et al. 2008). As ASS1 is the enzyme most responsible for the biosynthesis of agarwood sesquiterpenes and its expression is regulated at the transcriptional level by JA (Xu et al. 2013), we speculate a model, based on the data here, in which AsMYC2 regulates *ASS1* expression (**Fig. 7**): in healthy *A. sinensis*, AsMYC2 is repressed by the AsJAZ1 protein without targeting *ASS1*, but the expression of *ASS1* itself is very low and it may not even be expressed, resulting in a lack of synthesis of agarwood sesquiterpenes; in wounded *A. sinensis*, with the biosynthesis of endogenous JA, AsMYC2 is released and directly targets and activates *ASS1* expression, leading to biosynthesis of agarwood sesquiterpenes.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) and the *myc2-2* mutant were used in the generation of transgenic plants. The plants were grown in a growth chamber at 19–20°C on Murashige and Skoog (MS) medium (Sigma) at approximatley 80 μmol photons m^{-2} s^{-1} or in compost soil at approximately 120 μmol photons m^{-2} s^{-1} over a 16 h photoperiod. The tobacco *Nicotiana benthamiana* was cultivated in a greenhouse at 22°C and 1 μmol photons m^{-2} s^{-1} over a 16 h photoperiod. The *A. sinensis* calli were grown in MS medium at 25 °C in the dark.

AsMYC2 cloning with the RACE method

Total RNA was isolated from *A. sinensis* calluses using the Total RNA Purification Kit (Aidlab), supplemented with on-column DNA digestion according to the manufacturer's instructions. Single-stranded cDNA was synthesized from total RNA according to the SMART™ cDNA Library Construction Kit (Clontech) protocols. Using this cDNA as a template and based on the unigenes of 454 data (Xu et al. 2013), 5′-RACE and 3′-RACE were performed with *AsMYC2*-specific primers, following the manufacturer's instructions. The primers 3′-RACE CDS Primer A (3′-PA) and 5′-RACE CDS Primer A (5′-PA) were used as the primers to synthesize the 3′ and 5′ first-strand cDNAs, respectively. Gene-specific primers targeting *AsMYC2* were designed based on the transcriptome sequence. Antisense primers *AsMYC2*-5′GSP1 (5′-GGGTCGTTCTCGCCC TGATCCGGATTC-3′) and *AsMYC2*-5′GSP2 (5′-CAGGAACG CCTGACCCGGTA ATCCCG-3′) were synthesized for 5′-RACE, and sense primers *AsMYC2*-3′GSP1 (5′-CGCCTTGCGTGCTGTGGTCCCAAATGTG-3′) and *AsMYC2*-3′GSP2 (5′-CCC GGCCGCAAGACTCATGGCTGCATTG-3′) were synthesized for 3′-RACE. These two types of primers were both paired with 10 × Universal Primer A Mix (UPM) to amplify the 5′ and 3′ cDNA ends. The Nested Universal Primer A (NUP) was used as the nested primer. The PCR procedure employed Touchdown-PCRs: 9°C for 4 min and then 95°C for 30 s, 70°C for 30 s and 72°C for 90 s for the first cycle; the annealing temperature decreased by 1°C per cycle. After 10 cycles, the conditions were changed to 94°C for 30 s, 60°C for 30 s and 72°C for 90 s for 20 cycles. The duration of the 72°C elongation step was 10 min. The PCR product was cloned into the pGM-T vector (TIANGEN) and then sequenced.

Real-time PCR analysis

Total RNA was isolated using a Total RNA Rapid Extraction kit (BioTeke), then treated with RNase-free DNase I (TAKARA) at 37°C for 30 min to degrade genomic DNA, and purified using an RNA Purification kit (BioTeke). A 2 μg aliquot of RNA was subjected to first-strand cDNA synthesis using Moloney murine leukemia virus reverse transcriptase (Promega) and an oligo$(dT)_{18}$ primer. Real-time PCRs were performed with specific primers: for AsMYC2, forward 5′-ATGC ATGCCATCGTCCAATGG-3′ and reverse 5′-CCAGAAGCCGATGCGATTC-3′; for *ASS1*, forward 5′-CAGACATACAAGGCTGAAGAAAAG-3′ and reverse 5′-T TCTATCTTTGGTCACACCTTGG-3′; for *TPS11*, forward 5′-CACTTTGGGACAAC GACAGA-3′ and reverse 5′-CTTGGAAGTAATGAAGCTGCAAG-3′; and for *TPS21*, forward 5′-TCGCCTTGGTGTCT CCTATCAC-3′ and reverse 5′-CTTTGA ACTTCCCATTTTCGTCC-3′. The *A. sinensis Tubulin* gene (forward 5′-GCCAAGT GACACAAGCGTAGGT-3′ and reverse 5′-TCCTTGCCAGAAATAAGTTGCTC-3′) was used as an internal control. Analysis was performed using a Bio-Rad Real-Time System CFX96TM C1000 Thermal Cycler (Singapore).

Generation of *AsMYC2*-overexpressing plants

To generate a 35 S:*AsMYC2* construct, the coding sequence of the *AsMYC2* gene was amplified with the primer pair (*AsMYC2*-LF, 5′-gcTCTAGAATGACCGATT ACAGGCTCCC-3′; and *AsMYC2*-LR, 5′-cgGGATCCTTACCTTG CATCCCCCAC TTTG-3′) and inserted into a plant binary vector pBI121 at the restriction sites *Xba*I/*Bam*HI. The recombined plasmids were then introduced into *Agrobacterium tumefaciens* (LBA4404) and then transformed into *A. thaliana* plants using the floral dip method (Clough and Bent 1998). *Arabidopsis thaliana* ecotype Col-0 and the *AtMYC2* knockout mutant *myc2-2* were used in the generation of transgenic plants. The homozygous T_3 seeds of the transgenic plants were used for analysis.

In vitro pull-down assays

Coding sequences of full-length *AsMYC2* and *AsJAZ1* genes were PCR-amplified from *A. sinensis* cDNA templates, cloned into pGM-T and recombined in pGEX-4 T-1 or PET-28a to obtain GST–AsMYC2 and His-AsJAZ1 fusion proteins. Recombinant plasmids were expressed in *Escherichia coli* BL21 (DE3), and the fused proteins were purified according to standard protocols. For GST pull-down experiments, 200 μg of GST–AsMYC2 fusion protein or GST protein was added to 1 mg of His-AsJAZ1 fusion protein and incubated for 12 h at 4°C with rotation. After the samples were washed and denaturalized, the proteins were loaded on 10% SDS–polyacrylamide gels, transferred to nitrocellulose membranes and incubated with anti-His [HRP] mouse polyclonal antibody (Beijing ComWin Biotech Co., Ltd.). Similarly, for His pull-down experiments, 50 μg of His-AsJAZ1 fusion protein or His protein was added to 1 mg of GST–AsMYC2 fusion protein and incubated for 12 h at 4°C with rotation. After the samples were washed and denaturalized, they were loaded on 8% SDS–polyacrylamide gels, transferred to nitrocellulose membranes and incubated with anti-GST [HRP] mouse polyclonal antibody (Beijing ComWin Biotech Co., Ltd.).

AsMYC2–*ASS1* promoter interaction tested with yeast one-hybrid assay

Yeast one-hybrid assays were performed as described (R. Liu et al. 2013). The *ASS1* promoter was amplified by PCR using the following primer pairs: forward primer 5′-ATTGTCCGCCGCACCTGAG-3′ and reverse primer 5′-TCGGCGGCT AACATCTTCG-3′. The promoter DNA fragment was subcloned into the *Eco*RI/*Mlu*I sites of a pHIS2 vector. The one-hybrid assays were performed using the Y187 yeast strain according to the manufacturer's instructions. Yeast cells were co-transformed with a pHIS2 bait vector harboring the promoter of *ASS1* and a pGADT7 prey vector harboring the ORF of *AsMYC2*. As negative controls, the yeast cells were co-transformed with the combination of pGADT7-MYC2 and an empty pHIS2 vector, an empty pGADT7 vector and pHIS2 harboring the *ASS1* promoter, or two empty vectors, pGADT7 and pHIS2. Transformed yeast cells were first grown in SD–Trp–Leu medium to ensure that the yeast cells were successfully co-transformed, and the co-transformed yeast cells were then grown on SD–Trp–Leu–His medium plates. The SD–Trp–Leu and SD–Trp–Leu–His media were supplemented with 25 mM 3-amino-1,2,4-triazole (Sigma). The plates were then incubated for 3 d at 30 °C.

ChIP assay

For ChIP analyses, leaves (1.5 g) were cross-linked with 37 ml of formaldehyde in a vacuum for 20 min. A 2.5 ml aliquot of 2 M glycine was added to stop the cross-linking. The leaves were rinsed with water and then ground to powder with liquid nitrogen, and chromatin was extracted. The samples were then sonicated four times for 10 s at 20% power and kept on ice for 1 min during each interval to achieve an average fragment size ranging from 0.2 to 1.0 kb. Before precipitation, 1 : 10 dilutions of the supernatant were reserved as the 'input fraction'. Appropriate antibodies were added to each diluted sample, and each sample was then incubated overnight at 4°C with gentle rotation. The polyclonal antibody against MYC2 was mouse anti-MYC2 serum, and anti-acetyl-histone 3 and normal mouse IgG were used as positive and negative controls, respectively. Protein A–agarose beads (Beyotime Biotechnology) were added to the immune complex and vortexed to mix. This step was followed by standing the samples on a magnetic stand for 1 h, after which the supernatant was discarded. The beads were washed for 3–5 min each time with gentle rotation at 4°C and then centrifuged so that the supernatants could be discarded. The complexes were then eluted from the beads and centrifuged at 4°C. The elution step was repeated once, and the elution products were combined. Subsequently, 5 M NaCl was added, and the combined sample was incubated at 65°C overnight to reverse cross-linking. RNase was then added, and the sample was incubated at 37°C for 15 min. The protein was digested, and the DNA was purified using a DNA fragment purification kit (Genview). Finally, the DNA fragment concentration was determined by semi-quantitative PCR and quantitative RT-PCR using primers specifically to amplify sequences that contained the G-box (from −256 to −47) in the *ASS1* gene promoter (forward, 5′-ACAGCC CACGTGGTCATACAAG-3′; reverse, 5′-CAAGTTTGCTGTTTTGAGCGATG-3′) and the sequences outside the G-box (from −731 to −568) (forward, 5′-TGATG CGTATTTGTTCTTTCTTTTC-3′; reverse, 5′-TTGAATGGTATGAGAACCCGAA G-3′). The sequences of the three domains that were used in the ChIP assay are listed in **Supplementary Table S1**.

Transient expression assays in tobacco

Transient expression analysis was conducted by agroinfiltration of tobacco leaves according to the method of Yang et al. (2000). Six-week-old tobacco plants were used for infiltration. The recombinant plasmids containing GFP1 and AsMYC2 were individually transformed into the *A. tumefaciens* strain GV3101. *Agrobacterium tumefaciens* cells at an appropriate concentration (OD_{600} = 0.6) were collected and then resuspended in tobacco infiltration buffer (100 mM MES, 100 mM $MgCl_2$ and 10 mM acetosyringone, pH 5.7). In total, 100 μl of each bacterial suspension was infiltrated into the intercellular spaces of intact leaves using a 1 ml needleless syringe. After infiltration, tobacco plants were first grown in darkness for 12 h and then maintained in a growth chamber at 25°C for 60 h. The epidermis of the agroinfiltrated tobacco leaves was used for fluorescence observation via confocal microscopy (OLYMPUS V-TV0.5XC-3 n).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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