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NtAIDP1, a novel NtJAZ interacting protein, binds to an AT-rich region to activate the transcription of jasmonate-inducible genes in tobacco

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ABSTRACT

In plants, jasmonate ZIM-domain proteins (JAZs) act as critical regulators, interacting physically with transcription factors (TFs) and other transcriptional regulators to modulate jasmonate (JA)-responsive gene expression and participate in crosstalk with other hormone signalling pathways. Identifying novel JAZ-interacting proteins will provide new insights into JA signalling cascades in plants. Here, we performed yeast two-hybrid screening to identify 70 NtJAZ1-interacting proteins, including an A/T-rich interaction domain containing protein 1 (NtAIDP1) from JA-treated tobacco Bright Yellow-2 (BY-2) cells. NtAIDP1 is localised in the nucleus and interacts with NtJAZ1 via its C-terminal heat shock protein 20 (HSP) domain. Aside from NtJAZ1, NtAIDP1 also interacts with other JA-inducible NtJAZs, including NtJAZ2b, NtJAZ2b.2, NtJAZ5, NtJAZ7, NtJAZ11 and NtJAZ12, but not with NtJAZ3, NtJAZ3b or NtJAZ10, and interacts with NtNINJA, NtDELLA1 and NtDELLA2 in the yeast two-hybrid assay. Furthermore, NtAIDP1 binds to the AT-rich region of the GAG fragment of the putrescine *N*-methyltransferase 1a (*NtPMT1a*) promoter and activates the transcriptional activity of the GAG fragment, whereas NtMYC2a interacts with and competitively inhibits the transactivational activity of NtAIDP1 in *Arabidopsis* mesophyll protoplasts. Overexpression of *NtAIDP1* promotes the transcription of *NtPDF1.2* and *NtJAZ1*, but has little effect on the expression of *NtPMT1a*, quinolinate phosphoribosyltransferase 2 (*NtQPT2*), and *NtMYC2a* in tobacco. These results indicate that NtAIDP1 is a new component of the JA signalling pathway and is involved in JA-regulated gene expression.

1. Introduction

Jasmonates (JAs) are a class of polyunsaturated fatty-acid-derived phytohormones that regulate diverse aspects of plant development and immunity, including male sterility, sex determination of plants, growth inhibition, the production of secondary metabolites, defence responses to attack by herbivorous insects or necrotrophic pathogens, and biological responses to abiotic stresses (Ali and Baek, 2020; Browse, 2009; Chini et al., 2016; Goossens et al., 2016; Shukla et al., 2020; Xie et al., 1998; Zhai et al., 2017). In plants, JA is perceived by the co-receptor of the F-box protein coronatine insensitive 1 (COI1) and repressor protein jasmonate ZIM-domain (JAZ), triggering the ubiquitylation and degradation of JAZs to release basic helix–loop–helix transcription factors (bHLHs) and increasing the expression of JA-inducible genes (Chini et al., 2007; Sheard et al., 2010; Thines et al., 2007; Xie et al., 1998). In the absence of JA, JAZs recruit a co-repressor complex that includes the novel interactor of JAZ (NINJA) and TOPLESS proteins to repress the transactivational activity of bHLHs in *Arabidopsis*

Abbreviations: NtAIDP1, tobacco A/T-rich interaction domain containing protein 1; bHLH, basic helix–loop–helix transcription factors; BY-2, tobacco Bright Yellow-2 cells; COI1, coronatine insensitive 1; EMSA, electrophoretic mobility shift assay; GAG motif, G-box element AT-rich region and a GCC-motif; HSP domain, heat shock protein 20 domain; JA, jasmonate; JAZ, jasmonate ZIM-domain protein; LCI, firefly luciferase complementation imaging assay; PMT, *N*-methyltransferase; qPCR, real-time quantitative PCR; NtQPT2, quinolinate phosphoribosyl transferase 2.

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(Pauwels et al., 2010). Therefore, JAZ proteins play a dual role, acting as transcriptional repressors in the absence of JA and hormone co-receptors in the presence of JA.

JAZ proteins belong to the plant-specific TIFY family, which are characterised by two conserved motifs: a TIF[*F/Y*]XG motif (also called the zinc-finger protein expressed in the inflorescence meristem [ZIM] motif), which is vital for dimerisation and interaction with other JAZs and NINJA (Pauwels et al., 2010; Pauwels and Goossens, 2011), and a C-terminal Jas domain that distinguishes JAZs from other TIFY proteins and is responsible for interactions with COI1 and transcription factors, nuclear localisation of the JAZ proteins, and JAZ protein degradation (Chini et al., 2007; Melotto et al., 2008; Sheard et al., 2010; Thines et al., 2007; Yan et al., 2007). Previous studies have shown that plant genomes contain multiple members of the JAZ family, for example, 13 *Arabidopsis* JAZs (Chini et al., 2007; Thines et al., 2007; Thireault et al., 2015), 15 rice JAZs (Ye et al., 2009) and 17 tobacco JAZs (Yang et al., 2015). Most JAZ genes contain a conserved Jas intron that differentiates multiple truncated JAZ variants without an intact C-terminal Jas motif (Chung et al., 2010; Chung and Howe, 2009; Moreno et al., 2013; Yan et al., 2007). These splice variants have reduced capacity to form stable complexes with COI1 in the presence of JA, but retain the ability to repress MYC2, leading to dominant repression of JA signalling in transgenic *Arabidopsis* plants expressing these JAZ splice variants (Chung et al., 2010; Chung and Howe, 2009; Moreno et al., 2013). In addition, Shoji et al. (2008) showed that both RNA interference (RNAi)-mediated suppression of NtCOI1 and non-degradable NtJAZs strongly reduce JA-inducible putrescine *N*-methyltransferase (*PMT*) expression and total alkaloid accumulation in tobacco hairy roots and BY-2 cells, indicating that NtJAZs act as a transcriptional repressor to regulate *PMT* transcription and nicotine formation in tobacco. However, other studies have found that RNAi-mediated knockdown of JA-degradable *NaJAZh* or NtJAZ1, NtJAZ3a, NtJAZ7 and NtJAZ10 suppresses nicotine biosynthesis and significantly reduces nicotine levels in transgenic plants and tobacco Bright Yellow-2 (BY-2) cells (Oh et al., 2012; Yang et al., 2015). These results suggest that different members of the JAZ family play specific roles in JA signalling cascades. Therefore, it is essential to identify novel regulators that interact with NtJAZs in plants.

In plants, JAZ proteins directly interact with MYC and MYB transcription factors to suppress the expression of JA-responsive genes (Ali and Baek, 2020; Zhai et al., 2017). In addition, WRKY57 interacts with JAZ to integrate JA and auxin signalling and thus regulate JA-induced leaf senescence (Jiang et al., 2014). Zhu et al. (2011) showed that JAZ proteins physically interact with and repress the *Arabidopsis* ethylene-stabilised transcription factors (EIN3/EIL1), integrating the JA and ethylene signalling pathways in the regulation of plant development and defence. Moreover, DELLA proteins and JAZs interact with the WD-repeat/bHLH/MYB complex to mediate synergistic activity between gibberellic acid (GA) and JA signalling for the regulation of trichome development and anthocyanin biosynthesis in *Arabidopsis* (Qi et al., 2014; Xie et al., 2016). These results suggest that JAZ proteins act as a master regulator, modulating the JA signalling response and participating in crosstalk with other hormone signalling pathways in plants. In this study, using yeast two-hybrid screening techniques, we identified 70 NtJAZ1-interacting proteins in methyl jasmonate (MeJA)-treated BY-2 cells. Then, one NtJAZ1-interacting protein, A/T-rich interaction domain containing protein 1 (NtAIDP1), was characterised as a transcription factor that binds the AT-rich region to regulate the expression of JA-inducible genes in tobacco.

2. Materials and methods

2.1. cDNA library construction and yeast two-hybrid screening

Nicotiana tabacum L. cv. BY-2 suspension cultures were grown, maintained and treated with 100 μM MeJA as described previously (Yang et al., 2015). mRNA was extracted from MeJA-treated BY-2 cells using the FastTrack® MAG mRNA Isolation Kit (Thermo Fisher Scientific, USA) and a cDNA library was constructed using *pDONR™222* with the CloneMiner™ II cDNA Library Construction Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Then the cDNA library was recombined into the destination vector *pDEST-GADT7* for yeast two-hybrid screening. *pDEST-GBKT7-NtJAZ1* and *pDEST--GADT7-library* were co-transformed into *Saccharomyces cerevisiae* AH109 as described in the yeast protocols handbook (TaKaRa, Japan). The positive colonies were grown on yeast synthetic defined (SD) medium lacking His, Leu and Trp (SD-3). The yeast transformation efficiency was confirmed through enumeration of the yeast colonies grown on SD medium lacking His and Leu (SD-2). The plasmid was extracted from these colonies, transformed into *Escherichia coli*, and then sequenced to identify the inserted cDNA fragment.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from tobacco plants using TRIzol (Thermo Fisher Scientific) and purified with the PureLink RNA Mini Kit (Thermo Fisher Scientific) combined with the PureLink DNase kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The quality of the purified RNA extract was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) with a readout RNA integrity number (RIN) value of 9.2, and the quantity of RNA was measured using a Nanodrop ND-1000 spectrophotometer. The first-strand cDNA was synthesised with TransScript® II First-Strand cDNA Synthesis SuperMix (Transgen, China) for subsequent experiments.

2.3. DNA cloning and vector construction

The coding sequence (CDS) of *NtAIDP1*, *NtMYC2a*, tobacco *novel interactor of JAZ* (*NtNINJA*), *NtDELLA1*, *NtDELLA2*, tobacco *Groucho/Tup1-type co-repressor TOPLESS* (*TPL*) and *TOPLESS-related gene* (*TPR*) was amplified via polymerase chain reaction (PCR) and inserted into the *pE2c* (including His-tag) or *pE3c* (including Myc-tag) plasmid (Addgene, http://www.addgene.org/). The CDS of *NtAIDP1* was amplified and inserted into the *pE6c* (including EYFP-tag) plasmid. In addition, the N-terminal fragment of *NtAIDP1* (*NtAIDP1n*, 1–1311bp), the C-terminal fragment of *NtAIDP1* (*NtAIDP1c*, 1312–1707 bp), *NtJAZ1n* (1–540 bp) and *NtJAZ1c* (541–717 bp) were amplified and inserted into the *pENTR™/D-TOPO™* plasmid (Thermo Fisher Scientific).

To generate plant expression vectors, the target genes on *pE2c*, *pE3c* or *pE6c* were inserted into *pMDC32* using the Gateway LR II kit (Invitrogen, USA). To produce constructs for yeast two-hybrid assays, the target genes on pE2c or pENTR™/D-TOPO™ were separately inserted into *pDEST-GADT7* as prey or into *pDEST-GBKT7* as bait using the Gateway LR II kit. For transactivation activity assays, four copies of the GAG fragment (4 × GAG) from the promoter of *NtPMT* (Sears et al., 2014) were amplified and inserted into the *Bam*HI and *Asc*I restriction site of *pGPTV* to generate the P_{4*GAG}*:GUS* construct. To produce the MBP-NtJAZ1 fusion protein, full-length *NtJAZ1* was amplified and subcloned into the *Eco*RI and *Hind*III restriction sites of the plasmid *pHis-6p-MBP-RSFD*. To produce NtAIDP1-His fusion protein, *pE2c-NtAIDP1* was recombined with the binary vector *pET28a-DEST* using the Gateway LR II kit. For the firefly luciferase complementation imaging (LCI) assay, the *NtAIDP1*, *NtAIDP1n* and *NtAIDP1c* fragments were separately inserted at the *Bam*HI and *Xho*I restriction sites of *35S:nLUC* to generate the *35S:NtAIDP1-nLUC*, *35S:NtAIDP1n-nLUC* and *35S:NtAIDP1c-nLUC* vectors, and the *NtJAZ1* and *NtMYC2a* fragments were separately inserted at the *Kpn*I and *Sal*I restriction sites of *35S:cLUC* to generate the *35S:cLUC-NtJAZ1* and *35S:cLUC-NtMYC2a* vectors.

All clones and vectors were confirmed through sequencing. All primers used are listed in Supplementary Table S1.

2.4. Generation of transgenic plants

Tobacco plants were germinated and grown to maturity under 16/8 h light/dark illumination in pots in a greenhouse. For cultivation of aseptic seedlings, seeds are first surface-sterilised with 75 % alcohol for 2 min and washed with sterile water for 1 min, then deep-sterilised with 10 % sodium hypochlorite for 8–10 min and washed with sterile water five times, for about 1 min each time. The sterilised seeds were planted in Murashige and Skoog (MS) solid medium (containing 30 g/L sucrose, 8 g/L agar pH = 5.8) in a 50 mL flask and grown in a light incubator under 16/8 h light/dark at 25 °C for 4–5 weeks. The construct *35S:NtAIDP1-Myc* was introduced into *Agrobacterium* GV3101 and transfected into leaf disks of 4-week-old aseptic tobacco plants according to established protocols (Gallois and Marinho, 1995). The transformants were selected on MS medium containing 0.2 mg/L indole-3-acetic acid (IAA), 10 mg/mL hygromycin B and 500 mg/L cefotaxime (Sigma-Aldrich, USA), and then transferred to pots in a greenhouse for seed collection. T3-generation homozygous transformants carrying a single insertion were used for subsequent experiments.

2.5. Real-time quantitative PCR (RT-qPCR)

RT-qPCR was performed with GoTaq qPCR Master Mix (Promega, USA) and the ABI 7500 Real-time Detection System according to the manufacturers' protocols. The program was 2 min at 50 °C, 10 min at 95 °C, and then 40 cycles of 15 s at 95 °C and 60 s at 60 °C. *Actin* was used as an internal reference gene. Experiments were repeated at least three times. The primer sequences are listed in Supplementary Table S1.

2.6. Yeast two-hybrid assay

To confirm the interaction between NtAIDP1 and NtJAZ1, we inserted full-length *NtAIDP1*, *NtAIDP1n*, or *NtAIDP1c* into *pDEST-GBKT7* as bait and full-length NtJAZ1, *NtJAZ1n* or *NtJAZ1c* as prey into *pDEST-GADT7* for co-transformation of AH109. In addition, we inserted full-length *NtMYC2a* into *pDEST-GADT7*, and then co-transformed AH109 with *NtAIDP1*, *NtAIDP1n* or *NtAIDP1c* in *pDEST-GBKT7* to detect the interaction of NtAIDP1 with NtMYC2a. We further observed the interaction of NtAIDP1 fused to *pDEST-GADT7* as prey with other MeJA-inducible NtJAZs, including NtJAZ2b, NtJAZ2b.2, NtJAZ3, NtJAZ3b, NtJAZ5, NtJAZ7, NtJAZ10, NtJAZ11 and NtJAZ12, as well as with the other JA pathway proteins NtNINJA, NtTPL and NtTPR, and with the gibberellin acid (GA) signalling components NtDELLA1 and NtDELLA2, which were fused to GAL4-BD in pGBKT7 as bait. The positive yeast colonies were screened on SD-3 medium and on yeast SD medium lacking His, Leu, Trp and Ade (SD-4) with the addition of X-α-GAL.

2.7. Subcellular localisation of NtAIDP1

Arabidopsis (Col-0 ecotype) growth, mesophyll protoplast isolation and transformation were performed according published methods (Liang et al., 2015). The plant expression vector *35S:NtAIDP1-EYFP* and mCherry-labelled nucleus marker were co-transformed into *Arabidopsis* mesophyll protoplasts (2×10^5). After culturing at 22 °C for 18–24 h in the dark, EYFP and mCherry fluorescence was visualised using a Zeiss LSM 700 confocal microscope.

2.8. Electrophoretic mobility shift assay (EMSA)

The fusion protein His-NtAIDP1 was expressed in *E. coli* by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and purified using GE Healthcare Lifescience™ Ni Sepharose™ 6 Fast Flow (Thermo Fisher Scientific). The GAG oligonucleotide probe was synthesised and labelled with biotin fluorescein (FAM) at the 5′ end. The probe–protein complex was incubated in 1× binding buffer (5% glycerol, 5 mM $MgCl_2$) at room temperature for 20 min and separated via polyacrylamide gel electrophoresis. Unlabelled probe was used as a competitor in the probe–protein complex solution.

2.9. Recombinant protein production and pull-down assay

The fusion proteins His-NtAIDP1 and MBP-NtJAZ1 were expressed in *E. coli* BL21 (DE3) after being induced with IPTG. Briefly, *E. coli* BL21 cells containing *pDEST-ET28a-NtAIDP1* and *pHis-6p-MBP-NtJAZ1* were induced for 8 h with 0.1 mM IPTG at 16 °C, and then subjected to ultrasonic treatment in lysis buffer (50 mM Tris−HCl [pH 7.4], 200 mM NaCl, 1 mM β-mercaptoethanol, 0.5 % Triton X-100, 10 % glycerine). After centrifugation, the supernatant was used for a pull-down assay. Ni resin was used to pull down the complex of His-NtAIDP1 and MBP-NtJAZ1. MBP antibody (ZA-0634, ZSGB-BIO, China) was used to detect MBP-NtJAZ1 and MBP (used as the control).

2.10. LCI assay

The LCI assay was conducted as described previously (Liu et al., 2018). *Agrobacterium* cells containing *35S:NtAIDP1-nLUC*, *35S:NtAIDP1n-nLUC*, *35S:NtAIDP1c-nLUC*, *35S:nLUC*, *35S:cLUC-NtJAZ1*, *35S:cLUC-NtMYC2a* or *35S:cLUC* were suspended in infiltration medium (MS medium containing 10 mM 2-(N-morpholino)ethanesulfonic acid [MES, pH 5.6] and 150 mM acetosyringone) at an optical density at 600 nm (OD_{600}) of 1.0, and each pair of constructs was mixed in equimolar ratios for infiltration into 4-week-old *Nicotiana benthamiana* leaves. After incubation overnight, luciferase activity was assayed using the Lumazone FA1300 Imaging System (Roper Scientific, USA).

2.11. Transactivation analyses

The constructs *P_{4*GAG}:GUS* as a reporter, *NtAIDP1-c-myc* and *MYC2a-HA* as effector and *35S:LUC* (firefly luciferase) as an internal standard were co-transformed into *Arabidopsis* mesophyll protoplasts (2×10^5). After culturing at 22 °C for 18–24 h in the dark, the protoplasts were lysed and the supernatants were assayed for β-glucuronidase (GUS) and luciferase (LUC) activities. GUS activity was normalised to LUC activity in each transfection group, and the data are presented as the average of three biological replicates.

2.12. Chromatin immunoprecipitation–quantitative PCR (ChIP-qPCR) analyses

ChIP-qPCR was performed according to a published method (Liu et al., 2018) with some modifications. One gram of leaves from transgenic *Nicotiana benthamiana* plants overexpressing *NtAIDP1* was harvested and cross-linked in 1% (v/v) formaldehyde at 4 °C for 10 min, followed by neutralisation with 0.188 g glycine. The chromatin–protein complexes were isolated, resuspended and sonicated to reduce the average DNA fragment size to ~500 bp. Anti-MYC antibody (7 μg/sample) was incubated with Dynabeads Protein G (40 μL/sample) at 4 °C for at least 6 h, and then incubated with the chromatin–protein complexes overnight at 4 °C. The precipitated DNA fragments were recovered and quantified through qPCR using GoTaq qPCR Master Mix. The relative enrichment of DNA was calculated by normalising the amount of target DNA to the internal control, which is the first exon of *NtPMT1a* gene. IgG was used as the experiment control, and 50 μL sheared chromatin was used as the input control.

2.13. Statistical analyses

Two-tailed Student's *t*-tests were performed for comparisons of any two groups within each experiment. Probability values of <0.05 were considered statistically significant.

3. Results

3.1. Identification of NtJAZ1-interacting proteins through yeast two-hybrid screening

NtJAZ proteins are critical components of JA signalling cascades regulating the transcription of JA-inducible genes in *Nicotiana* species (Oh et al., 2012; Shoji et al., 2008; Yang et al., 2015). Therefore, it is essential to explore the novel NtJAZ-interacting proteins and characterise their functions in tobacco. Here, we constructed a cDNA library of MeJA-treated BY-2 cells and performed yeast two-hybrid screening using *BD-NtJAZ1* as the bait. After the first round of screening on SD-4 medium with X-α-GAL, we obtained 102 candidate NtJAZ1-interacting clones from 2.15×10^7 total cDNA clones. Then, we used the extracted plasmids from these clones as prey for co-transformation with *BD-NtJAZ1* into yeast for the second round of screening. Next, the repeat clones were removed via sequencing, and finally, 70 NtJAZ1-interacting genes were identified (Supplementary Table S2). Among these 70 NtJAZ1-interacting proteins, we found that NtJAZ1/3/3b/12 interacts with NtJAZ1, as previously reported (Yang et al., 2015). We also found a homologue of NINJA, which was previously characterised as a negative regulator of jasmonate responses in *Arabidopsis* (Pauwels et al., 2010). Interestingly, an A/T-rich interaction domain-containing protein, designated NtAIDP1, was found to interact with NtJAZ1. Therefore, we characterised the role of NtAIDP1 in mediating JA signalling.

3.2. NtAIDP1 is localised in the nucleus and interacts with NtJAZ1

The CDS of full-length *NtAIDP1* encodes a protein composed of 569 amino acids (aa) and containing two conserved domains, the A/T-rich interaction domain (ARID) and heat shock protein 20 (HSP) domain (Figure S1). Then, we searched the tobacco genome sequence with the protein sequence of NtAIDP1 using the Basic Local Alignment Search Tool (BLAST) and found 12 homologues containing ARID and other conserved domains, such as the ELM2 (Egl-27 and MTA1 homology 2) domain, SANT (SWI3, ADA2, N-CoR and TFIIIB) domain, plant homeodomain zinc finger (PHD), and high-mobility group (HMG) box (Fig. 1). In addition, we found 10 and 6 homologues in the *Arabidopsis* and rice genomes that exclusively contain ARID, and generated a phylogenetic tree using the protein sequences of these 28 AIDPs, which could be separated into four distinct clades designated HSP, PHD, HMG and ELM2 (Fig. 1). As some homologues have two domains of ELM2 and SANT, these proteins show a closer phylogenetic relationship than other proteins, merging into the ELM2 clade (Fig. 1).

To characterise the interaction domain between NtAIDP1 and NtJAZ1 in detail, we constructed full-length *NtAIDP1*, *NtAIDP1n* (1–1311 bp) and *NtAIDP1c*, (1312–1707 bp) as the bait and full-length *NtJAZ1*, *NtJAZ1n* (1–540 bp) and *NtJAZ1c* (541–717 bp) as prey for yeast two-hybrid assays (Figure S1). We found that only NtAIDP1c interacts with full-length NtJAZ1 in the yeast two-hybrid system (Fig. 2A). Next, we performed an *in vitro* pull-down experiment using purified MBP-NtJAZ1 and His-NtAIDP1 to reveal the interaction of His-NtAIDP1

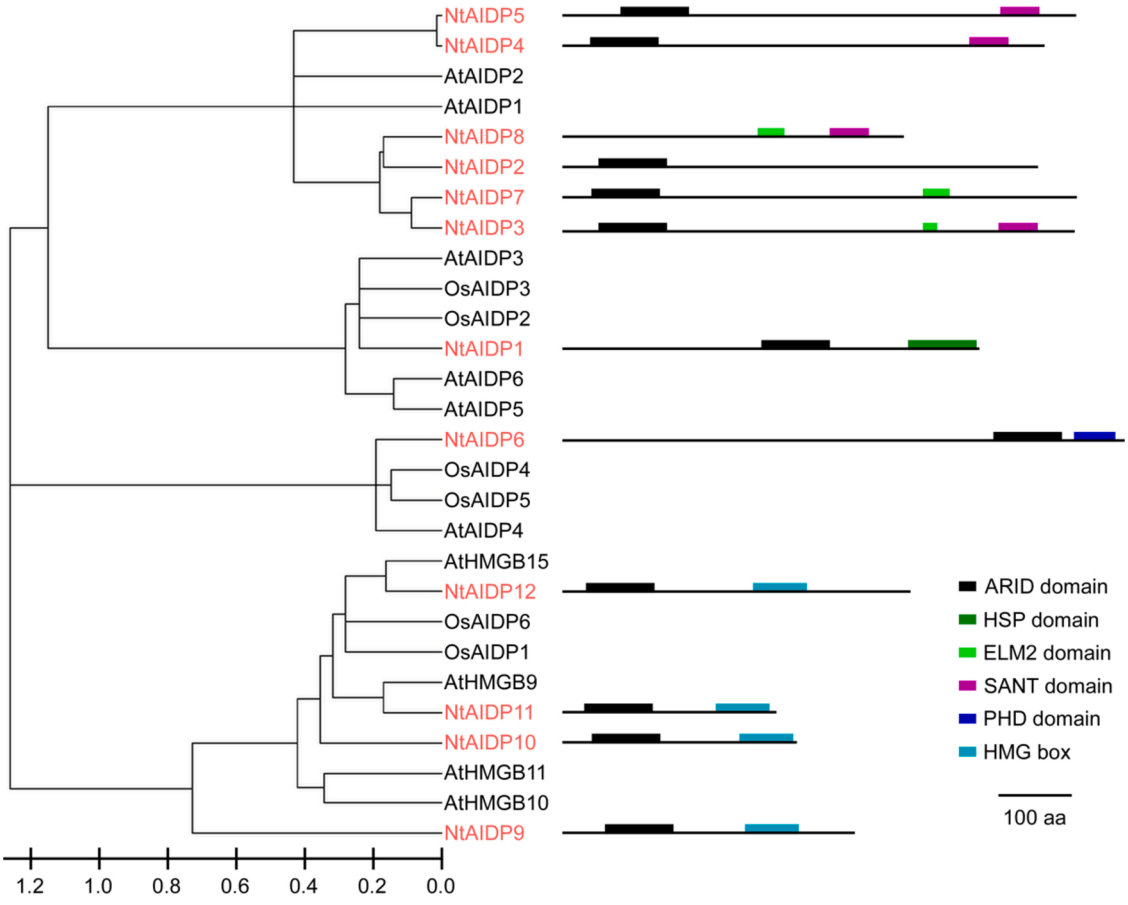

Fig. 1. Phylogenetic and primary structure analyses of AT-rich interaction domain-containing protein (AIDP) family in tobacco. **(left)** Phylogenetic tree constructed using the amino acid sequences of 12 members of the AIDP family in tobacco, 10 members in *Arabidopsis*, and 6 in rice. The tree was generated using MEGA-X software with the neighbour-joining algorithm. Scale bar indicates the number of amino acid substitutions per site. **(right)** Primary structure of NtAIDP1 and its 11 homologues in tobacco. ARID indicates the A/T-rich interaction domain. HSP indicates the heat shock protein 20 domain. ELM2 indicates the Egl-27 and MTA1 homology 2 domain. SANT indicates the SWI3, ADA2, N-CoR and TFIIIB domain. PHD indicates the plant homeodomain zinc finger. HMG box indicates the high-mobility group box.

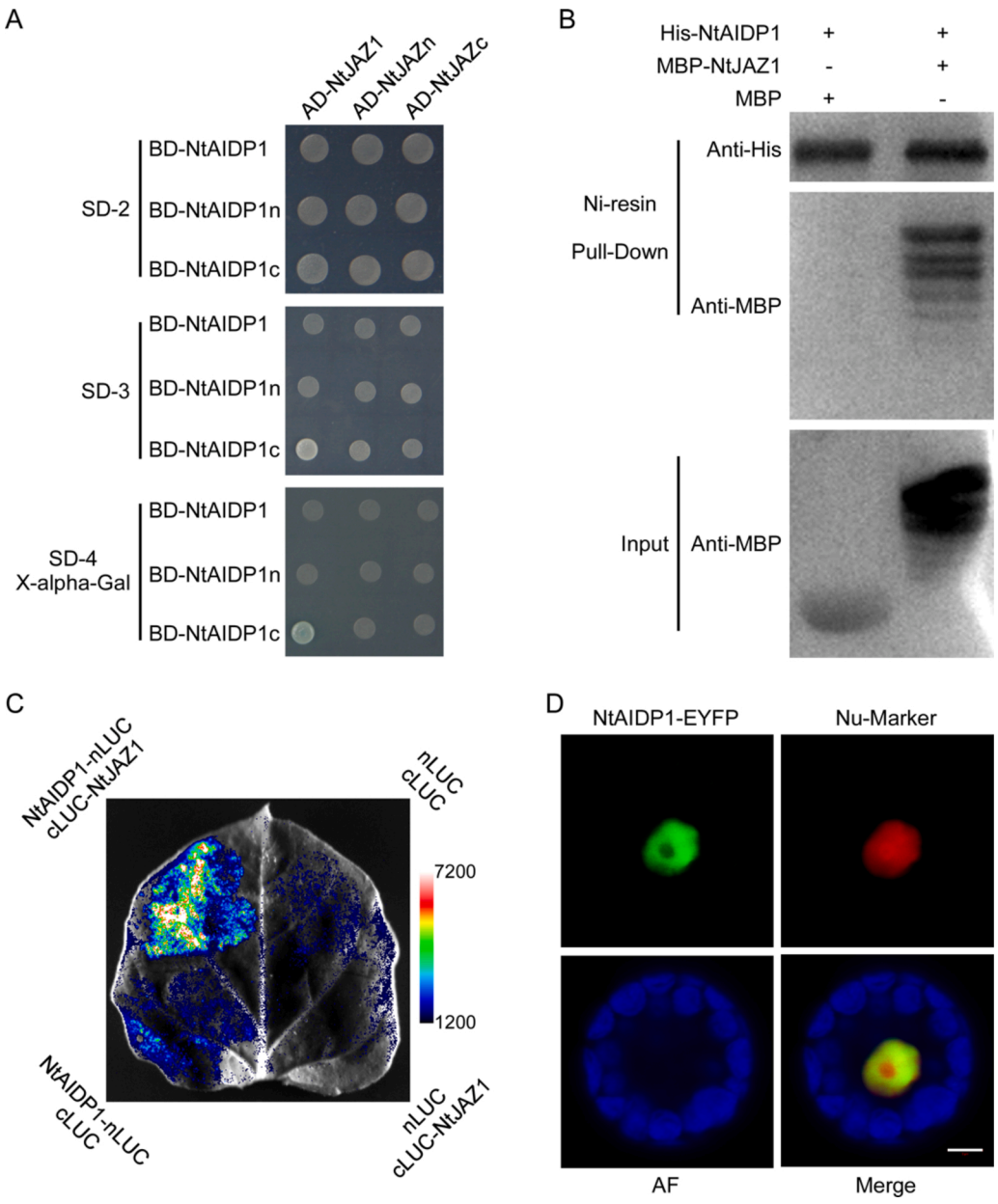

Fig. 2. NtAIDP1 interacts with NtJAZ1 and is localised in the nucleus. **(A)** Pairwise interactions between full-length NtAIDP1, N-terminal fragment of NtAIDP1 (NtAIDP1n) or C-terminal fragment of NtAIDP1 (NtAIDP1c) as the bait and full-length NtJAZ1, N-terminal fragment of NtJAZ1 (NtJAZ1n) or C-terminal fragment of NtJAZ1 (NtJAZ1c) as the prey detected using the yeast two-hybrid assay. SD-2 indicates yeast synthetic drop-out (SD) medium lacking Leu and Trp; SD-3 indicates SD medium lacking Leu, Trp and His; SD-4 indicates SD medium lacking Leu, Trp, His and Ade. X-α-Gal was added to SD-4 for blue–white screening. **(B)** *in vitro* interaction of NtAIDP1 with NtJAZ1 detected through the pull-down assay. His-NtAIDP1, MBP-NtJAZ1 and MBP were separately expressed in *E. coli* and purified using Ni or amylose resin. Then, His-NtAIDP1 was incubated with MBP-NtJAZ1 or MBP and pulled down with anti-His antibody. MBP-NtJAZ1 or MBP was detected through immunoblotting using anti-MBP antibody. **(C)** *in vivo* interaction between NtAIDP1 and NtJAZ1 monitored through the firefly luciferase complementation imaging (LCI) assay in tobacco leaves. Full-length *NtAIDP1* was fused to the 5′ end of the N-terminus of luciferase (*NtAIDP1-nLUC*), and full-length *NtJAZ1* was fused to the 3′ end of the C-terminal fragment of luciferase (*cLUC-NtJAZ1*). Empty vectors were used as negative controls. **(D)** Subcellular localisation of NtAIDP1-EYFP fusion protein in *Arabidopsis* mesophyll protoplast. EYFP indicates fluorescence of NtAIDP1-EYFP; Nu-marker indicates fluorescence of mCherry-labelled nuclear marker; AF indicates chloroplast auto-fluorescence; Merge is a superposition of the green, red and blue channels. Scale bar: 5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

with MBP-NtJAZ1, but not MBP (Fig. 2B). Moreover, an LCI assay in tobacco leaves using *35S:NtAIDP1-nLUC* and *35S:cLUC-NtJAZ1* showed a fluorescence signal for the combination of *NtAIDP1-nLUC* and *cLUC-NtJAZ1*, indicating that NtAIDP1 interacts with NtJAZ1 *in vivo* (Fig. 2C). In addition, we co-expressed NtAIDP1-EYFP fusion protein with mCherry-labelled nuclear marker (Nu-Marker) in *Arabidopsis* mesophyll protoplasts and found that the fluorescence of NtAIDP1-EYFP merges with Nu-Marker in the nucleus, demonstrating that NtAIDP1, similar to NtJAZ1, is localised in the nucleus (Fig. 2D). These results indicate that NtAIDP1 interacts with NtJAZ1 in the nucleus of plant cells.

3.3. In addition to NtJAZ1, NtAIDP1 interacts with other MeJA-inducible NtJAZs, NtNINJA and NtDELLAs

In a prior study, we characterised nine MeJA-inducible NtJAZs, including NtJAZ1, some of which exhibit homo- and heteromeric interactions in the yeast two-hybrid assay (Yang et al., 2015). Therefore, we explored the interactions between NtAIDP1 and eight other MeJA-inducible NtJAZs. We found that NtJAZ2b, NtJAZ2b.2, NtJAZ5, NtJAZ7, NtJAZ11 and NtJAZ12 interact with NtAIDP1c, whereas NtJAZ3, NtJAZ3b and NtJAZ10 do not, in the yeast two-hybrid assay (Fig. 3). In addition, previous studies have shown that JAZ proteins interact with NINJA in recruiting TPL and TPR to regulate JA signalling, and also interact with DELLA proteins to mediate the crosstalk between JA and GA signalling in *Arabidopsis* (Hou et al., 2010; Pauwels et al., 2010). Therefore, we performed a yeast two-hybrid assay to monitor the interaction between NtAIDP1 and the tobacco homologues of NINJA, TPL, TPR and DELLA, and found that NtAIDP1 interacts with NtNINJA, NtDELLA1 and NtDELLA2, but not with NtTPL or NtTPR (Fig. 3). These results suggest that NtAIDP1 is involved in the crosstalk between JA and GA signalling at the transcriptional and post-translational levels.

3.4. NtAIDP1 binds to the AT-rich region of the GAG motif in the NtPMT1a promoter and activates the transcriptional activity of the GAG motif

Previous research identified a GAG motif containing a G-box element, AT-rich region and a GCC-motif located –103 to –56 bp from

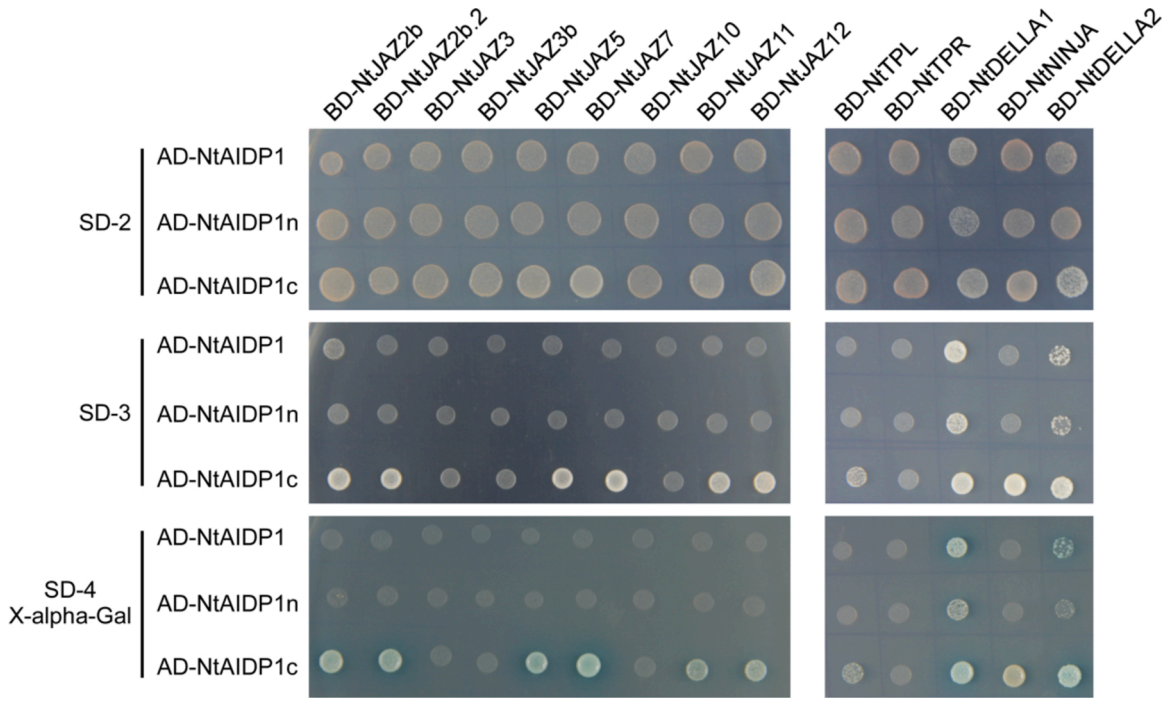

Fig. 3. Interactions between NtAIDP1 and other MeJA-inducible NtJAZs and JA signalling-associated proteins in yeast-two hybrid assay.
The pairwise interactions of NtJAZ2b, NtJAZ2b.2, NtJAZ3, NtJAZ3b, NtJAZ5, NtJAZ7, NtJAZ10, NtJAZ11, NtJAZ12, NtTPL (TOPLESS), NtTPL (TOPLESS-related protein), Novel Interactor of JAZ (NtNINJA), NtDELLA1 and NtDELLA2 as bait with NtAIDP1, NtAIDP1n and NtAIDP1c as prey detected using the yeast two-hybrid assay, respectively. SD-2 indicates yeast synthetic drop-out (SD) medium lacking Leu and Trp; SD-3 indicates SD medium lacking Leu, Trp and His; SD-4 indicates SD medium lacking Leu, Trp, His and Ade. X-α-Gal was added to SD-4 for blue–white screening.

the transcriptional start site of putrescine *N*-methyltransferase 1a (*NtPMT1a*) that is required for MeJA-inducible transcription of *NtPMT1a* (Xu and Timko, 2004). In addition, bHLH transcription factors specifically bound to the G-box element and ethylene response factors (ERFs) bound to the GCC box to separately regulate the expression of *NtPMT1a* (Sears et al., 2014; Shoji and Hashimoto, 2012; Shoji et al., 2010; Sui et al., 2019; Todd et al., 2010; Zhang et al., 2012). However, the transcription factors that bind to the AT-rich region remain uncharacterised. Therefore, we hypothesized that NtAIDP1 binds to the AT-rich region and activates the transcriptional activity of GAG. The results of EMSA showed that His-NtAIDP1 specifically binds to the biotin FAM-labelled AT-rich probe, which competes with unlabelled AT-rich probe (Fig. 4A). Furthermore, ChIP-qPCR revealed that the GAG fragment was significantly enriched due to NtAIDP1-Myc in transgenic tobacco plants overexpressing *NtAIDP1-Myc* (Fig. 4B). These results suggest that NtAIDP1 is a transcription factor that binds to the AT-rich region to regulate the expression of JA-inducible genes.

MYC2 interacts with JAZ proteins to mediate JA signalling cascades (Chini et al., 2007; Zhang et al., 2012). Therefore, we monitored the interaction between NtAIDP1 and NtMYC2a. Yeast two-hybrid assays using NtAIDP1, NtAIDP1n and NtAIDP1c as bait showed that these proteins have no apparent interactions with NtMYC2a (Figure S2). Then, we performed the LCI assay with tobacco leaves and observed strong fluorescence signals between NtAIDP1c-nLUC and cLUC-NtMYC2a, and NtAIDP1-nLUC and cLUC-NtMYC2a, but not between NtAIDP1n-nLUC and cLUC-NtMYC2a (Fig. 4C), indicating that the interaction between NtAIDP1 and NtMYC2a depends on its C-terminal fragment, in accordance with the interaction between NtAIDP1 and NtJAZs. Moreover, using *p4XGAG:GUS* as a reporter vector and firefly luciferase (fLUC) as an internal control for transient transformation of *Arabidopsis* mesophyll protoplasts (Fig. 4D), we found that NtAIDP1 or NtMYC2a alone can activate the transcriptional activity of the GAG motif but their co-transformation strongly inhibited the transcriptional activity of the GAG motif (Fig. 4E). These results indicate that NtAIDP1 activates the transcription of *NtPMT1a* by binding to the AT-rich region, while MtMYC2a competitively inhibits the transactivational function of NtAIDP1.

3.5. *Overexpression of NtAIDP1 promotes the transcription of NtPDF1.2 and NtJAZ1, but not NtPMT1a, NtQPT2 and NtMYC2a*

To further explore the role of NtAIDP1 in regulating JA-inducible genes, we first observed the expression of *NtAIDP1* in plants. In BY-2 cells, we found that the expression of *NtAIDP1* is not MeJA-inducible (Figure S3A). In tobacco plants, transcription of *NtAIDP1* is generally distributed among the different tissues, including the root, stem, leaf and flower, although the relative expression level of *NtAIDP1* is higher in the root and stem than the leaf and flower (Figure S3B). Next, we generated transgenic tobacco plants overexpressing *NtAIDP1-Myc* to explore the role of NtAIDP1 on the transcription of specific JA-inducible genes. Plant defensin 1.2 (PDF1.2) is one marker gene of JA-dependent signalling pathways and responsible for fungal pathogen resistance (Pieterse et al., 2009; Seo et al., 1997). In addition, another key gene in nicotine biosynthesis, quinolinate phosphoribosyl transferase 2 (*NtQPT2*), is also a JA-inducible gene (Yang et al., 2015). The promoter region of NtQPT2 contains a GAG motif (Figure S4). Here, we found that overexpression of *NtAIDP1* activates transcription of *NtPDF1.2* and *NtJAZ1,* but does not promote the expression of *NtPMT1a, NtQPT2* or *NtMYC2a* in three independent *35S:NtAIDP1-Myc* lines, namely #5, #13 and #15 (Fig. 5). These results suggest that NtAIDP1 functions as a transcription factor to activate the transcription of JA-responsive genes.

4. Discussion

In this study, NtAIDP1 was identified as a novel interactor of NtJAZ1, which functions as a transcription factor, binding to the AT-rich region of the promoters of JA-responsive genes to activate their expression in tobacco. NtAIDP1 is composed of two conserved domains: ARID, which

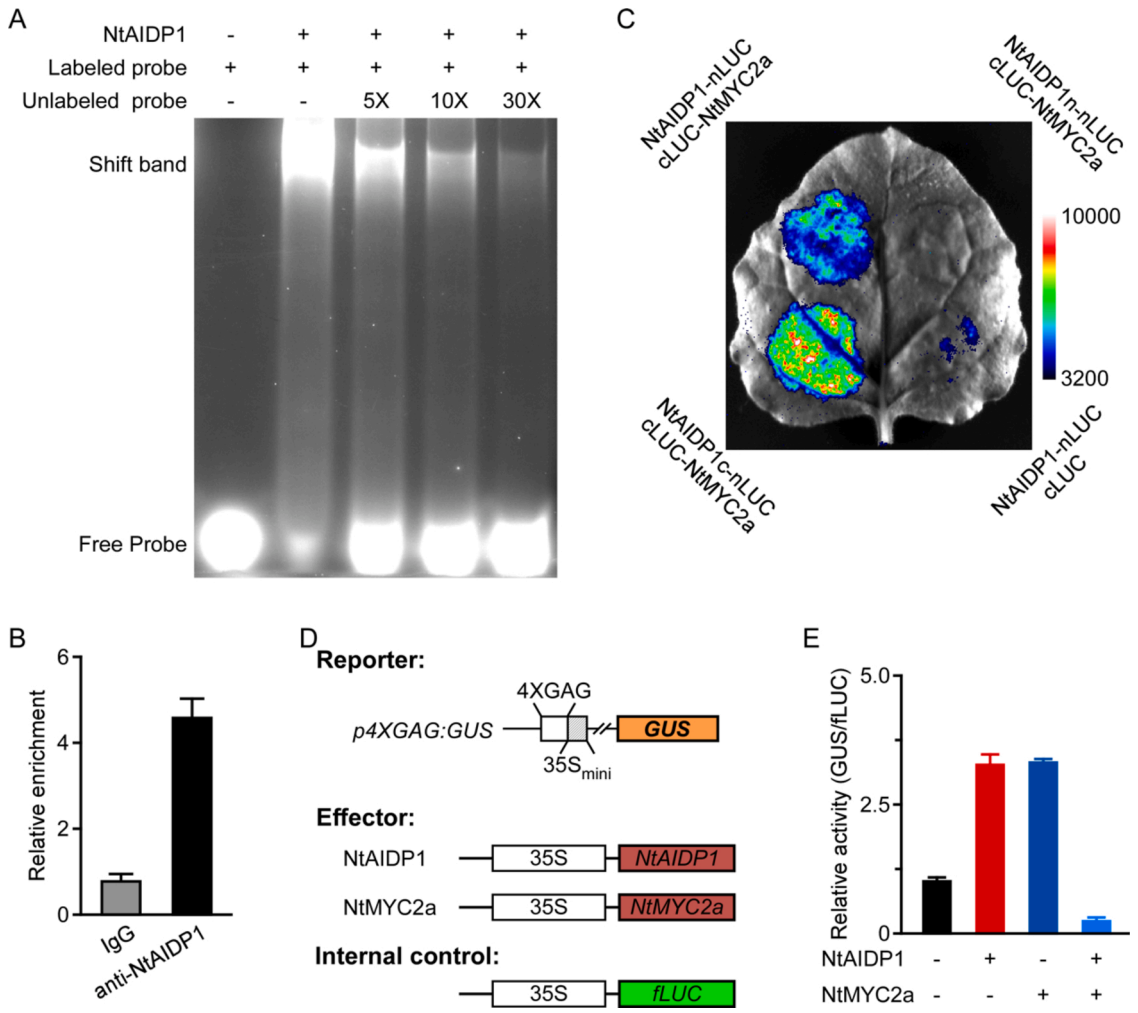

Fig. 4. NtMYC2a interrupts NtAIDP1 binding and activation of the AT-rich region in the GAG fragment of the NtPMT1a promoter.
(A) EMSA showed His-NtAIDP1 binding to the AT-rich region labelled with biotin-FAM at the 5′ end. Five-, 10- and 30-fold excesses of unlabelled probes used for binding competition. GAG fragment in the NtPMT1a promoter contains G-box, AT-rich region and GCC-box (Sears et al., 2014). **(B)** The GAG fragment was enriched by NtAIDP1 activity based on ChIP-qPCR. Chromatin of *35S:NtAIDP1-MYC* plants was immunoprecipitated using anti-MYC antibody and IgG, and the immunoprecipitated DNA was quantified via qPCR. The first exon of *NtPMT1a* was used as a nonspecific target. Enrichment of the GAG fragment is presented as multiples of input DNA. Data are mean ± SD ($n = 3$). **(C)** *in vivo* interaction between NtAIDP1 and NtMYC2a monitored using the LCI assay in tobacco leaves. Full-length *NtAIDP1*, *NtAIDP1n* and *NtAIDP1c* were separately fused to the 5′ end of the N-terminus of luciferase (*NtAIDP1-nLUC*, *NtAIDP1n-nLUC* and *NtAIDP1c-nLUC*), and full-length *NtMYC2a* was fused to the 3′ end of the C-terminal fragment of luciferase (*cLUC-NtMYC2a*). Empty vectors were used as negative controls. **(D)** Diagram of the construct used for transactivational activity measurement. The 4 × GAG fragment fused with $35S_{mini}$ promoter was used as the reporter, NtAIDP1 and NtMYC2a driven by 35S as the effector, and fLUC driven by 35S as the internal control. GUS, beta-glucuronidase. fLUC, firefly luciferase. **(E)** Effect of NtAIDP1, NtMYC2a, and both on transcriptional activity of the GAG fragment in *Arabidopsis* mesophyll protoplasts under constant light conditions for 48 h. The relative transactivational activity of the GAG fragment is indicated as the ratio of GUS to fLUC activity. Data are mean ± SD ($n = 3$).

is essential for DNA binding, and the HSP domain, which is responsible for interaction with NtJAZs. Based on our results, a model was proposed to explain how NtAIDP1 interacts with NtJAZs to mediate JA signalling cascades in tobacco. Under normal conditions, NtAIDP1 is repressed by NtJAZs due to a low level of JA, thereby preventing their activation of the promoters of JA-responsive genes. In the presence of high JA levels, NtJAZ-NtCOI1 acts as a co-receptor to bind JA, triggering NtJAZ degradation, which in turn leads to the release of bound NtAIDP1 from the AT-rich region or increased expression of *NtAIDP1*, resulting in the expression of JA-responsive genes. In addition, the interactions between NtAIDP1 and NtDELLAs and NtNINJA suggest that NtAIDP1 is involved in crosstalk between the GA and JA signalling pathways in tobacco.

The bHLH TF MYC2 is a master regulator of JA signalling cascades that specifically bind the G-box element to activate the expression of JA-responsive genes in plants (Ali and Baek, 2020; Chini et al., 2007; Thines et al., 2007; Zhai et al., 2017). Xu and Timko (2004) showed that a GAG fragment of about 50 base pairs (bp) containing a G-box element, AT-rich region and a GCC-motif in the promoter region of *PMT1a* was sufficient to confer MeJA-responsive expression of *PMT1a* in BY-2 cells. Zhang et al. (2012) showed that NtMYC2a and NtMYC2b bind to the G-box in the GAG fragment to activate its transcriptional activity and that RNAi-mediated suppression of *NtMYC2a* and *NtMYC2b* reduces JA-inducible transcriptional levels of *NtPMT*, *NtQPT* and *NtA622* in BY-2 cells. In addition, MYC TF activates the expression of ERFs via binding the G-box in the promoter region of ERFs, and ERFs can then bind to the GCC-motif of JA-responsive genes to regulate their transcription in plants (De Boer et al., 2011). Herein, we showed that NtAIDP1 is a novel regulator of JA signalling cascades that specifically binds to the AT-rich region to upregulate the transcription of JA-responsive genes. Moreover, we found that the interactions between NtJAZs or NtMYC2a with NtAIDP1 depend on its C-terminal HSP domain, which causes the transcription activity of the GAG fragment to be separately activated by NtAIDP1 or NtMYC2a, and inhibited by both in combination. In addition, overexpression of *NtAIDP1* in tobacco plants leads to upregulated

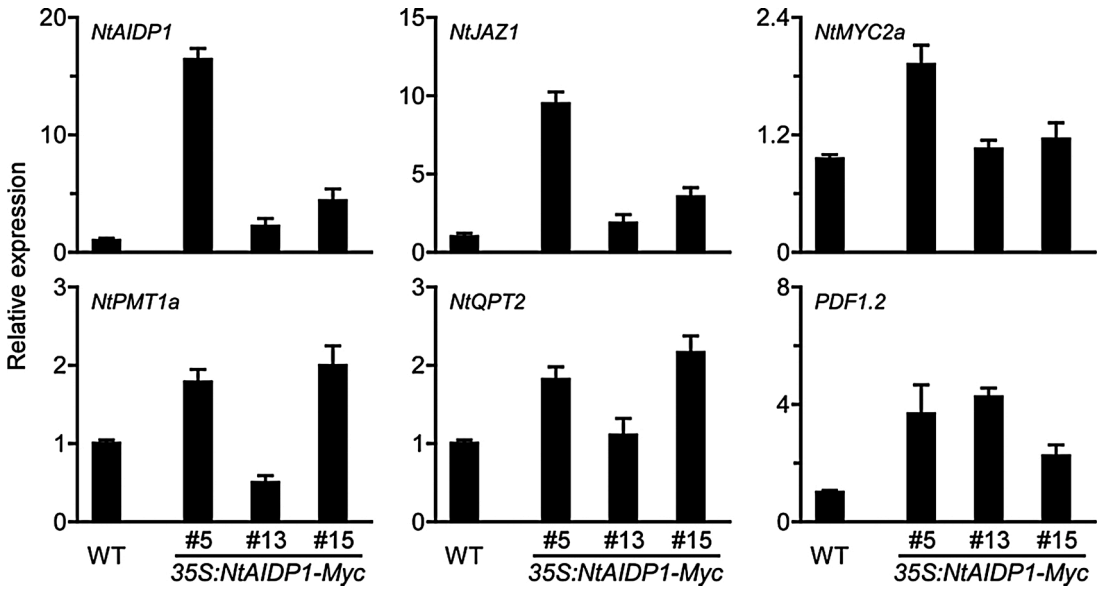

Fig. 5. Transcriptional level of *NtAIDP1* and several JA-inducible genes in tobacco plants overexpressing *NtAIDP1*.
RT-qPCR was performed to measure the expression of *NtAIDP1*, *NtPDF1.2*, *NtPMT1a*, *NtQPT2*, *NtJAZ1* and *NtMYC2a* in transgenic tobacco lines overexpressing *NtAIDP1-Myc* #5, #13 and #15. WT indicates wild-type tobacco. *Actin* was used as an internal control. Bars represent mean ± SD ($n = 3$).

expression of *NtPDF1.2* and *NtJAZ1*, but not *NtPMT1a*, *NtQPT2* and *NtMYC2a*, suggesting that transgenic plants present the high resistance to fungal pathogen, but no change of nicotine content, and further supporting our hypothesis of a complex regulatory network for JA-induced nicotine biosynthesis in tobacco (Yang et al., 2015).

Plant genomes contain multiple members of the AIDP family, including 12 NtAIDPs, 10 AtAIDPs and 6 OsAIDPs, which contain a common ARID domain and various C-terminal conserved domains. Our results revealed that an AIDP family member functions as a transcription factor regulating target gene expression through ARID binding to the AT-rich region of the promoter of the target genes. Based on the C-terminal domain, they can be divided into four distinct clades: the HSP, PHD, HMG, and ELM2 and SANT clades. Here, we showed that the interaction between NtJAZ1 and NtAIDP1 depends on its C-terminal HSP domain, indicating that different C-terminal domains of AIDP1 regulate the expression of different genes involved in diverse biological functions. Characterising the protein interactions of the other three C-terminal domain types of AIDP proteins will help elucidate their regulatory mechanisms and biological functions.

5. Conclusions

NtAIDP1 was identified as a NtJAZ1-interacting protein from JA-treated BY-2 cDNA library with yeast two-hybrid screening. In addition, NtAIDP1 interacted with other JA-inducible NtJAZs, including NtJAZ2b, NtJAZ2b.2, NtJAZ5, NtJAZ7, NtJAZ11 and NtJAZ12, but not with NtJAZ3, NtJAZ3b or NtJAZ10, and interacts with NtNINJA, NtDELLA1 and NtDELLA2 in the yeast two-hybrid assay. Moreover, NtAIDP1 binds to the AT-rich region in the promoter of JA-responsive genes to activate their transcription. These results indicate that NtAIDP1 is a new component of the JA signalling pathway and is involved in JA-regulated gene expression.

CRediT authorship contribution statement

Caiyun Zhao: Conceptualization, Data curation, Investigation, Methodology, Writing - original draft. **Xiaoqing Geng:** Conceptualization, Data curation, Investigation, Methodology, Writing - original draft. **Yuping Yang:** Conceptualization, Data curation, Methodology, Validation. **Yuhui Chai:** Conceptualization, Formal analysis, Methodology. **Zhongbang Song:** Methodology, Validation. **Chao Xi:** Investigation. **Kai Liu:** Methodology. **Heping Zhao:** Resources. **Yingdian Wang:** Conceptualization. **Bingwu Wang:** Conceptualization, Project administration, Supervision. **Michael P. Timko:** Conceptualization, Project administration, Supervision. **Shengcheng Han:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jplph.2021.153452.

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