





Arabidopsis C-terminal binding protein ANGUSTIFOLIA modulates transcriptional co-regulation of *MYB46* and *WRKY33*

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Summary

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- The apparent antagonism between salicylic acid (SA) and jasmonic acid (JA)/ethylene (ET) signalling resulting in trade-offs between defence against (hemi)biotrophic and necrotrophic pathogens has been widely described across multiple plant species. However, the underlying mechanism remains to be fully established.
- The molecular and cellular functions of ANGUSTIFOLIA (AN) were characterised, and its role in regulating the pathogenic response was studied in Arabidopsis.
- We demonstrated that AN, a plant homologue of mammalian C-TERMINAL BINDING PROTEIN (CtBP), antagonistically regulates plant resistance to the hemibiotrophic pathogen *Pseudomonas syringae* and the necrotrophic pathogen *Botrytis cinerea*. Consistent with phenotypic observations, transcription of genes involved in SA and JA/ET pathways was antagonistically regulated by AN. By interacting with another nuclear protein TYROSYL-DNA PHOSPHODIESTERASE1 (TDP1), AN imposes transcriptional repression on *MYB46*, encoding a transcriptional activator of *PHENYLALANINE AMMONIA-LYASE* (*PAL*) genes which are required for SA biosynthesis, while releasing TDP1-imposed transcriptional repression on *WRKY33*, a master regulator of the JA/ET signalling pathway.
- These findings demonstrate that transcriptional co-regulation of *MYB46* and *WRKY33* by AN mediates the coordination of SA and JA/ET pathways to optimise defences against (hemi)biotrophic and necrotrophic pathogens.

Introduction

Rapid and selective transcriptional reprogramming is central for launching effective host immune responses to microbial infection (Tsuda & Somssich, 2015). Plants have sophisticated regulatory mechanisms to fine-tune transcriptional responses, depending on the nature and virulence mechanism of the pathogen. The coordinated actions of transcription factors and interactions among hormone signalling pathways contribute to the selective activation of defence-related genes (Tsuda & Somssich, 2015). Despite great progress, molecular mechanisms modulating pathogen-specific immune responses remain to be fully elucidated.

In general, the salicylic acid (SA) pathway triggers defences against biotrophic and hemibiotrophic pathogens, which acquire nutrients from living host tissues, whereas the jasmonic acid (JA) and ethylene (ET) pathways cooperate to activate defences against necrotrophic pathogens, which feed on the remains of

dead host cells. In addition to the isochlorismate pathway, studies in Arabidopsis have demonstrated that SA is also synthesised via the phenylpropanoid pathway (Mauch-Mani & Slusarenko, 1996). PHENYLALANINE AMMONIA-LYASE (*PAL*) catalyses the first step of the phenylpropanoid pathway. Arabidopsis knockout mutants of *PAL* genes have reduced SA accumulation and exhibit the enhancement of disease symptoms induced by the hemibiotrophic bacteria *Pseudomonas syringae* (*P. syringae*) (Huang *et al.*, 2010). The accumulation of SA promotes the translocation of the NONEXPRESSER OF PR GENES1 (*NPR1*) monomer into the nucleus and acts as a transcriptional co-activator to trigger the expression of certain stress-responsive genes (e.g. *PATHOGENESIS-RELATED PROTEIN1* (*PR-1*)) to limit the growth of biotrophs (Pieterse & Van Loon, 2004). In resistance to necrotrophs, two transcription factors, ETHYLENE INSENSITIVE3 (*EIN3*) and ETHYLENE INSENSITIVE3-LIKE1 (*EIL1*), form a regulatory hub of the JA/ET signalling

pathway to activate APETALA2/ETHYLENE-RESPONSIVE FACTOR (AP2/ERF) transcription factors, such as *OCTAD ECANOIDRESPONSIVE ARABIDOPSIS APETALA2/ETHYLENERESPONSIVE FACTOR59 (ORA59)* and *ERF1* (Solano *et al.*, 1998; Zhu *et al.*, 2011). *ORA59* and *ERF1* then activate the expression of stress-responsive genes (e.g. *PLANT DEFENSIN1.2 (PDF1.2)*) against necrotrophs (Lorenzo *et al.*, 2003; Zarei *et al.*, 2011). *WRKY* transcription factors are major players in plant immunity. *WRKY33* is required for defence against necrotrophs (e.g. *Botrytis cinerea (B. cinerea)*) (Zheng *et al.*, 2006). In Arabidopsis, *WRKY33* targets *ORA59*, *PDF1.2*, as well as ethylene biosynthetic genes *ACC SYNTHASE (ACS2)* and *ACS6* (Birkenbihl *et al.*, 2012; Datta *et al.*, 2015; Liu *et al.*, 2015; Wang *et al.*, 2015).

Trade-offs exist in plant defences against (hemi)biotrophic and necrotrophic pathogens. Elevated resistance to (hemi)biotrophs is often correlated with increased susceptibility to necrotrophs and vice versa (Robert-Seilaniantz *et al.*, 2011). The antagonism between SA and JA/ET pathways has been identified to be involved in trade-offs between (hemi)biotroph and necrotroph defences (Robert-Seilaniantz *et al.*, 2011). At the molecular level, transcription factors involved in SA and JA/ET pathways play crucial roles in this antagonism. For example, *NPR1*, the master activator of SA signalling, was shown to suppress the JA signalling pathway (Spoel *et al.*, 2003). By contrast, overexpression of *ERF1* is capable of promoting JA/ET-dependent defences and reducing the defence against biotrophs (Berrocal-Lobo *et al.*, 2002). In addition to activating genes involved in JA/ET signalling, *WRKY33* was found to repress some genes involved in SA signalling and host cell death pathway (Birkenbihl *et al.*, 2012; Liu *et al.*, 2015).

C-TERMINAL BINDING PROTEIN (CtBP) is an ancient protein that exists widely in mammals, flies, worms and plants. Animal CtBPs play a central role in development and disease via transcriptional co-repression (Chinnadurai, 2009). By physically interacting with transcription factors and subsequently recruiting histone modification enzymes in the nucleus, CtBPs efficiently silence the expression of numerous tumour suppressor genes and proapoptotic genes to promote cellular survival and tumorigenesis (Chinnadurai, 2009). Phylogenetic analysis has illustrated that plant and animal CtBPs are in different subfamilies, suggesting that plant CtBPs may have unique functions not shared by animal CtBPs (Kim *et al.*, 2002). *ANGUSTIFOLIA (AN)* is the singular homologue of CtBP in Arabidopsis and the first plant CtBP that has been studied. *AN* is involved in the morphogenetic development of leaves and floral organs by controlling the arrangement of cortical microtubules. The Arabidopsis *an* mutant has a narrow-leaf phenotype with fewer and smaller cells in the leaf-width direction than those of wild-type plant (Tsuge *et al.*, 1996; Kim *et al.*, 2002). In addition, *AN* has been shown to affect plant responses towards biotic and abiotic stresses. The *an* mutant has enhanced tolerance to drought and *P. syringae* that is accompanied by induced expression of stress-responsive genes (Gachomo *et al.*, 2013). Collectively, *AN* is involved in development and disease tolerance as found for animal CtBPs. However, the nuclear function of *AN* remains unclarified. To date, only

physical and genetic associations with cytosolic proteins, including a receptor-like kinase *STRUBBELIG (SUB)*, RNA-binding proteins in stress granules, *SPIKE1 (SPK1)* and *DUAL-SPECIFICITY TYROSINE PHOSPHORYLATION-REGULATED KINASEs (DYRKPs)*, have been validated experimentally for *AN* (Bai *et al.*, 2013; Bhasin & Hulskamp, 2017; Iwabuchi *et al.*, 2019). Despite the lack of direct evidence, a potential transcriptional function of *AN* cannot be ruled out, as the dysregulation of several genes has been detected in the Arabidopsis *an* knockout mutants (Kim *et al.*, 2002; Gachomo *et al.*, 2013; Bryan *et al.*, 2018). Moreover, dimerisation of Arabidopsis *AN* has been reported (Kim *et al.*, 2002), which is an essential feature for the transcriptional role of animal CtBPs.

In the present study, we demonstrated that *AN* is involved in the trade-offs between (hemi)biotroph and necrotroph defences. Alteration of *AN* expression in transgenic plants oppositely affected resistance to *P. syringae* DC3000 (hemibiotroph) and *B. cinerea* (necrotroph). Gene expression analysis revealed that alternation of *AN* expression also antagonistically altered gene expression in the SA and JA/ET pathways during pathogen infection. Molecular studies illustrated that *AN* has nuclear accumulation and transcriptional repressor activity that are associated with the physical interaction with TYROSYL-DNA PHOSPHODIESTERASE 1 (TDP1), an enzyme involved in DNA damage repair in plants and animals (Lee *et al.*, 2010; Stingle *et al.*, 2017). *AN* directly targets and represses the transcription of *MYB46*, which encodes a master regulator of the phenylpropanoid pathway (Xie *et al.*, 2018b). Moreover, *AN* displayed the capability to release the TDP1-imposed transcriptional repression on *WRKY33*. The antagonistic transcriptional regulation of *MYB46* and *WRKY33* by *AN* suggested a transcriptional co-regulatory mechanism of genes involved in the SA (e.g. *PR-1*, *PALs*) and JA/ET pathways (e.g. *PDF1.2*, *ACS*, *ERF1* and *ORA59*), demonstrating a transcriptional node regulating the trade-offs between (hemi)biotrophic and necrotrophic defences.

Materials and Methods

Plant materials and growth conditions

Arabidopsis plants used in this study were grown in a growth chamber under 12 h : 12 h, light : dark, 23°C : 20°C conditions with 60% relative humidity. The T-DNA insertional mutants *an-t1* (Gachomo *et al.*, 2013) and *tdp1-2* (Enderle *et al.*, 2019) were obtained from the ABRC (<https://abrc.osu.edu/>). To generate *an-1 35S:AN* and *35S:TDP1* transgenic plants, full-length *AN* and *TDP1* were cloned into the pENTR vector and then subcloned into binary vectors pGWB520 and pGWB515, respectively, for transformation.

Pathogen infection and analysis

To analyse disease symptoms, detached 4-wk-old Arabidopsis leaves were inoculated with 5 µl *B. cinerea* conidiospore suspension (5×10^5 spores ml⁻¹ in potato dextrose broth) as previously described (Ingle & Roden, 2014). Leaf lesions were pictured and

measured using a Zeiss dissecting microscope. For each genotype, 20 leaves (40 spots) from independent plants were used for statistical analysis. Inoculation with *P. syringae* DC3000 was performed as previously described (Katagiri *et al.*, 2002). Here, *c.* 10 μl of *P. syringae* DC3000 bacteria suspension (1×10^6 CFU ml^{-1} in 10 mM MgCl_2) was used to infiltrate the leaves from 3-wk *Arabidopsis* plants. For each genotype, 15 leaves from five different plants were infiltrated. After 3 d, infiltrated leaves were pictured and measured for total weight. Leaves were then washed three times with sterile water and homogenised in 500 μl sterile water. Diluted lysis samples were plated onto LB medium containing 50 $\mu\text{g ml}^{-1}$ rifampicin. After growing at 28°C for 2 d, the bacterial colony forming units (CFU) were counted and normalised with total weight to obtain the bacterial titer value ($\log_{10}(\text{CFU mg}^{-1})$).

To measure gene expression, 4-wk-old *Arabidopsis* plants were sprayed with *B. cinerea* conidiospore suspension (2.5×10^5 spores ml^{-1}) or infiltrated with *P. syringae* DC3000 bacteria suspension (1×10^7 CFU ml^{-1}). Leaves were collected at 0, 24, 48 and 72 h post infection (hpi) for *B. cinerea* treatment. Leaves were collected at 0 and 6 hpi for *P. syringae* treatment. Then, total RNAs were extracted using a Plant RNA extraction kit (Sigma, St Louis, MO, USA). Next, 2 μg of total RNA was used for cDNA synthesis. qRT-PCR was then performed using cDNA as the template and following the Maxima SYBR Green qPCR Master Mixes manual (Thermo Scientific, Waltham, MA, USA). Gene expression was calculated using the $\Delta\Delta C_T$ method with expression of a housekeeping gene (*EF1 α*) for template normalisation. Three biological replicates were included in each gene expression analysis. Primers used for qPCR are listed in Supporting Information Table S1.

To analyse the nuclear accumulation changes of AN, protoplasts co-transformed with HA-TDP1 of AN-Myc plasmids were incubated overnight then treated with *P. syringae* (1×10^6 CFU ml^{-1}) or *B. cinerea* (5×10^5 spores ml^{-1}) for 6 h. After treatment, protoplasts were collected for cell fractionation assay.

Protoplast isolation and transfection

Arabidopsis mesophyll protoplasts were isolated and transfected as described previously (Yoo *et al.*, 2007). Protoplasts were isolated from fully expanded leaves from 3–4-wk-old Col-0 plants. For subcellular localisation analysis, 8 μg of AN-YFP plasmid DNA and 2 μg of CFP-SHY2 or Golgi-mCherry plasmid DNA were co-transfected into 100 μl of protoplasts. For co-localisation analysis, 5 μg of AN-YFP plasmid DNA was co-transfected with 5 μg of either mCherry-TDP1 or mCherry-TDP1 Δ 1-122 plasmid DNA into 100 μl of protoplasts. For cell fraction analysis, 20 μg of AN-Myc plasmid DNA were transfected into 200 μl of protoplasts. Five reactions were combined to obtain 1 ml of protoplasts for subsequent cell fraction and western blotting analyses. To determine the effect of TDP1 on AN nuclear accumulation, 15 μg of HA-TDP1 and 5 μg of AN-Myc plasmid DNA were co-transfected into 200 μl of protoplasts. To balance the protein level of AN-Myc, an AN-Myc only reaction was performed by

co-transfecting 15 μg of blank vector and 5 μg of AN-Myc plasmid DNA into 200 μl of protoplasts. For transcriptional activity assays, in total 10 μg plasmid DNA including reporter, effector, and/or transactivator plasmid DNA were co-transfected into 100 μl of protoplasts. To compare the effects of different effector constructs, the same amount of effect constructs was used. For single protein chromatin immunoprecipitation (ChIP), 20 μg of plasmid DNA was transfected into 200 μl of protoplasts. For AN and TDP1 co-expression, 10 μg of plasmid expressing AN-Myc and 10 μg of plasmid expressing HA-TDP1 were co-transfected into 200 μl of protoplasts. To balance the protein level of AN-Myc, an AN-Myc only reaction was performed by co-transfecting 10 μg of blank vector and 10 μg of AN-Myc construct into 200 μl of protoplasts.

Transactivation assay

A protoplast transfection-based transcriptional activity assay was performed in accordance with the method described by Tiwari *et al.* (2004). Reporter and effector constructs were co-transfected into protoplasts and incubated in the dark for 18–20 h at room temperature. GUS activity assay was performed as described previously (Yoo *et al.*, 2007). GUS activity was measured using a Fluoroskan microplate reader. To normalise GUS activity, 100 ng of 35S:luciferase plasmid was co-transfected for each reaction. Luciferase activity was measured using the Promega Luciferase Assay System in accordance with the manufacturer's manual. GUS activity in individual samples was normalised against luciferase activity (GUS/LUC). Three replicates were performed for statistical calculation.

Subcellular localisation analysis

AN-YFP construct was co-transfected with the nuclear marker into protoplasts to determine the subcellular localisation of AN. For co-localisation analysis, paired constructs were co-transfected into protoplasts. After 14 h incubation under weak light at room temperature, protoplasts were collected and resuspended in cold W5 solution (2 mM MES pH 5.7, 154 mM NaCl, 125 mM CaCl_2 , and 5 mM KCl) and subjected to microscopy. Images were collected using a Zeiss LSM 710 confocal microscope, equipped with 458, 514 and 561 nm laser lines for excitation of CFP, YFP and mCherry fluorescent markers, respectively. Images were processed using ZEN software (Zeiss).

Cell fractionation and protein gel blots

One ml of transfected protoplasts was incubated at room temperature for 14 h to express protein and then collected by centrifugation. Cytosolic and nuclear fractions were separated as previously described (Xie *et al.*, 2018a). Cytosolic and nuclear proteins were separated by SDS/PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Anti-Myc (C3956; Sigma), anti-HA (H3663; Sigma), anti-histone H3 (ab1791; Abcam), and anti-UGPase (AS05 086; Agrisera) were used as primary antibodies. Anti-rabbit IgG peroxidase antibody (A9169; Sigma)

was used as the secondary antibody for anti-Myc, anti-histone H3 and anti-UGPase. Anti-mouse IgG peroxidase antibody (A9044; Sigma) was used as the secondary antibody for anti-HA. Chemiluminescence signals were generated using ECL Western Blotting Detection Reagents (GE Health) and detected using the ChemiDoc XRS + system (Bio-Rad). Western blotting signals were quantified using IMAGE LAB software (Bio-Rad).

ChIP–qPCR and μ ChIP–qPCR

ChIP using transgenic plants was performed as previously described (Zhang *et al.*, 2013). Anti-Myc (C3956; Sigma) and anti-HA (H6908; Sigma) were used to immunoprecipitate AN-Myc and HA-TDP1. qPCR was performed to quantify DNA enrichment. Three biological replicates were included in each analysis. Primers used for PCR are listed in the Table S1.

μ ChIP was performed as described previously (Xie *et al.*, 2018a). AN-Myc and HA-TDP1 plasmid DNAs were expressed transiently in protoplasts. After 14 h incubation at room temperature, *c.* 40 000 transfected protoplasts were used for μ ChIP. ChIPed DNA and the input DNA were then cleaned and concentrated using a Qiagen MinElute PCR Purification Kit (Qiagen). qPCR was performed to quantify DNA enrichment. Three biological replicates were included in each analysis. Primers used for PCR are listed in Table S1.

Electrophoretic mobility shift assay

AN was cloned into the pGEX-6P-1 vector (GE Healthcare, Chicago, IL, USA) using *Bam*HI restriction enzyme for GST fusion constructs. The constructs were transformed into *E. coli* strain BL21(DE3) pLysS (Invitrogen) for protein expression. GST fusion proteins were extracted and purified as previously described using Glutathione Sepharose 4B beads (GE Healthcare) (Xie *et al.*, 2012). For DNA probes, the –447-bp to –300-bp promoter region of *AtMYB46* was amplified by PCR from Col-0 genomic DNA, gel purified, and end labelled with biotin using a DNA 3' End Biotinylation Kit (Thermo Scientific) in accordance with the manufacturer's manual. The DNA binding reaction was incubated at room temperature for 20 min as previously described (Xie *et al.*, 2018a). The reaction mixtures were then resolved in 6% DNA retardation gel (Novex) by electrophoresis at 100 V for 1–2 h and transferred to Nylon membrane. Biotin signals were detected using the Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific) as suggested by the manufacturer.

Yeast two-hybrid assay

pGADT7 and pGBKT7 plasmid pairs containing various genes were co-transformed into yeast Y2H Gold (Clontech Laboratories, Mountain View, CA, USA). SD–Leu–Trp plates were used to select yeast containing both constructs. The resulting clones were diluted in 50 μ l water and 5 μ l was used for spot assay on SD–Leu–Trp–Ade–His plates. Interactions between AN and TDP1 were based on the growth of yeast cells in the selection

medium without Ade and His. Three independent clones were tested for each plasmid pair.

SA measurement

Here, *c.* 85–100 mg of frozen ground plant material was twice extracted overnight with 2 ml 80% ethanol. The extracts were combined before drying in a 1 ml aliquot under a stream of nitrogen. Sorbitol (50 μ l; 1 mg ml^{–1}) was added before extraction as an internal standard. Dried extracts were silylated by addition of 500 μ l of acetonitrile (TS-20062; ThermoFisher) and 500 μ l of MSTFA plus 1% TMCS (TS-48915; ThermoFisher) and heated for 1 h at 70°C to produce trimethylsilyl (TMS) derivatives. After 2 d, 1 μ l was analysed by injection into an Agilent 7890A gas chromatograph coupled to an Agilent 5975C inert XL mass spectrometer (Tschaplinski *et al.*, 2019). SA and SA 2-*O*-glucoside metabolite peaks were quantified by extraction of the key *m/z* 267 and then scaled back up to the total ion current using predetermined scaling factors. Data were normalised to extracted plant mass, internal standard recovered, extraction volume, derivatisation volume and injection volume. For each genotype, at least two biological replicates were measured for statistical analysis.

Accession numbers

Sequence data from this article can be found under the following Arabidopsis Genome Initiative accession numbers: *AN* (AT1G01510), *TDP1* (AT5G15170), *MYB46* (AT5G12870), *WRKY33* (AT2G38470), *PR-1* (AT2G14610), *PDF1.2* (AT5G44420), *PAL1* (AT2G37040), *PAL2* (AT3G53260), *PAL3* (AT5G04230), *PAL4* (AT3G10340), *ACS2* (AT1G01480), *ACS6* (AT4G11280), *ERF1A* (AT4G17500), *ORA59* (AT1G06160), *ACT10N* (AT5G09810), *EF1 α* (AT5G60390), *ICS1* (AT1G74710), *PBS3* (AT5G13320) and *EDS5* (AT4G39030).

Results

AN antagonistically affects plant resistance to *P. syringae* and *B. cinerea*

In a previous RNA-seq analysis, we found the enrichment of genes involved in hormone and defence pathways among differentially expressed genes in the Arabidopsis T-DNA insertional mutant of *AN* (*an-t1*) (Bryan *et al.*, 2018). Knockout of *AN* was also shown to enhance plant tolerance to the hemibiotrophic bacteria *P. syringae* (Gachomo *et al.*, 2013). These results prompted us to investigate AN's involvement in plant immunity.

Firstly, we generated transgenic Arabidopsis overexpressing AN-Myc in *an-t1*. As shown in Fig. S1, overexpression of Myc-tagged AN (AN-Myc) in *an-t1* (*an-t1 35S:AN*) reversed its narrow-leaf phenotype to wild-type morphology, indicating that AN-Myc is functional in Arabidopsis plants. By assessing the responses of *an-t1* and *an-t1 35S:AN* plants to pathogen infection, we observed that AN oppositely affected the resistance to the hemibiotrophic bacteria *P. syringae* DC3000 and the necrotrophic fungi *B. cinerea*. In our analysis, *an-t1* displayed

weaker disease symptoms (e.g. chlorosis, water-soaked) and lower bacterial populations than Col-0 after 72 h of inoculation with *P. syringae* (Fig. 1a), consistent with a previous study (Gachomo *et al.*, 2013). By contrast, *an-t1 35S:AN* had more severe disease symptoms than Col-0 (Fig. 1a). Opposite to the resistance to *P. syringae*, *an-t1* exhibited clear lesion areas of dead cells after 72 h of inoculation with *B. cinerea*, indicative of its increased susceptibility towards *B. cinerea* infection (Fig. 1b). Compared with Col-0, *an-t1 35S:AN* displayed limited lesions, suggesting that it had enhanced resistance to *B. cinerea* (Fig. 1b).

These results demonstrated that AN antagonistically regulates resistance towards *P. syringae* and *B. cinerea*, implying that AN may be involved in the regulation of the trade-offs between (hemi)biotroph and necrotroph defences.

AN antagonistically affects gene expression of SA and JA/ET pathways

Host cell death limits (hemi)biotrophic but benefits necrotrophic pathogens. These distinct lifestyles led to trade-offs between defence responses against these two types of pathogens, with elevated resistance to (hemi)biotrophs being correlated with increased susceptibility to necrotrophs and vice versa. The SA pathway is primarily responsible for defence against (hemi)

biotrophic pathogens, whereas the JA/ET pathway is primarily responsible for defence against necrotrophic pathogens. The antagonism between SA and JA/ET pathways has been shown to be central to the regulation of these trade-offs (Robert-Seilanianz *et al.*, 2011).

qPCR results showed that AN antagonistically affected the expression of SA-responsive marker gene *PR-1* and JA/ET-responsive marker gene *PDFI.2*. As shown in the Fig. S2, *P. syringae* infection activated *PR-1* expression at 6 hpi. In *an-t1*, *P. syringae* infection induced *PR-1* expression to a higher level than that in Col-0 whereas *PR-1* expression in *an-t1 35S:AN* was lower than that in Col-0 at 6 hpi. By contrast, *PDFI.2* expression was lower in *an-t1* and higher in *an-t1 35S:AN* than that in Col-0 at 24 hpi of *B. cinerea* infection (Fig. S2).

In addition to defence genes (*PR-1* and *PDFI.2*), in our analysis AN exhibited antagonistic regulation on the expression of other genes involved in the SA and JA/ET pathways. As the enzyme catalysing the first step of the phenylpropanoid pathway, PAL was shown to be involved in SA biosynthesis in Arabidopsis (Mauch-Mani & Slusarenko, 1996; Huang *et al.*, 2010). Arabidopsis has four genes that encode PAL (*PAL1-4*). Among these, *PAL1* and *PAL2* were shown to be highly induced under environmental stress (Rohde *et al.*, 2004; Olsen *et al.*, 2008). In our qPCR analysis, *PAL1* and *PAL2* showed a significant induction at 6 hpi of *P. syringae* infection (seven-fold and two-fold in Col-0, respectively; Fig. S2a). *PAL1* and *PAL2* were upregulated to a higher level in *an-t1*, but to a lower level in *an-t1 35S:AN* compared with that in Col-0 (Fig. S2a). Although the majority of plant defence-related SA is synthesised via the isochorismate pathway, our qPCR analysis revealed that AN only has minor effect on this pathway. ISOCHORISMATE SYNTHASE 1 (*ICS1*), avrPphB SUSCEPTIBLE 3 (*PBS3*), and ENHANCED DISEASE SUSCEPTIBILITY 5 (*EDS5*) are essential components of the isochorismate pathway (Rekhter *et al.*, 2019). During *P. syringae* infection, AN knockout and overexpression plants exhibited no significant change in *ICS1* or *PBS3* expression (Fig. S2a). In *an-t1*, we observed the reduction of *P. syringae*-induced *EDS5* expression. Although *an-t1 35S:AN* showed higher *EDS5* induction than that in *an-t1*, its *EDS5* expression was still slightly lower than that in Col-0 at 0 and 6 hpi of *P. syringae* infection (Fig. S2a). In the JA/ET pathway, ACS catalyses the rate-limiting step of ET biosynthesis (Kende, 1993). Transcription factors ERF1 and ORA59 are involved in JA/ET signalling to induce *PDFI.2* expression (Lorenzo *et al.*, 2003; Zarei *et al.*, 2011). We observed that *ACS2*, *ERF1A* and *ORA59* exhibited a similar expression pattern to that of *PDFI.2* during infection with *B. cinerea*, with lower expression in *an-t1* and higher expression in *an-t1 35S:AN* (Fig. S2b).

Collectively, results from gene expression analysis suggested that AN is involved in the regulation of trade-offs between defence responses to *P. syringae* and *B. cinerea* that are associated with the antagonism between SA and JA/ET pathways. Differential gene expression induced by AN implies that AN may be

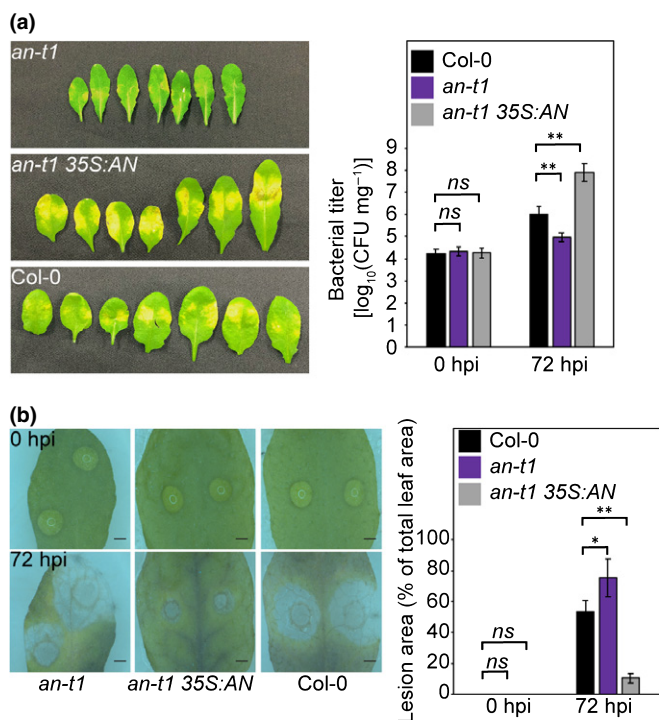


Fig. 1 AN antagonistically regulates Arabidopsis resistance to *Pseudomonas syringae* and *Botrytis cinerea*. (a) *P. syringae* inoculation of Col-0, *an-t1*, and *an-t1 35S:AN*. Bacteria titers in 0 h post infection (hpi) and 72 hpi are shown in the bar graph. Values represent means \pm SE, $n = 15$. (b) *B. cinerea* inoculation of Col-0, *an-t1* and *an-t1 35S:AN*. Bars, 1 mm. Lesion areas (% of total leaf area) in 0 hpi and 72 hpi are shown in the bar graph. Values represent means \pm SE, $n = 40$. Statistical significance was determined by two-tailed Student's *t*-tests against Col-0 (**, $P < 0.01$; *, $P < 0.05$; ns, $P > 0.05$).

involved in transcriptional regulatory mechanisms, which prompted us to examine AN's potential nuclear function.

AN has nuclear accumulation

To determine whether AN has nuclear functions, we first examined the subcellular localisation of yellow fluorescent protein (YFP)-tagged AN (AN-YFP) using the Arabidopsis mesophyll protoplast transient expression system. Signals of AN-YFP (green colour) were present in both the cytoplasm and the nucleus (Fig. 2a). In 38.75% transfected cells ($N=80$), AN-YFP signals partially overlapped with signals from the nuclear marker mCherry-VirD2NLS (Lee *et al.*, 2008), as indicated by the yellow colour (Fig. 2a). Consistently, using western blotting, Myc-tagged AN (AN-Myc) was detected in both cytosolic and nuclear fractions (Fig. 2b).

The observed nuclear accumulation of AN is not likely to be due to its nuclear localisation signal (NLS; KKRH), which was demonstrated in a previous study to be nonfunctional (Minamisawa *et al.*, 2011). To determine the mechanism translocating AN into the nucleus, we sought to identify proteins interacting with AN. Gachomo *et al.* (2013) identified nine potential interacting partners of AN using the high-throughput integrated knowledge-based Arabidopsis protein interaction network analysis (ANAP) (Gachomo *et al.*, 2013). Among these nine proteins, only At5G15170 (TDP1) has been reported to localise in the nucleus (Lee *et al.*, 2010), which prompted us to evaluate its interaction with AN. As shown in Figs 2(c), S3, we observed the interaction between AN and TDP1 using the yeast two-hybrid (Y2H) assay. Consistent with the Y2H result, AN-YFP showed co-localisation with mCherry-TDP1 in both the cytoplasm and the nucleus (Fig. 2d). TDP1 is a key enzyme catalysing DNA repair in both plants and animals. In addition to the TDP domain that is present in all TDP1 proteins, Arabidopsis TDP1

contains an FHA domain at its N-terminal region (Kim *et al.*, 2012). The FHA domain of TDP1 contains an NLS and is indispensable for its nuclear localisation (Kim *et al.*, 2012). Our Y2H and co-localisation analyses of AN and truncated TDP1 showed that AN was specifically associated with the FHA domain (TDP1 Δ 123–605), whereas it had no interaction with the TDP domain (TDP1 Δ 1–122) (Fig. 2c,d).

Given the result that AN interacts with the NLS-containing domain of TDP1 (FHA domain), we hypothesised that TDP1 may be involved in the nuclear accumulation of AN. To test this hypothesis, AN-Myc and HA-tagged TDP1 (HA-TDP1) were co-expressed in the protoplasts and their accumulations in the cytoplasm and nucleus were detected using western blotting and then quantified using IMAGE LAB software (Bio-Rad). Western blotting analysis of total proteins showed that co-expression of HA-TDP1 did not affect AN-Myc expression (Fig. S4). Compared with the empty vector control, co-expression of HA-TDP1 increased the percentage of AN nuclear signals from 37.2% to 59.0% (Fig. 2b), suggesting that TDP1 is capable of enhancing the nuclear localisation of AN. Moreover, the presence of a small amount of TDP1 protein in the cytosolic fraction (Fig. 2b) implied that TDP1 may bind to AN in the cytoplasm and translocate AN into the nucleus.

AN has transcriptional repressor activity and targets the transcription factor gene *MYB46*

The nuclear accumulation of AN suggested potential transcriptional functions. We then analysed whether AN could regulate transcription using the transactivation assays (Xie *et al.*, 2018a). In this assay, the promoter region of β -glucuronidase (GUS) reporter gene contains two DNA binding sites, Gal4 and LexA (Fig. 3a). The LexA binding domain (LD)-fused Herpes simplex virus VP16 (LD-VP16) was used to constitutively activate GUS

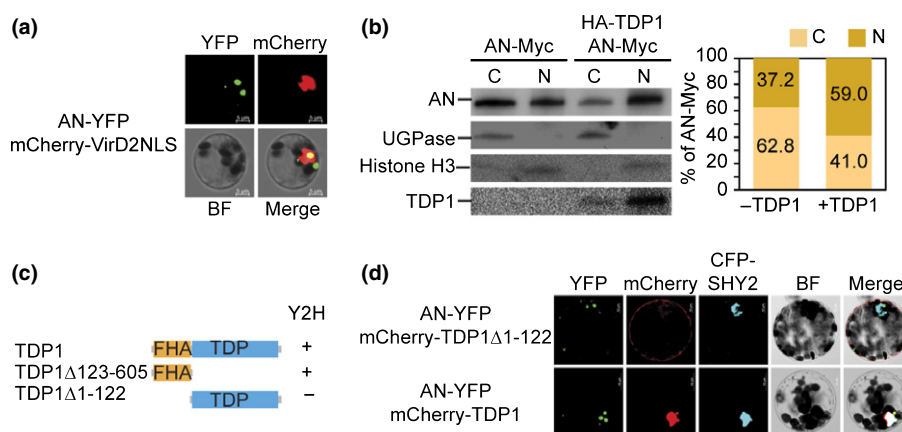


Fig. 2 TDP1 enhances the nuclear accumulation of AN in Arabidopsis. (a) Subcellular localisation analysis showing the partial nuclear localisation of AN in the Arabidopsis protoplasts. Overlapping of AN-YFP (green) and mCherry-VirD2NLS (nuclear marker, red) is indicated as a yellow colour. BF, bright field. Bars, 5 μ m. (b) Immunoblot analysis of the nuclear accumulation of AN with or without TDP1. AN-Myc, HA-TDP1, UGPase (cytosolic marker), histone H3 (nuclear marker) are examined in cytosolic (C) and nuclear (N) fractions. AN-Myc signals were quantified using IMAGE LAB software (Bio-Rad) and then normalised against the corresponding nuclear or cytosolic marker to calculate the relative ratio of AN signals in cytosolic and nuclear fractions. (c) Yeast two-hybrid analysis of the interaction between AN and TDP1. Domain structures of truncated TDP1 are displayed. '+' indicates interaction; '-' indicates no interaction. (d) Co-localisation analysis of AN with full-length TDP1 and truncated TDP1 without FHA domain (TDP1 Δ 1–122). CFP-SHY2 (blue) is a nuclear marker. Bars, 10 μ m.

expression (Fig. 3a). The finding that Gal4-binding-domain (GD)-fused AN (GD-AN), but not GD only, reduced GUS expression by *c.* 25% suggested that AN has moderate transcriptional repressor activity (Fig. 3a). No transcriptional activator activity of AN was detected (Fig. S5).

Subsequently, we attempted to identify target genes of AN using microchromatin immunoprecipitation (μ ChIP) following PCR (Xie *et al.*, 2018a). Given that the expression levels of a number of transcription factor genes were altered in the *an* knockout mutant (*an-t1*) (Bryan *et al.*, 2018), we hypothesised that AN may function upstream of these transcription factors. We focused on transcription factors with significant gene expression changes (>2-fold) in *an-t1* (Fig. S6). Among 12 tested transcription factors (seven upregulated genes: *MYB58*, *MYB46*, *MYB63*, *MYB55*, *MYB20*, *MYB103* and *NAC073*; and five downregulated genes: *WRKY33*, *WRKY40*, *WRKY53*, *WRKY26* and *WRKY22*), AN-Myc showed association with only *MYB46* promoter in the ChIP assays (Fig. 3b, c). Subsequently, an *in vitro* DNA binding assay (electrophoretic mobility shift assay, EMSA) was used to determine the direct binding of AN to the *MYB46* promoter. Purified GST-tagged AN protein (GST-AN), but not the GST tag alone, was found to bind to the biotin-labelled 148-bp *MYB46* promoter fragment (–447 bp to –300 bp) (Fig. 3d), suggesting that AN has DNA binding activity and directly targets *MYB46*. A competition assay using 100 \times unlabelled *MYB46* promoter DNA abolished the shifted band, suggesting that the binding of AN to *MYB46* promoter is specific (Fig. 3d).

AN–TDP1 interaction enhances transcriptional repression on *MYB46*

The association of AN with the *MYB46* promoter was further confirmed via ChIP assays using transgenic plants expressing AN-Myc (*an-t1* 35S:AN) (Fig. 4a). As TDP1 was shown to enhance the nuclear accumulation of AN, it is possible that TDP1 may also impact the transcriptional repressor effect of AN. To test this hypothesis, a transactivation assay was performed to measure the transcriptional repression of AN on *MYB46* promoter. Because AN was shown to act as a transcriptional repressor (Fig. 3a), VND6 (Zhong *et al.*, 2007), a transcriptional activator for the *MYB46* gene was used to constitutively activate *MYB46* expression in the protoplast transient expression system. As shown in Fig. 4(b), AN exhibited moderate repression on *MYB46* promoter activity (*c.* 25% reduction). Addition of full-length TDP1 was capable of enhancing the repression of AN on *MYB46* (*c.* 40% reduction). However, the truncated TDP1 lacking the FHA domain (TDP1 Δ 1–122) had little effect on the repression of *MYB46* in the transactivation assay (Fig. 4b). Collectively, these results suggested that the nuclear accumulation of AN (enhanced by TDP1) is important for its transcriptional repressor function.

We then investigated whether TDP1 could increase the binding of AN to the *MYB46* promoter *in vivo*. AN-Myc and HA-TDP1 were co-expressed in the protoplasts and subjected to μ ChIP-qPCR assay. Consistent with the increased nuclear

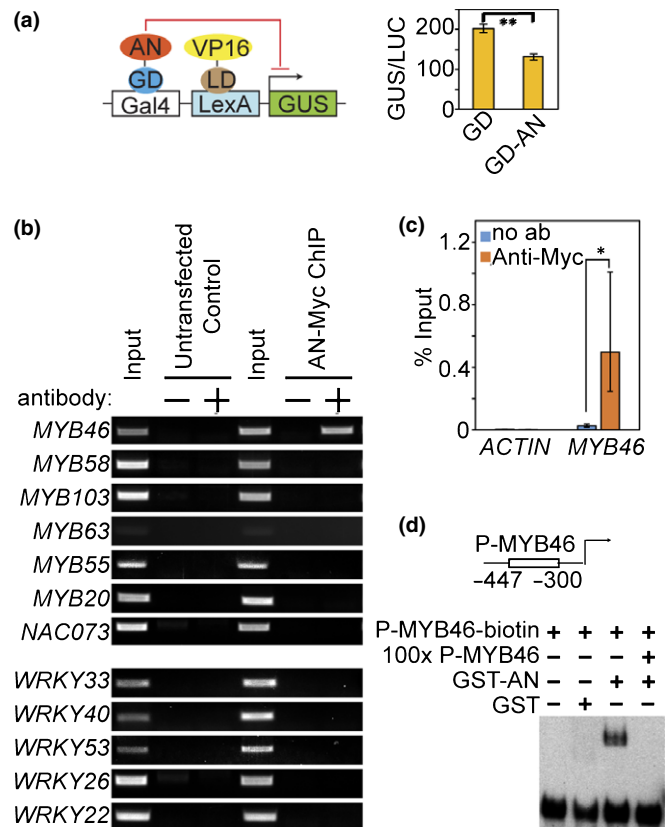


Fig. 3 AN has transcriptional repressor activity and directly targets *MYB46* in Arabidopsis. (a) Transactivation analysis of the repressor activity of AN. Top scheme displays the three vectors used in transactivation analyses: reporter construct containing Gal4 binding site and LexA binding site upstream of the GUS reporter gene; transactivation construct expressing LexA binding domain (LD) fused VP16; effector construct expressing Gal4 binding domain (GD) fused AN. GUS activity in individual samples was normalised against luciferase activity (GUS/LUC). Values represent means \pm SE, *n* = 3. Statistical significance was determined by two-tailed Student's *t*-tests (**, *P* < 0.01). (b) μ ChIP-PCR analyses of AN association with promoters of genes upregulated in *an-t1* (*MYB46*, *MYB58*, *MYB63*, *MYB55*, *MYB20*, *MYB103*, *NAC073*) and genes downregulated in *an-t1* (*WRKY33*, *WRKY40*, *WRKY53*, *WRKY26* or *WRKY22*). Two negative controls are shown: no antibody control and protoplasts without expression of AN-Myc (untransfected control). +, indicates reactions with anti-Myc antibody. –, indicates reactions without anti-Myc antibody. (c) μ ChIP-qPCR analyses of AN association with *MYB46* promoter. No antibody assays were used as negative controls. Values represent means \pm SE, *n* = 3. Statistical significance was determined by two-tailed Student's *t*-tests (*, *P* < 0.05). (d) EMSA analysis showing the direct binding of AN to the *MYB46* promoter. The 148-bp *MYB46* promoter (–447 to –300 bp from the start codon) was labelled with biotin as the probe (P-MYB46-biotin). The competition assay was performed using 100 \times unlabelled *MYB46* promoter DNA (100 \times P-MYB46).

accumulation, the chromatin association of AN with *MYB46* promoter was significantly increased (*P* < 0.05) when co-expressed with TDP1 (Fig. 4c). Meanwhile, co-expression of AN and TDP1 also significantly increased the chromatin association of TDP1 with *MYB46* promoter (Fig. S4b). Conversely, AN-Myc expressed in the *TDP1* T-DNA insertional mutant *tdp1-2*

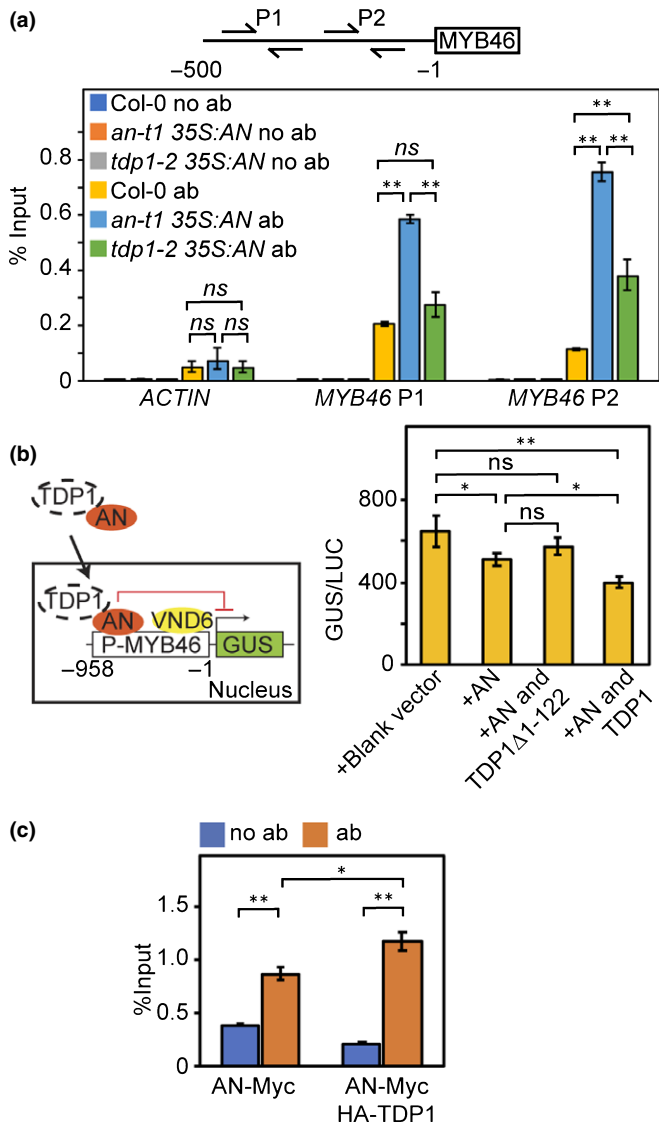


Fig. 4 TDP1 is required for AN-mediated transcriptional repression on *MYB46* in Arabidopsis. (a) ChIP-qPCR analysis on *an-t1* and *tdp1-2* backgrounds. Upper scheme indicates primers for the amplification of *MYB46* promoters (*MYB46* P1 and P2). Nontransgenic plants (*Col-0*) and no antibody assays were used as negative controls. (b) Transactivation analysis showing that TDP1 enhances AN transcriptional repression on *MYB46*. Left scheme displays the four vectors used in transactivation analyses: reporter construct containing *MYB46* promoter and GUS reporter gene; transactivation construct expressing VND6; effector construct expressing AN; effector construct expressing TDP1. GUS activity in individual assays was normalised against luciferase activity (GUS/LUC). (c) μ ChIP-qPCR analysis demonstrating the enhancement of association of AN with *MYB46* promoter by TDP1. Assays without antibody (no ab) were performed as negative controls. Values represent means \pm SE, $n = 3$. Statistical significance was determined by two-tailed Student's *t*-tests (**, $P < 0.01$; *, $P < 0.05$; ns, $P > 0.05$).

(Enderle *et al.*, 2019) exhibited dramatically reduced association with *MYB46* promoter in the ChIP analysis (Fig. 4a).

Collectively, these molecular and genetic studies support that physical interaction with TDP1 is required for AN to fully execute its transcriptional repressor function on *MYB46*.

AN–TDP1 interaction releases the TDP1-imposed transcriptional repression on *WRKY33*

WRKY transcription factors play critical roles in plant immunity. Although multiple WRKY transcription factors were downregulated in the *an-t1* mutant, we did not detect the association of AN with promoters of these WRKY transcription factors (Fig. 3b). However, previous studies in *Medicago* suggested that TDP1 may regulate the expression of *WRKY33* in plants. Deletion of *TDP1* in *Medicago* was shown to activate the expression of *WRKY33* (*Medtr2g033820* and *Medtr3g031220*) (Dona *et al.*, 2013). Given that Arabidopsis and *Medicago* TDP1 proteins share high amino acid identity (62.8%) and a similar structure (both of them have the FHA domain and TDP domain; Fig. S7), we hypothesised that Arabidopsis TDP1 may negatively regulate *WRKY33* expression. To examine this possibility, we found that Arabidopsis *TDP1* and *WRKY33* genes had a negative co-expression relationship across different developmental stages and pathogen infections (Fig. 5a). Furthermore, transient expression of TDP1 in Arabidopsis protoplasts was capable of reducing the transcriptional activity of the *WRKY33* promoter by 34.9% and reducing the transcript level of endogenous *WRKY33* by 46.0% (Fig. 5b,c). In addition, co-expression of AN and TDP1 alleviated the repression of *WRKY33* expression by TDP1 (Fig. 5c), suggesting that AN may negatively affect the transcriptional role of TDP1.

To understand the molecular mechanisms underlying AN–TDP1 regulation on *WRKY33* expression, we measured the association of TDP1 with *WRKY33* promoter using transgenic plants expressing HA–TDP1 (*35S:TDP1*). Relative to the nontransgenic control and no antibody control, *WRKY33* promoter fragments were enriched in HA–TDP1 immunoprecipitates (Fig. 5d). A similar result was obtained via μ ChIP-qPCR using protoplasts transiently expressing HA–TDP1 (Fig. 5e). Moreover, the association between TDP1 and *WRKY33* promoter was significantly reduced by the co-expression of AN and TDP1 (Fig. 5e), suggesting that AN may release the TDP1-imposed transcriptional repression on *WRKY33* by sequestering TDP1 away from the *WRKY33* promoter.

Discussion

Being continuously challenged by pathogens with different lifestyles in the natural environment, plants have evolved a sophisticated regulatory network to fine-tune the activation of specific immune genes in response to a given pathogen. This network includes complex interconnections of hormones and transcription factors. Due to their distinct lifestyles, plants activate contrasting mechanisms in defence against (hemi)biotrophic and necrotrophic pathogens that depend on SA and JA/ET pathways, respectively. The antagonism between SA and JA/ET pathways has been demonstrated to be critical for the selective activation of defence against biotrophic and necrotrophic pathogens. However, the mechanisms coordinating SA and JA/ET pathways remain largely unknown. Using well characterised pathogens with either biotrophic or necrotrophic lifestyle (*P. syringae* and

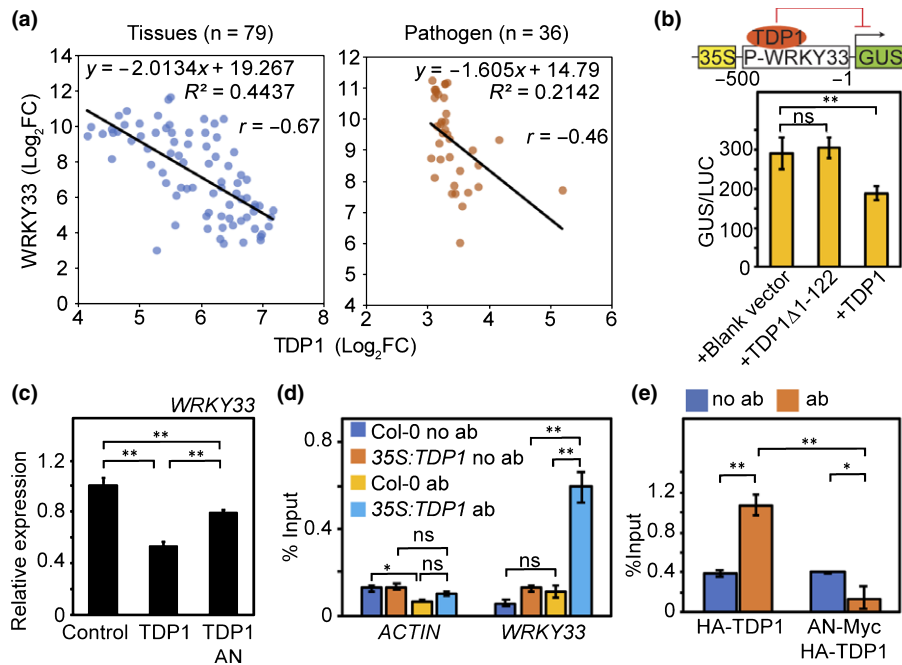


Fig. 5 The AN–TDP1 interaction releases the TDP1-imposed transcriptional repression on *WRKY33* in Arabidopsis. (a) Negative co-expression of *TDP1* and *WRKY33* across various tissues and pathogen infections. Gene expression data were obtained from the AtGenExpress Visualization Tool (AVT) (<http://jsp.weigelworld.org/expviz/expviz.jsp?experiment=development&normalization=absolute&probesprobe=At5G61850&action=Run>). (b) Transactivation analysis of TDP1 repression of *WRKY33*. Upper panel is a scheme of two constructs used in the transactivation analysis: reporter constructs containing 35S promoter, *WRKY33* promoter and GUS reporter gene; effector construct expressing TDP1. (c) qRT-PCR results showing effects of AN and TDP1 on the expression of *WRKY33*. Gene expression was normalised against the expression of *EF1α*. *WRKY33* expression in Col-0 was set as 1. Values represent means \pm SE, $n = 3$. (d) ChIP-qPCR analysis of HA–TDP1 in transgenic plants. Nontransgenic plants (Col-0) and no antibody assays were used as negative controls. (e) μ ChIP-qPCR analysis demonstrated that AN reduces TDP1 association with *WRKY33* promoter. Values represent means \pm SE, $n = 3$. Statistical significance was determined by two-tailed Student's *t*-tests (**, $P < 0.01$; *, $P < 0.05$; ns, $P > 0.05$).

B. cinerea), we found that ANGUSTIFOLIA (AN) antagonistically regulated Arabidopsis resistance to biotrophic and necrotrophic pathogens. Our pathogen inoculation analyses demonstrated that AN is capable of enhancing resistance to necrotrophic pathogen and reducing the resistance to biotrophic pathogens (Fig. 1). By measuring the expression levels of marker genes for plant defence during pathogen infection, we found that AN was involved in the antagonistic transcriptional regulation of SA pathway genes (*PR-1* and *PALs*) and JA/ET pathway genes (*PDF1.2*, *ACS2*, *ERF1A* and *ORA59*) (Fig. S2). These results suggested that the AN-mediated regulation of host immune responses was associated with regulation of SA–JA/ET antagonism, a well known mechanism involved in trade-offs between (hemi)biotroph and necrotroph defences (Robert-Seilaniantz *et al.*, 2011). In Arabidopsis, SA is synthesised via the isochorismate pathway and the phenylpropanoid pathway. Our results that AN knockout and overexpression altered *PAL* expression, but not *ICS1* or *PBS3* expression, during *P. syringae* infection demonstrated that AN is likely to regulate the phenylpropanoid pathway rather than the isochorismate pathway. Although the expression of *EDS5* in the isochorismate pathway was reduced in the *an-t1* mutant, AN overexpression did not increase *EDS5* expression to a level higher than that in Col-0, suggesting that the change in *EDS5* expression may not be due to the direct effect of AN disruption.

Through investigating the transcriptional function of AN (Figs 2–4), we found that AN directly targeted and repressed the expression of *MYB46*, a transcription factor directly activating *PAL* genes in Arabidopsis (Zhong & Ye, 2012). Conversely, the results of our molecular analyses suggested that AN is capable of releasing transcriptional repression of *WRKY33* by sequestering TDP1 away from the *WRKY33* promoter (Fig. 5). *WRKY33* is a transcription factor that directly targets JA/ET pathway genes (e.g. *ACS2*, *ORA59* and *PDF1.2*) and is critical for plant defences against *B. cinerea* (Zheng *et al.*, 2006; Birkenbihl *et al.*, 2012; Datta *et al.*, 2015; Liu *et al.*, 2015; Wang *et al.*, 2015). These results suggested that AN is involved in the antagonistic transcriptional regulation of *MYB46* and *WRKY33*, and may explain how AN antagonistically regulates SA and JA/ET pathway genes. Collectively, our discoveries indicated that AN mediates the transcriptional co-regulation of *MYB46* and *WRKY33*, which play an important role in SA–JA antagonism and trade-offs between (hemi)biotroph and necrotroph defences (Fig. S8). Consistent with our model, the *an-t1* mutant had increased *P. syringae*-induced SA accumulation and *an-t1* 35S:AN had reduced *P. syringae*-induced SA accumulation compared with Col-0 (Fig. S9). In this study, we have provided multiple lines of evidence supporting AN's transcriptional regulatory role and determining its target genes as well as its interacting partner (TDP1). However, we do not rule out the possibility that AN/TDP1 may

also target other genes. In a previous study, knockout of *MYB46* via T-DNA insertion did not exhibit any phenotypic effect on the Arabidopsis response to *P. syringae* although pathogen-induced expression levels of bacterial disease marker gene *Ep5C* and SA pathway stress-responsive gene *PR-1* were altered in the *myb46* mutants (Ramírez *et al.*, 2011). Interestingly, the transcriptomic analysis did not reveal significant change in *PAL* gene expression (Ramírez *et al.*, 2011). As an essential enzyme involved in phenylpropanoid and lignin biosynthesis, the expression of *PALs* is controlled by a NAC–MYB transcriptional network in which MYB46 is a major player (Nakano *et al.*, 2015). Within this NAC–MYB transcriptional network, redundant, feed-back and feed-forward regulations exist widely (Xie *et al.*, 2018b). This may possibly explain why knocking-out a single transcription factor (i.e. MYB46) within the NAC–MYB transcriptional network did not result in the alteration of *PAL* expression and phenotypic response to *P. syringae*. Together with the results in the present study, we speculated that, in addition to *MYB46*, AN may target and regulate other members in the NAC–MYB transcriptional network to control *PAL* gene expression and pathogenic responses. In future studies, genome-wide AN-based and TDP1-based ChIP-seq together with RNA-seq analysis upon pathogen infection may help to uncover additional target genes for AN and TDP1, and reveal both overlapping and nonoverlapping target genes in response to pathogen infection. This is a fruitful area for future investigations. Conversely, AN seems to be under the regulation of plant–microbe interaction mechanisms, because we observed the alteration of AN transcription and nuclear accumulation by *P. syringae* and *B. cinerea* (Fig. S10). As shown in Fig. S10, the alteration of AN transcription occurred at the early stages of *P. syringae* (6 hpi) and *B. cinerea* (24 hpi) infections. We also found that *P. syringae* can induce the reduction of AN nuclear accumulation at the early stage of infection (6 hpi). Because nuclear accumulation of AN is essential for its transcriptional function, we speculated that this regulation occurred at the early stage of pathogen infection. However, answers to the questions which factors trigger the interaction between AN and TDP1, at which point the AN–TDP1 protein complex turns ON or OFF the promoter of their target genes, and which factor controls the ON or OFF, are completely unknown. Now that we have established the transcriptional regulatory role of AN, future studies focusing on addressing each of these questions may help to reveal the precise role of the AN–TDP1 complex and AN–TDP1 interaction in response to pathogen infection.

Although CtBP is an ancient protein, widely existing in mammals, flies, worms and plants, the evolution of its molecular and biological roles remains poorly studied. AN protein has only 26.6% identity with animal CtBPs, and this was thought to result from high diversity in protein structure and the complete loss of nuclear function of AN. AN was believed to only have cytosolic function, because previous localisation analyses using stable transgenic plants expressing green fluorescent protein-tagged AN failed to detect AN in the nucleus (Minamisawa *et al.*, 2011). However, using the transient expression system, we were able to detect the nuclear accumulation of AN protein (Fig. 2). Furthermore, we

identified the transcriptional repressor function of AN and the physical interaction of AN with the nuclear protein TDP1 (Figs 2,3). Our results suggested that AN has a nuclear function as found in animal CtBPs. It is plausible that AN may have rapid nucleocytoplasmic shuttling as found for CtBP2 (Zhao *et al.*, 2006), which forms occasional nuclear localisation and may lead to the lack of nuclear localisation in stable transgenic plants. Our molecular analyses of AN nuclear function further indicated that the divergence in protein sequences of AN and animal CtBPs may have given rise to their different mechanisms of action, which can be specified by two aspects:

- (1) Different from animal CtBPs, the NLS of AN is nonfunctional (Minamisawa *et al.*, 2011). Our results showed that AN physically associates with the NLS-containing FHA domain of TDP1, suggesting that AN may utilise the NLS of TDP1 rather than its own NLS for nuclear localisation.
- (2) AN lacks residues to bind to PxDLS-containing transcription factors (Stern *et al.*, 2007).

In this study we have provided results from yeast two-hybrid assays, cell fractionation, co-localisation and transactivation to demonstrate that TDP1 is capable of enhancing AN nuclear accumulation and function. ChIP assay of AN in the *tdp1-2* mutant further illustrated that TDP1 is required for AN binding to the *MYB46* promoter, which is crucial for AN transcriptional function. Instead of depending on associated transcription factors to repress target genes, we found that AN itself was sufficient to execute transcriptional repressor function and directly bind to the *MYB46* promoter. Despite these distinct molecular functions, our discoveries of the involvement of AN in the regulation of host tolerance to pathogens, which is analogous to animal CtBPs involvement in tumour progression, suggested that plant and animal CtBPs are evolutionary conserved from the point of their biological function. This point is supported by the fact that AN is involved in the morphogenetic development of plants (Kim *et al.*, 2002) analogous to animal CtBP role in development. In conclusion, our studies on the nuclear function and the involvement in plant immune responses of AN provide new insights into the molecular and biological functions of AN, which enable a better understanding of the evolution of CtBP in animals and plants.

TDP1 is a tyrosyl-DNA phosphodiesterase hydrolysing DNA–protein cross-links to prevent double-stranded DNA damage in plants and animals (Lee *et al.*, 2010; Stinglee *et al.*, 2017). However, little information is known about its role in transcriptional regulation. The existence of cross-talk between DNA damage mechanisms and the plant immune responses has been previously reported, such as the dual role of SUPPRESSOR OF NPR1 INDUCIBLE1 (SNI1), RAD51, and BREAST CANCER2 (BRCA2) in the DNA damage response and the transcriptional regulation of defence response genes (Durrant *et al.*, 2007; Wang *et al.*, 2010). However, the complex interaction between these two fundamental biological processes is still poorly studied. Our results suggested that TDP1 negatively regulates the expression of *WRKY33*, encoding an essential transcription factor activating plant defence mechanisms against necrotrophic pathogens. Although TDP1 has been reported to be involved in plant

responses to abiotic stresses (Sabatini *et al.*, 2017), its role in biotic stress responses has not been reported previously. Our genetic analyses using the *tdp1-2* mutant demonstrated that TDP1 is involved in plant responses towards *P. syringae* and *B. cinerea*, because the *tdp1-2* mutant exhibited enhanced tolerance towards *P. syringae* and *B. cinerea* (Fig. S11). These results are consistent with our proposed model that TDP1 is involved in the transcriptional repression of both *MYB46* and *WRKY33* (Fig. S8).

In summary, our study revealed that AN is involved in plant immunity against biotrophic and necrotrophic pathogens. We demonstrated that the AN–TDP1 interaction is essential for the antagonistic transcriptional co-regulation of *MYB46* and *WRKY33*, which in turn regulates the expression of genes in the SA and JA/ET pathways.





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Author contributions

WM and J-GC initiated the project and coordinated data analysis and manuscript preparation; MX performed all biochemical assays; JZ, ACB and GAT contributed to bioinformatics analysis; MX, NE, TY, YP, AJR and TJT contributed to physiological analysis; MX, JL and DAP contributed to the pathogenic response assay; MX, JLM contributed to imaging analysis; MX, JZ, TY, ACB and JS contributed to expression analysis; MX, FC, WM and J-GC drafted the manuscript and all authors critically reviewed and approved the final version of the manuscript for publication.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Overexpression of Myc-tagged AN (AN-Myc) in *an-t1* (*an-t1 35S:AN*) reverses its narrow-leaf phenotype to wild-type morphology.

Fig. S2 AN antagonistically regulates gene expression in SA and JA/ET pathways.

Fig. S3 Yeast two-hybrid analysis of AN and TDP1 interaction.

Fig. S4 μ ChIP-qPCR analysis using protoplasts co-expressing AN-Myc and HA-TDP1.

Fig. S5 AN has no transcriptional activator activity.

Fig. S6 Transcription factors upregulated and downregulated in the *an-t1* mutant.

Fig. S7 Amino acid sequence alignment of Arabidopsis TDP1 (AT5G15170) and *Medicago* TDP1 (XM_003622639).

Fig. S8 Model showing AN-mediated transcriptional regulation of plant resistance to *P. syringae* and *B. cinerea*.

Fig. S9 Total amounts of salicylic acid (SA) and salicylic acid 2-*O*-glucoside (SAG) in Col-0, *an-t1*, and *an-t1 35S:AN* in 0 hpi and 24 hpi of *P. syringae* inoculation.

Fig. S10 Pathogen infection changes the transcription and nuclear accumulation of AN.

Fig. S11 *tdp1-2* mutant has increased resistance to *P. syringae* and *B. cinerea*.

Table S1 Primers used in this study.

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