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ORIGINAL ARTICLE

NtRAV4 negatively regulates drought tolerance in *Nicotiana tabacum* by enhancing antioxidant capacity and defence system

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Abstract

Key message ***NtRAV4*** **is a nucleus-localised protein and no self-activation effect.** ***ntrav4*** **mutants maintain the steady state of the ROS system under drought stress by enhancing antioxidant capacity and defence system.**

Abstract The APETALA2/ethylene response factor (AP2/ERF) transcription factor (TF) family plays an important role in plant responses to environmental stresses. In this study, we identified a novel NtRAV4 TF, a member of RAV subfamily among AP2/ERF gene family, which have AP2 and B3 domain in its N- and C-terminus, respectively. Subcellular localisation and self-activation activity analysis revealed that NtRAV4 localised in the nucleus and had no self-activation effect. The overexpression and gene editing vectors of *NtRAV4* were constructed by homologous recombination and CRISPR/Cas9 gene editing methods, and transformed into tobacco by *agrobacterium*-mediated method. *ntrav4* led to the appearance of termination codon in advance and lacked the unique B3 domain of RAV subfamily protein. Further analysis displayed that knockout of the *NtRAV4* in tobacco increased drought tolerance with high relative water content, accompanied by reduced stomatal aperture, density, and stomatal opening ratio compared to overexpression lines and WT. Moreover, *ntrav4* knockout plants also exhibited increased osmotic tolerance with low malondialdehyde (MDA) and ion leakage (EL), less accumulation of $O_2^{\bullet-}$ and H_2O_2, and high enzymatic antioxidant (SOD, POD, CAT) activities, non-enzymatic antioxidant (AsA-GSH cycle) contents and hormone (IAA, ABA, GA3, and ZR) levels under drought stress. Furthermore, *ntrav4* mutants in tobacco improved the expression levels of ROS-related proline synthesis and stress-responsive genes under osmotic stress. Our results indicate that NtRAV4 negatively regulates plant tolerance to drought stress by reducing water loss and activating the antioxidant system and stress-related gene expression to maintain the steady state of the ROS system.

Keywords Tobacco · Overexpression · *ntrav4* mutants · Drought tolerance · Antioxidant system

Abbreviations

ABA	Abscisic acid
MeJA	Methyl Jasmonate
EL	Electrolyte leakage
GFP	Green fluorescent protein
GR	Glutathione reductase
IAA	Indole acetic acid
NBT	Nitro blue tetrazolium

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NLS	Nuclear localisation signal
ROS	Reactive oxygen species
RWC	Relative water content
SD	Standard deviation
SOD	Superoxide dismutase
TF	Transcription factor
GA3	Gibberellin A3
ZR	Zeatin riboside

Introduction

As sessile organisms, plants are exposed to adverse conditions frequently, such as drought, high or low temperatures, and high salinity, which can influence the growth and development of plants. During evolution, plants have developed sophisticated mechanisms to adapt to stress. TFs can bind to promoters *cis*-acting elements or interact with other proteins to regulate (activate or repress) the expression of specific stress-related genes. AP2/ERF transcription factors are among the largest plant TF families. AP2/ERF TFs are divided into three subfamilies according to the number of domains and their binding motifs: AP2 (containing two AP2 domains), ERF/DREB (having one AP2 domain), and RAV (with an AP2 and a B3 domain) (Mizoi et al. 2012). For example, the RAV (ABI3/VP1) transcription factor has two distinct DNA-binding domains with an AP2 domain in the N-terminal region and a B3 domain in the C-terminal region, which can bind to CAACA and CACCTG sequence elements, respectively, therefore unique (Swaminathan et al. 2008).

Previous studies have found that proteins with AP2 and B3 domains are involved in plant stress responses and play a vital role in plant growth and hormone signal transduction (Park et al 2001; Swaminathan et al. 2008; Zhang et al. 2005; Gao et al. 2020). In *Arabidopsis*, *AtRAV1* suppresses flowering and hypocotyl elongation, and its overexpression of *AtRAV1* inhibits lateral roots and leaf growth (Hu et al. 2004). Similarly, soybean *GmRAV* overexpression showed a dwarfing phenotype, lower photosynthetic rate, shorter root length, and delayed flowering time (Lu et al. 2014; Zhao et al. 2008). The cotton *RAV1* overexpression in *Arabidopsis* confers plants with high salinity resistance and drought sensitivity compared to wild type (Li et al. 2015). *AtRAV1* and *AtRAV2* overexpression enhance drought tolerance in transgenic cotton (Mittal et al. 2014). In addition, in *Arabidopsis,* RAV TF modulates drought and salt stress response independent of ABA (Fu et al. 2014), AtTEM1 and AtTEM2 (TF with B3 domain) inhibit the production of two florigenic molecules and gibberellin production, delaying flowering (Matias-Hernandez et al. 2014; Osnato et al. 2012). Another research also displayed that *TEM* overexpressing plants in *Arabidopsis* were hypersensitive, whereas the *tem1tem2* double mutants were more tolerant to salinity (Osnato et al. 2021). Furthermore, in transgenic tomatoes, overexpressing the *Arabidopsis CBF1* gene, tomato RAV interacted with *AtCBF1* to regulate the expression of PR genes conferring bacterial wilt tolerance (Li et al. 2011). Although previous researches showed that the expression of *RAV* genes from other species in tobacco affects tobacco growth (Lu et al. 2014; Zhao et al. 2008), their role in tobacco is not known.

Tobacco is an economic crop, also used as an essential model in gene experiments. However, its growth and development were influenced largely by environment factors, such as drought stress. Previous studies have found that when tobacco suffered from drought stress, numerous physiological and molecular adaptations to drought were observed including abscisic acid and amino acid accumulation, stomatal closure, reactive oxygen species (ROS) homeostasis, and regulation the expression of drought-responsive gene (Lim et al. 2015; Jha et al. 2022; Xie et al. 2022). Studying the molecular mechanisms of stress adaptation and tolerances as well as mining regulatory genes is essential for tobacco. In this study, the NtRAV4 TF encoding a RAV DNA-binding protein was identified in tobacco. By overexpression and knockout of *NtRAV4* in *Nicotiana tabacum*, we found that NtRAV4 plays a negative role in drought tolerance by regulating the tobacco's antioxidant capacity and defence system. Our findings will improve the understanding of the molecular mechanisms underlying transcriptional regulation in response to abiotic stress.

Materials and methods

Plant materials and stress treatment

NC89 tobacco seeds were planted in soilless media (Pindstrup substrate) and cultured at 28 °C for 16 h / 8 h light and dark cycle in a controlled environment. At approximately 4-week tobacco seedlings were treated in 200 mM mannitol solution and incubated for 72 h to simulate drought stress. For hormone treatment, seedlings were sprayed with 100 µmol ABA and methyl jasmonate (MeJA), and data on three seedling replicates of the same leaf position were collected separately at 0, 1, 3, 6, 9, 12, 24, 48, and 72 h after treatment administration. Samples from root, stem, lugs, middle leaf, upper leaf, flower, and seed were obtained from NC89 tobacco plants of following anthesis in the field. Samples were immediately frozen in liquid nitrogen and stored at -80 °C until use.

Promoter analysis

The promoter sequence of *NtRAV4* (1500 bp upstream of ATG) was retrieved from the restricted Tobacco Genome

Database, China, and *cis*-acting elements of the promoter is predicted by online software PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

RNA extraction, reverse transcription and quantitative reverse transcription (qRT) PCR

Total RNA was extracted using the Plant Total RNA Isolation Kit (Foregene Co., Ltd, Chengdu, China) and then reverse transcribed into cDNA using a HiScript II Q RT SuperMix for qPCR Kit (Vazyme, Nanjing, China) for further qRT-PCR analysis. The qRT-PCR mix included 1 µL cDNA, 5 µL MonAmp™ SYBR® Green qPCR Mix (Monad, Wuhan, China), 0.2 µL forward primer, 0.2 µL reverse primer, and 3.6 µL Nuclease-Free Water. The reaction was carried out in a 96-well plate, qRT-PCR instrument (Eppendorf, Hamburg, Germany). The PCR procedure was 95 °C for 30 s, 40 cycles (95 °C for 10 s, 60 °C for 10 s, 72 °C for 30 s), and dissolution curve (use the instrument default acquisition program). The qRT-PCR analysis was performed using gene-specific primers for *NtRAV4*, ROS-related (*NtSOD, NtPOD, NtCAT*), and stress-responsive genes (*NtP5CS, NtOAT, NtNCED3, NtLEA5,* and *NtLTP1*). The mRNA levels were normalised to those of the L25. The specific primers used are shown in Supplementary Table S1, and gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Subcellular localisation

The full-length cDNA of *NtRAV4* in tobacco leaves was amplified with specific primers (NtRAV4-Sub-F/R), according to the sequence data in the tobacco Genome Database of *NtRAV4* (GenBank accession: XM_016589940), and the amplified PCR products were connected to the pBWA (V) HS-ccdb-GLosgfp vector to produce the pBWA (V) HS-NtRAV4-GFP vector fused with the green fluorescent protein (GFP) reporter gene. *NtRAV4* was linked to nuclear localisation signal protein (NLS) and cloned into the pBWA (V) HS-ccdb-GLosgfp vector to produce the pBWA (V) HS-NtRAV4-NLS fusion protein as a nuclear labelling vector. The positive clones were confirmed by sequencing and then transferred into *Agrobacterium tumefaciens* (EHA105) by electrotransformation. The 30-day-old quality seedlings were selected, injected from the leaf lower epidermis, cultured in low light for 2 days, and observed and imaged under a laser confocal microscope (Nikon C2-ER, Tokyo, Japan) with the corresponding empty carrier as a control.

Transactivational and binding activity analysis

The coding sequence (CDS) of NtRAV4 was subcloned into the GAL4 DNA-binding domain at the *Eco*RI/*Bam*HI site of the pGBKT7 vector. Transfecting pGBKT7-NtRAV4 (plasmid), pGBKT7-p53 (positive control), and pGBKT7 (negative control) into AH109 cells independently. The yeast cells were selected in SD-Trp/-Leu (DDO), SD-Trp/-Leu/-His + X-α-Gal (TDO/X), SD-Trp/-Leu/-His/-Ade + X-α-Gal (QDO/X) culture medium after transformation. The yeast cells were selected for 3 days and then cultured with 20 $g{\cdot}mL^{-1}$ X-α-Gal in blue. The transcriptional activity of NtRAV4 was verified based on its growth status and α-galactosidase activity.

Generation and identification of transgenic plants

According to the NovoRec® PCR, one-step directional cloning kit, the overexpression vector was constructed, and the positive overexpression vector was transferred into the competent cells of Agrobacterium GV3101. Then, tobacco was transformed by leaf disc method, kanamycin resistance was screened, well-developed wild-type and transgenic strains were selected, DNA was extracted, and primers were designed for PCR positive identification.

The *ntrav4* mutants were generated using the CRISPR/Cas9 system in the tobacco background. The 22 bp targeting sequences (including 3 bp PAM sequences) (AGG ACATGTTGTGAAGATGGGG) were used to construct the CRISPR/Cas9 genome editing vector. The CRISPR/Cas9 plasmids were introduced into *Agrobacterium tumefaciens* strain EHA105. The generation and identification of mutants were conducted as described previously (Li et al. 2017). Genomic DNA was extracted and primer pairs flanking the designed target site were used for PCR amplification. And, the PCR products were sequenced to confirm the homozygous mutants.

Drought treatment

For drought treatment, approximately 9-week-old tobacco seedlings were cultured as described above. The seedling irrigation was cut for 12 days until the soil moisture content to approximately 8% with normal irrigation for the control seedlings. After 12 days of moisture stress, parts of the tobacco seedlings were collected for analysis, and other parts of the seedlings were watered to observe the phenotype, and detect the stomatal characteristics, gene expression level using the first young leaf from the top of the seedlings, enzyme activity, hormone concentration and malondialdehyde (MDA), $O_2^{\bullet-}$ and H_2O_2 concentrations using the 2rd leaf counted from top to bottom of the seedlings, as well as proline content using the 3rd leaf counted from top to bottom of the seedlings.

Stomatal measurement

Ten 1 cm^2 square leaf discs were cut from the back of 3rd tobacco leaves from top to bottom, and the leaf discs were placed in 4% glutaraldehyde PBS buffer, dehydrated by ethanol, and freeze-dried. The pores were observed and imaged using scanning electron microscopy, and the stomatal pore diameter and density were measured using Photoshop software.

Physiological measurements and histochemical staining

Ion leakage (EL) was detected according to the method by previous study (Dahro et al. 2016). Proline, MDA content, ROS level ($O^{2\bullet-}$ and H_2O_2), and antioxidant enzyme activities including superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) as well as AsA-GSH cycle substances contents including ascorbic acid (AsA), glutathione (GSH), ascorbate peroxidase (APX) and glutathione reductase (GR) were determined using an enzyme activity test kit (Nanjing Jian Cheng Bioengineering Institute, Nanjing, China). The accumulation of $O_2^{\bullet-}$ and H_2O_2 in the first leaves was detected by histochemical staining with NBT and DAB (Yang et al. 2014).

Hormone measurement

The indole acetic acid (IAA), abscisic acid (ABA), gibberellin (GA3), and zeatin riboside (ZR) contents in plants were determined by enzyme-linked immunosorbent assays (Nanjing Jian Cheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Statistical analysis

The SPSS software (version 23.0; IBM Analytics, NY, USA) was used for data analysis, and the Student's t-test was used to evaluate the significance of differences. All data were obtained from at least three biological replicates with standard deviation (SD).

Results

Expression pattern analysis of *NtRAV4* in tobacco

Based on the genome sequence of *N. tabacum*, 1500 bp promoter of *NtRAV4* was obtained, and *cis*-regulatory elements were predicted using the PlantCARE software. The results showed that the *cis*-acting elements included low-temperature response element (LTR), MeJA-responsive element (CGTCA-motif), MYB binding site involved in drought inducibility (MBS), light-responsive elements (SP1 and MRE), stress-responsive elements (TC-rich repeats), and abscisic acid-responsive elements (G-box, ABRE) (Fig. 1a). Based on this prediction, qRT-PCR was used to detect the expression level of *NtRAV4* in different tissues under abiotic stress, and in hormonal treatments. Tissue-specific expression analysis displayed that *NtRAV4* was expressed in all examined tissues, the stems and lugs had the highest expression level, and the lowest expression level was in the flowers (Fig. 1b). And, the expression levels of *NtRAV4* firstly increased after drought-induced stress, ABA, and MeJA treatments, then decreased, and reached a peak at different points with different degrees (Fig. 1c–e).

Subcellular localisation and trans-activation activity analysis of NtRAV4 Protein

The NtRAV4 protein cellular distribution was detected using the *Agrobacterium*-mediated transient expression method labelled with green fluorescent protein (GFP) in tobacco epidermal cells. Fluorescence microscopic imaging showed that in the transformed control vector leaves, GFP was uniformly distributed throughout the epidermal cells. Furthermore, after transient expression of NtRAV4-GFP fusion proteins in tobacco epidermal cells, the GFP signals were merged with the nucleus-anchored localisation signal marker (NLS) (Fig. 2), suggesting that NtRAV4 was localised in the nucleus.

Transcriptional activity is an essential feature of the transcription factors. A yeast two-hybrid system was used to analyse the transcriptional activity of NtRAV4. The results showed that PGBKT7-P53, PGBKT7, and PGBKT7-NtRAV4 could grow plaques on DDO, which indicated that the plasmid was successfully transferred into yeast cells and is not toxic to the yeast cells. However, only PGBKT7-P53 developed plaques and turned blue on QDO/X. The pGBKT7 and pGBKT7-NtRAV4 did not develop plaques, suggesting that they could not activate the transcription of a downstream reporter gene and could not interact with X-α-Gal to turn plaque blue. Therefore, pGBKT7- NtRAV4 had no self-activation effect (Fig. 3).

Generation and identification of transgenic plants

NtRAV4 overexpression vector was constructed by homologous recombination method and transformed into tobacco by *Agrobacterium tumefaciens*. The successful transformation of *NtRAV4* was confirmed in nine tobacco plants using PCR (Fig. 4a). The relative expression of *NtRAV4* gene in each positive plant was detected by qRT-PCR. The OE-6 and OE-9 lines with relative higher expression levels were selected for the follow-up experiments (Fig. 4b).

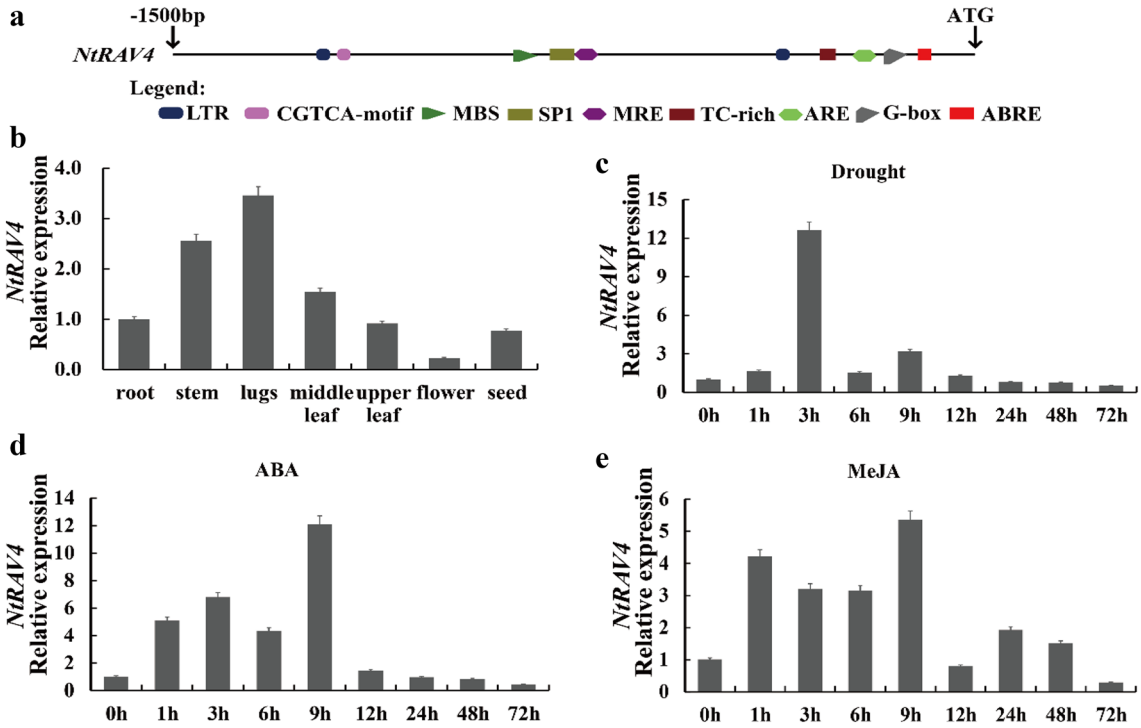

Fig. 1 Expression profiles of *NtRAV4* in tobacco. **a** *Cis*-elements analysis of *NtRAV4* promoter. **b** Expression of the *NtRAV4* genes in seven different tissues at various tobacco developmental stages. **c–e** Relative gene expression levels of *NtRAV4* in tobacco leaves under drought, ABA, and MeJA treatments. Relative expression level refers to normalization with ribosomal L25. Data represent the mean ± SD of three replicates

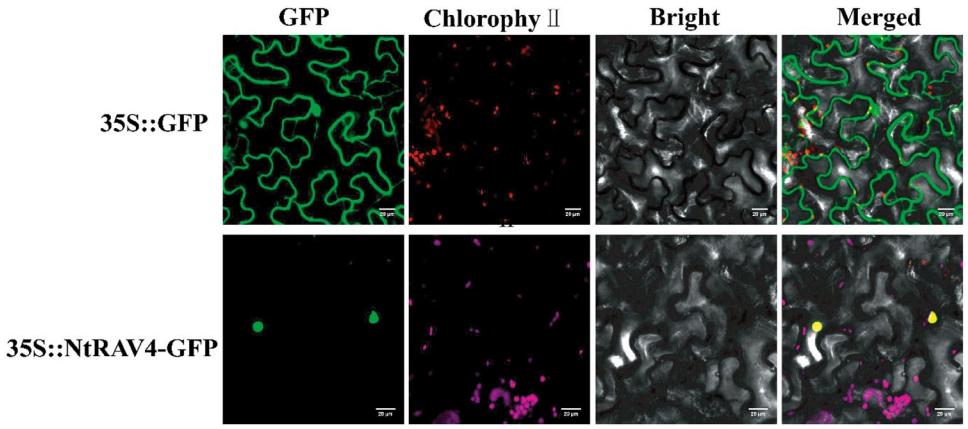

Fig. 2 Subcellular localisation analysis of NtRAV4. NtRAV4-GFP fusion protein was transiently expressed in tobacco epidermal cells. The images were observed by a laser confocal microscope (scale = 20 μm)

To further confirm NtRAV4 function, we employed the CRISPR/Cas9 system to generate mutants. After identification by sequencing, two independent homozygous mutants, *ntrav4-2* and *ntrav4-5*, were obtained in tobacco. The *ntrav4-2* mutant plants contained a 2 bp (CT) deletion of NtRAV4, and the *ntrav4-5* mutant plants contained a 1 bp (T) insertion of NtRAV4 (Fig. 4c), which led to the appearance of termination codon in advance (Fig. 4d). The progeny of these homozygous mutants were used in the subsequent experiments. It is worth mentioning that compared to NtRAV4 protein with both AP2 and B3 domains in WT, the *ntrav4* mutant protein lacked the unique B3 domain of RAV transcription factor (Table 1), which comprises seven beta-strands and two short alpha-helices (Yamasaki et al. 2013). Lack of B3 domain may lead to the corresponding function changes in mutant plants.

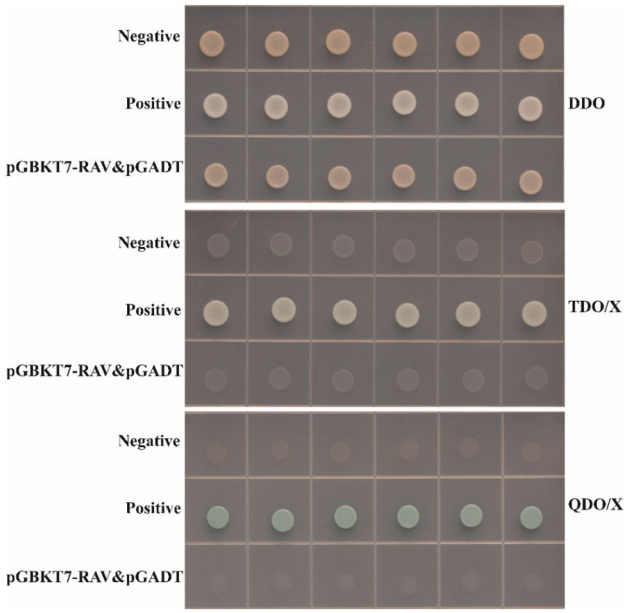

Fig. 3 Transcriptional activity analysis of NtRAV4 in yeast. PGBKT7- NtRAV4, PGBKT7-P53, and PGBKT7 were independently transfected into AH109 cells. The transformed yeast cells were cultured on DDO, TDO/X, QDO/X selective medium for 3 days, and then cultured into blue colour with 20 g mL-1 X-α-Gal

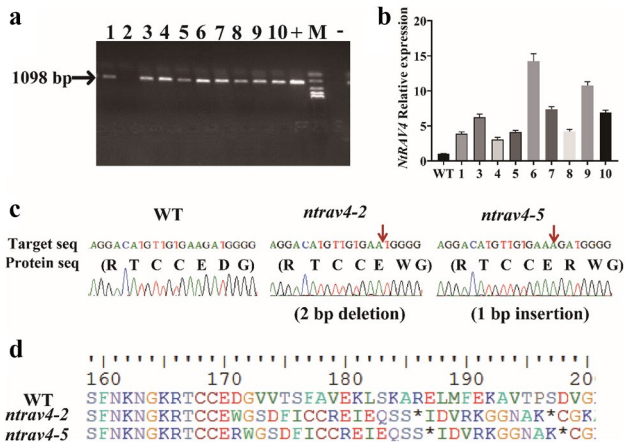

Fig. 4 Molecular characterization *NtRAV4* transgenic lines. **a** Positive identification of overexpression. M, 2000 bp DNA marker; +, positive control; -, negative control; 1–10, transgenic lines. **b** The expression level of *NtRAV4* gene in transgenic and WT plants. The expression level of wild-type *NtRAV4* gene of tobacco variety WT was normalized to one. **c** Analysis of the *ntrav4* mutants and sequencing detection peak. **d** The resulting proteins sequences of the *ntrav4* mutant. *, termination codon

ntrav4 mutants enhanced drought stress tolerance and affected osmotic tolerance

To determine whether *NtRAV4* is involved in the regulation of drought stress response. Six true leaves of WT and transgenic lines were subjected to natural drought for 12 days with normal seedling watering as a control. There were no significant morphological and RWC differences between the WT and transgenic lines before drought stress. After drought stress for 12 days, *ntrav4* mutant plants enhanced stress response capacity by reducing water loss in tobacco, and showed reduced withering and chlorotic phenotyping, and higher RWC (Fig. 5a, b).

We also measured the EL, MDA, and proline content. EL is a significant indicator of the cell membrane permeability. A higher EL value indicates more electrolyte leakage and a higher cell membrane damage. MDA is the final metabolic product of membrane lipid peroxidation. MDA content reflects the extent to which plants suffer adversity. On the other hand, under adverse conditions, plant cells make regular efforts to accumulate osmotic adjustment substances to reduce osmotic stress. Proline, an osmotic adjustment substance, plays a regulatory role in regulating cytoplasmic osmotic balance. *ntrav4* mutant plants displayed lower EL and MDA content but higher proline content than WT plants and OE lines under drought treatment(Fig. 5c–e), indicating that WT plants and OE lines had more severe damage than *ntrav4* plants.

The contents of chlorophyll a, chlorophyll b and total chlorophyll in tobacco leaves under drought stress were also measured (Fig. 5f–h). The results showed that under normal conditions, the contents of chlorophyll a, chlorophyll b and total chlorophyll in leaves of *ntrav4* mutant plants were significantly higher than those of WT and OE lines. After drought treatment, the contents of chlorophyll a, chlorophyll b and total chlorophyll in leaves of all lines decreased, however, the contents in *ntrav4* mutant plants were significantly higher than in WT and OE lines, which showed that *NtRAV4* knockout plants could keep higher chlorophyll content and better photosynthesis capacity under drought stress condition.

ntrav4 mutants affected stomatal characteristics in tobacco

As more than 95% of water loss in plants occurs via transpiration (Schroeder et al. 2001), stomata are the primary organization for gas exchange and transpiration. Water loss was investigated by observing stomatal closure. Under standard seedling irrigation, no significant differences were recorded between the WT and transgenic lines about stomatal aperture and density. However, drought stress resulted in reduced, stomatal apertures and density of WT and transgenic lines when compared with standard seedling irrigation conditions (Fig. 6a–c). And, the *ntrav4* plants showed more reduced stomatal aperture and density than WT and OE plants under drought stress, which resulted in that the stomatal opening ratio decreased more obviously in *ntrav4* plants after drought treatment (Fig. 6d–e).

Table 1 Knockout of *ntrav4* mutants' domain

Seq ID	Alignment start	Alignment end	Hmm acc	Hmm name	pfam
WT	190	287	PF02362.24	B3 DNA binding domain	AP2 B3 366
	60	107	PF00847.23	AP2 domain	
ntrav4-2	60	107	PF00847.23	AP2 domain	AP2 365
ntrav4-5	60	107	PF00847.23	AP2 domain	AP2 366

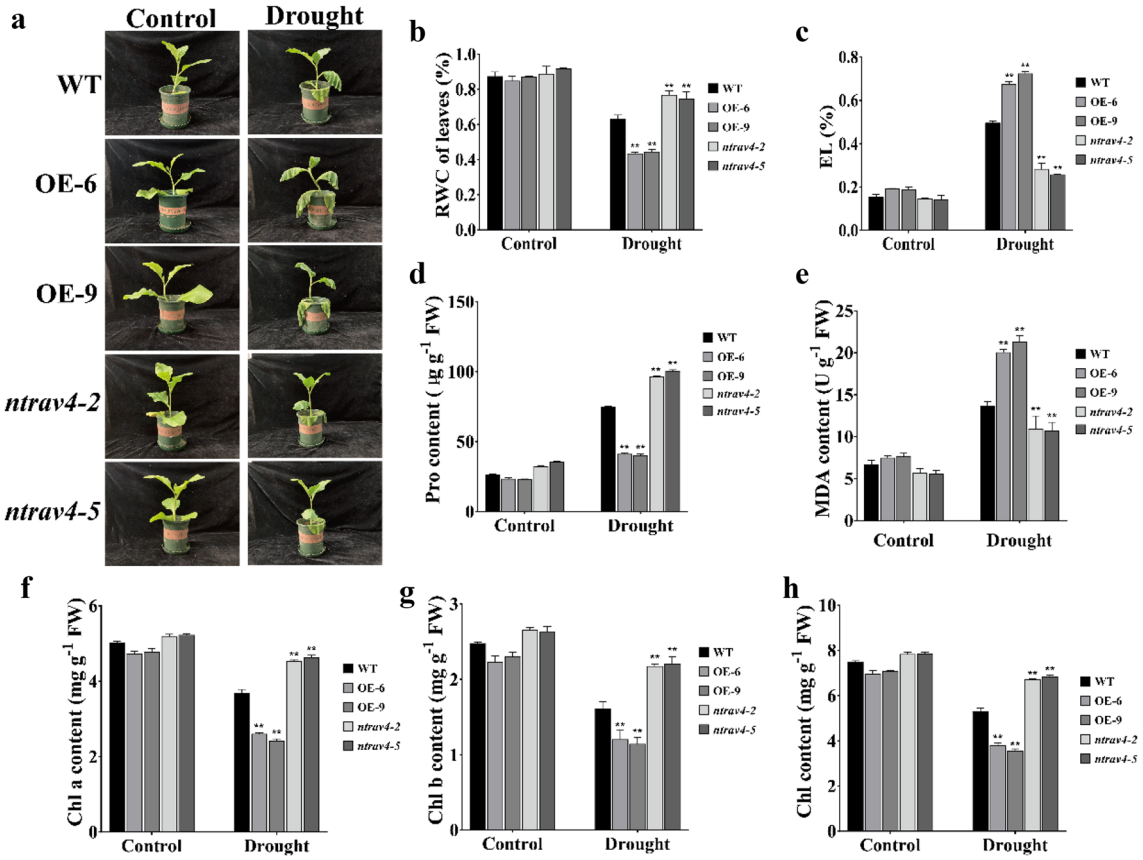

Fig. 5 Characterization of tobacco transgenic lines and WT under normal watered conditions and drought stress. **a** Morphology of transgenic lines and WT plants growth situation. **b** The relative water content of leaves was measured at 25 °C. **c** Electrolyte leakage (EL). **d** Proline content. **e** MDA content. **f** Chlorophyll a content. **g** Chlorophyll b content. **h** Total Chlorophyll content. Values represent mean ± SE of three independent replicates. Significant differences (*$p < 0.05$, **$p < 0.01$)

These results indicated that *ntrav4* plants enhanced tobacco drought tolerance by reducing water loss through stomatal closure, thereby reducing stomatal aperture and density.

ntrav4 mutants affected antioxidant metabolism under drought stress

To analyse the transgenic plants' antioxidant capacity under standard and drought stress conditions, we detected NBT and DAB staining conditions and the quantitative accumulation of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). The results showed that seedling leaves were slightly and heavily stained under drought stress. The degree of WT and OE seedlings staining was significantly deeper (Fig. 7a, b), suggesting that reactive oxygen species (ROS) levels were higher in the leaves of WT and OE than in *ntrav4* plants. Quantitative analyses of $O_2^{\bullet-}$ and H_2O_2 levels further validated these results (Fig. 7c, d).

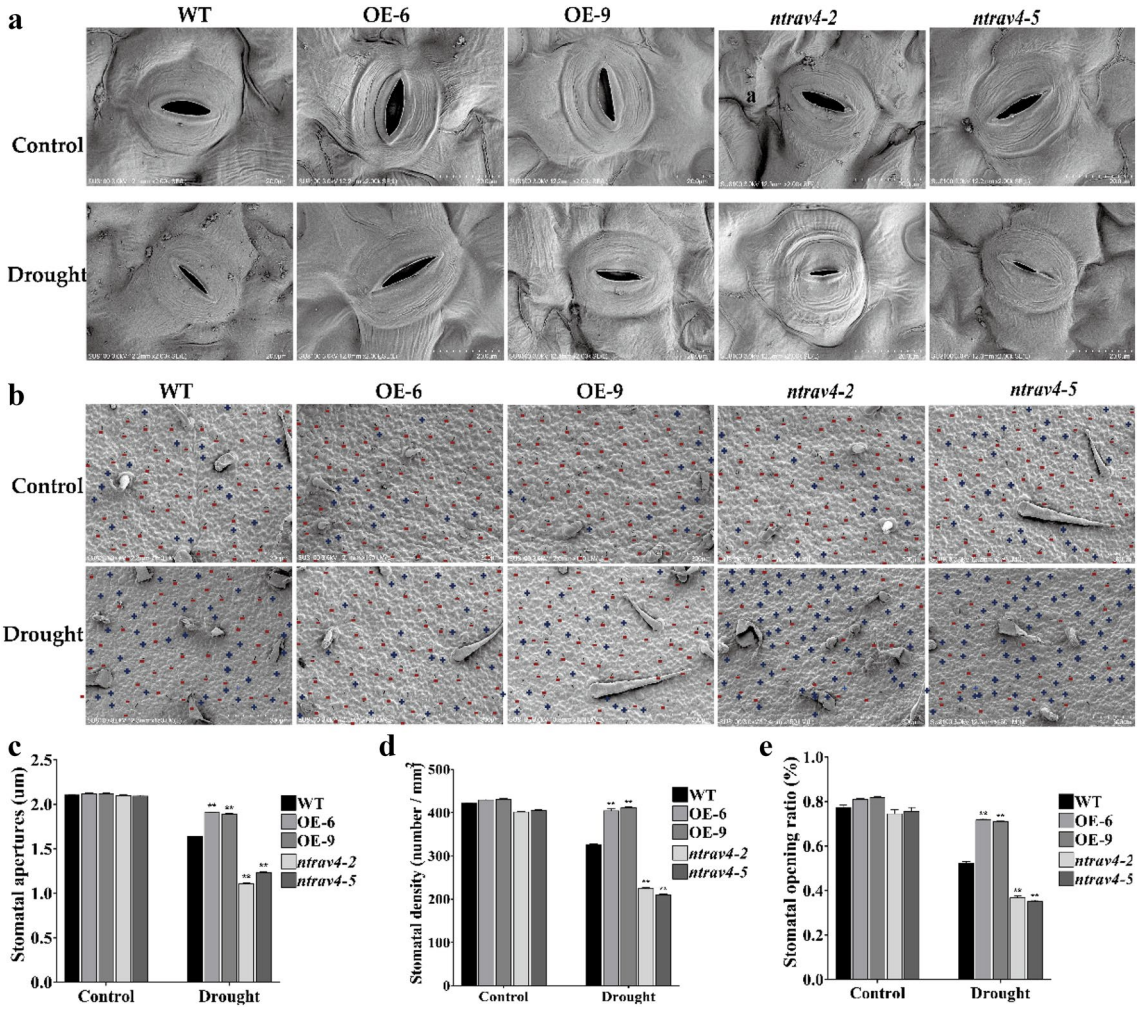

Fig. 6 Characterization of stomatal characteristics of transgenic lines and WT plants in tobacco under normal watered conditions and drought stress. **a** Scanning electron microscope images of stomatal apertures of transgenic lines and WT plants. **b** Scanning electron microscope images of the stomatal density of transgenic lines and WT plants. The blue symbol "+" represents closed stomata and the red symbol "-" represents open stomata. **c** Stomatal apertures in transgenic lines and WT plants. **d** Stomatal density in transgenic lines and WT plants. **e** Stomatal opening ratio (%) in transgenic lines and WT plants. Values represent mean ± SE of three independent replicates. Significant differences (*$p < 0.05$, **$p < 0.01$)

We examined the activities of ROS-scavenging enzymes, including SOD, POD, and CAT. The results showed that the activities of SOD, POD, and CAT antioxidant enzymes were significantly higher in *ntrav4* plants than in WT and OE plants under drought treatment (Fig. 7e–g). AsA and GSH play an essential role in maintaining protein stability, integrity of the biomembrane system structure, and defence against membrane lipid peroxidation, which, together with APX and GR, constitute the AsA-GSH cycle, can effectively remove free radicals. The accumulation of AsA-GSH cycle antioxidants was also significantly higher in *ntrav4* than in WT and OE plants (Fig. 7h–k). These results suggest that *NtRAV4* negatively regulates drought tolerance in tobacco by regulating antioxidant capacity.

ntrav4 mutants affected hormone levels under drought stress

Drought stress often causes changes in hormone levels. Therefore, the IAA, ABA, GA3, and ZR contents were measured. As shown in Fig. 8, the increases of IAA, ABA, GA3, and ZR levels were generally observed in *ntrav4* compared to WT and OE plants under normal conditions. Among which, ABA and GA3 reached extremely significant levels. After drought treatment, the levels of these hormones significantly increased, the IAA, ABA, GA3, and ZR contents were higher in *ntrav4* plants than in WT and OE plants under drought treatment.

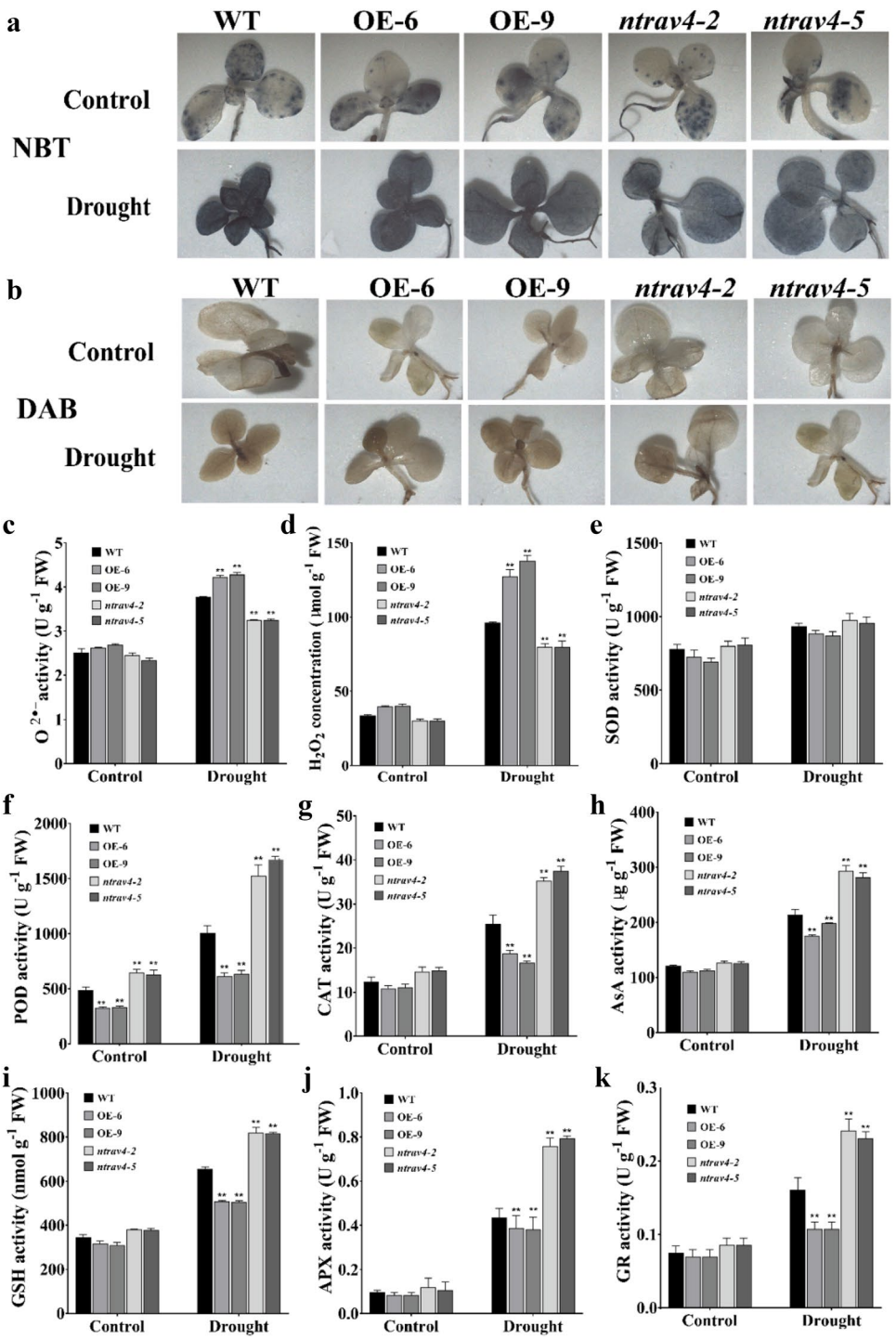

Fig. 7 Biochemical analyses of WT and transgenic lines under normal conditions (control) and drought treatment. **a**, **b**, In vivo localisation of $O_2^{\bullet-}$ and H_2O_2 by nitro blue tetrazolium (NBT) and diaminobenzidine (DAB) staining, respectively. **c**, **d**, Determination of $O_2^{\bullet-}$ production and H_2O_2 levels. **e**–**g**, Activities of antioxidant enzyme (SOD, POD and CAT); **h**–**k**, contents of AsA-GSH cycle, AsA, GSH, APX, and GR. Values represent mean ± SE of three independent replicates. Significant differences (*$p < 0.05$, **$p < 0.01$)

NtRAV4 regulates the expressions of stress-related genes under drought stress

To explain the function and molecular mechanisms of *NtRAV4*, we analyzed the stress-related metabolic pathway genes expression levels, including the antioxidant enzyme system, critical genes involved in proline synthesis, and key genes involved in ABA biosynthesis as well as other drought-related genes by qRT-PCR. The expression of these genes is strongly induced by drought stress. Consistent with the activity results of three antioxidant enzymes (SOD, POD, and CAT) in *ntrav4* plants, the relative expression levels of *NtSOD, NtPOD*, and *NtCAT* were significantly higher than those in WT and OE plants under drought stress. Similarly, *NtP5CS* and *NtOAT* genes encoding critical enzymes in proline synthesis were upregulated under drought stress,

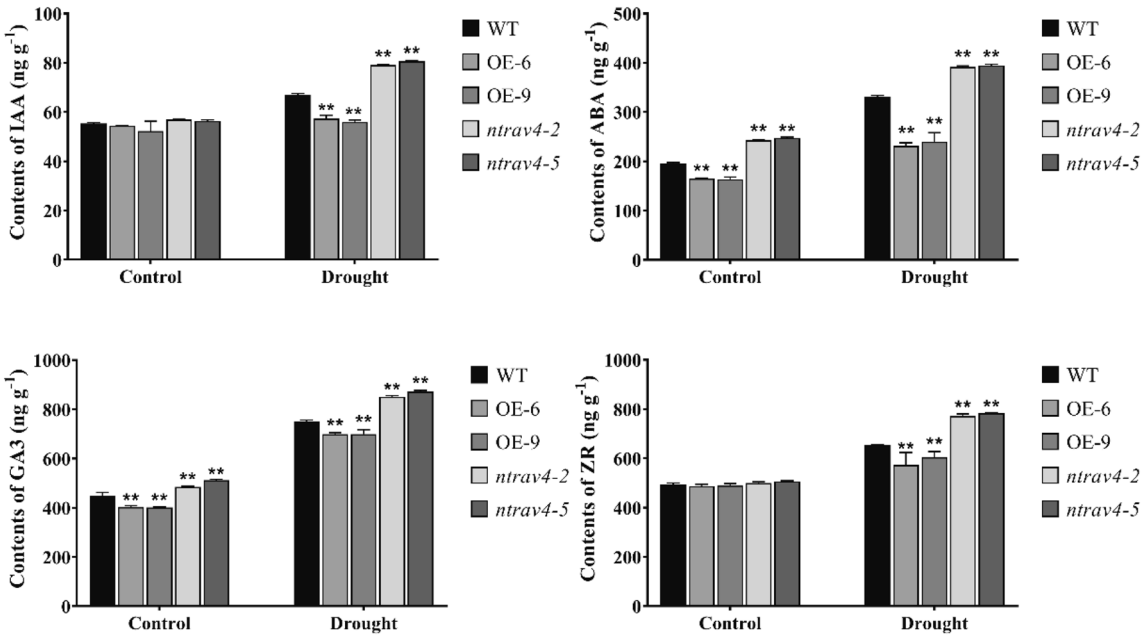

Fig. 8 Hormone (IAA, ABA, GA3, and ZR) contents in WT and transgenic lines under normal conditions (control) and drought treatment. The experiment was replicated three times (*$p < 0.05$, **$p < 0.01$)

particularly in mutant plants. Furthermore, the *NtNCED3*, a key regulatory gene upstream of the ABA metabolic pathway, in addition to *NtLEA5* and *NtLTP1*, which are related to stress defence and drought tolerance, were significantly induced by drought treatments. In agreement herewith, the gene expression of *ntrav4* mutant plants was significantly higher than that of WT and OE plants (Fig. 9).

Discussion

The RAV transcription factor is unique because it has AP2 and B3 DNA-binding domains (Kagaya et al. 1999). Previous studies have reported that TFs with B3 domain, such as FUS3, LEC2, and ABI3 function in seed development and ABA signal transduction pathway (Giraudat et al. 1992; Stone et al. 2006); TFs with AP2 domain play central roles in plant growth and development and response to stress and hormone signalling pathways. Also, some researches have shown that the foreign *RAV* gene could confer stress tolerance in tobacco (Lu et al. 2014; Zhao et al. 2008), but the biological functions and molecular mechanisms of *NtRAV* in tobacco have not yet been documented. This study identified a novel *NtRAV4* TF, a member of RAV subfamily among AP2/ERF gene family, from *N. tabacum*. Subcellular localisation and self-activation activity analysis revealed that NtRAV4 is localised in the nucleus and has no self-activation effect.

Through the analysis of *cis*-elements suggested that *NtRAV4* may be participate in abiotic stress responses in plants. The *NtRAV4* promoter contains several droughts stress response-related *cis*-elements such as MBS (MYB binding site involved in drought inducibility), G-box, and ABRE elements (abscisic acid-responsive element), TC-rich repeats (stress-responsive element), and CGTCA-motif (MeJA-responsive element) (Fig. 1A). The upregulated *NtRAV4* expression after drought, ABA, and MeJA treatments in tobacco suggested that *NtRAV4* was involved in stress response in plants. Consequently, to explain the *NtRAV4* function, the *NtRAV4* overexpressed plants and CRISPR/Cas9—mediated knockout of *NtRAV4* transgenic tobacco was obtained. Sequence and domain analysis results showed that the translation of *ntrav4* mutant was terminated in advance and *ntrav4* mutant protein lacked the B3 domain of RAV transcription factor. The deletion of B3 domain may cause the corresponding function change. Then the drought tolerance ability was evaluated by examining various physiological indicators and clarifying its regulated molecular mechanism. Subsequent experiments suggested that *ntrav4* mutant plants confer drought tolerance in transgenic tobacco with higher RWC and lower water loss in leaves.

Plants can control water loss by reducing stomatal conductance and evapotranspiration. Transpiration is the principal pathway that leads to water loss. The stomatal operation state reflects the metabolism of plants to a certain extent. Therefore, the rapid adjustment of the stomatal aperture is an essential plant drought-tolerant characteristic (Jezek and Blatt 2017; Zoulias et al. 2018). The TF-mediated stomatal closure is

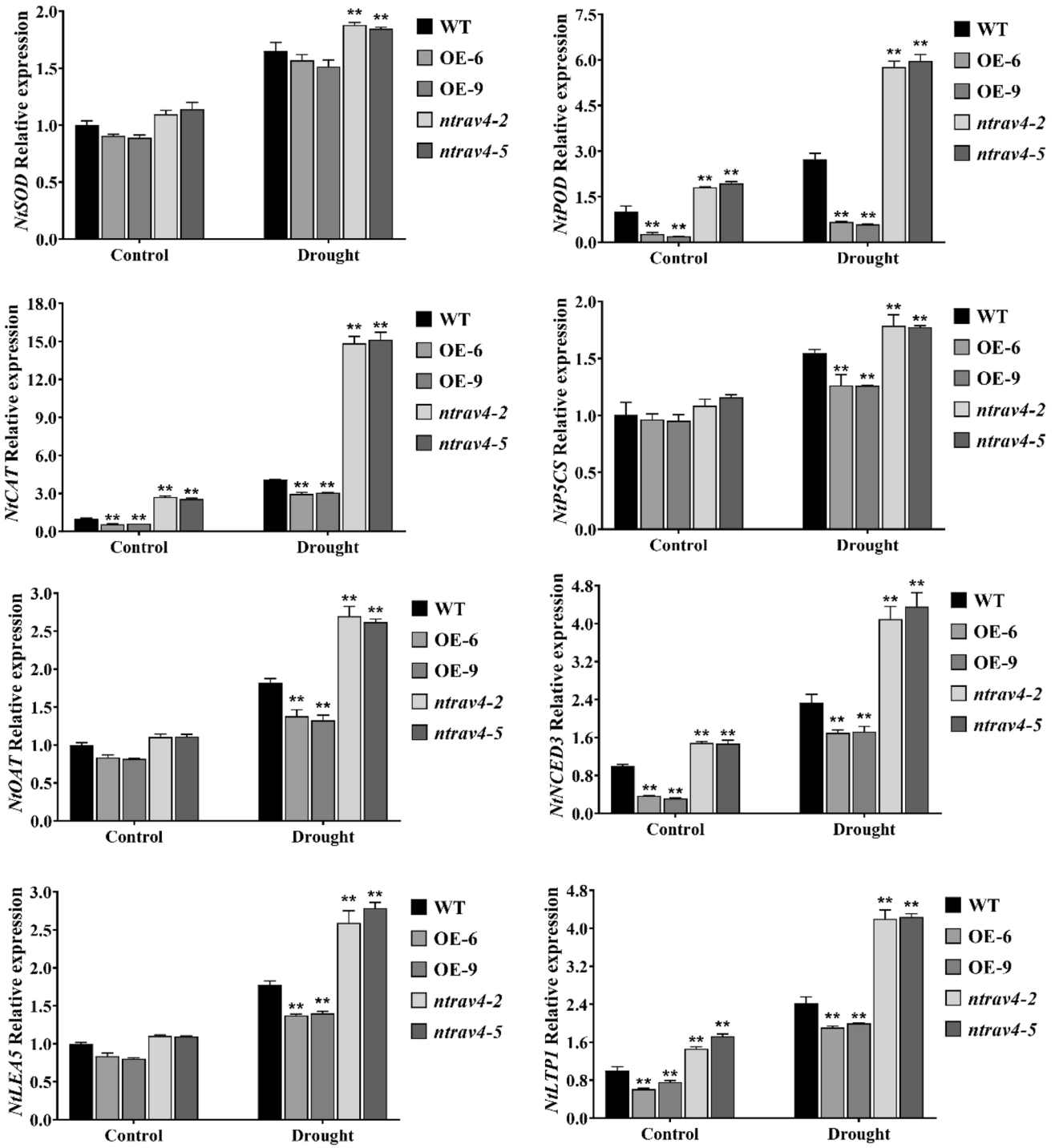

Fig. 9 Analysis of expression levels of ROS-related and stress-responsive genes in WT and transgenic lines by qRT-PCR under normal and drought stress conditions. Relative expression level refers to normalization with ribosomal L25. Values represent mean ± SE of three independent replicates. The experiment was replicated three times (*$p < 0.05$, **$p < 0.01$)

essential for reducing water loss, this mechanism is crucial for plant survival under drought stress. Our results showed that *ntrav4* mutant plants had better water retention capacity under drought stress than WT and OE plants due to stomatal closure and reduced stomatal aperture and density. Consistent with these results, *NtERF172* or *NtHSP70* overexpression enhances drought tolerance by decreasing stomatal apertures and transpiration rates with higher water use efficiency in tobacco (Song et al. 2021; Zhao et al. 2020). Other studies also illuminated that ABA signalling-induced stomatal closure triggered

changes in guard cells, thus reducing transpiration in response to drought stress (Raghavendra et al. 2010; Lee et al. 2012; Yuan et al. 2021). In our research, after drought treatment, the content of ABA and the expression level of *NtNCED3* and key regulatory genes of the ABA metabolic pathway increased significantly. These results suggest that *NtRAV4* might be involved in ABA-mediated stomatal closure during drought stress.

Most abiotic stress signals can generate redundant ROS accumulation, especially $O_2^{\bullet-}$ and H_2O_2, bring about badly oxidative damage in plants (Skopelitis et al. 2006). Furthermore, ROS accumulation may cause biomembrane lipid peroxidation resulting in MDA accumulation and severe EL. Under drought stress, *ntrav4* mutant plants had a significantly lower quantitative accumulation of $O_2^{\bullet-}$and H_2O_2, MDA, and EL than WT plants. To eliminate ROS accumulation and reduce osmotic stress, plants form an antioxidase system composed of ROS-scavenging enzymes (such as SOD, POD, and CAT), and actively accumulated osmotic adjustment substances (such as proline) to regulate the cytoplasmic osmotic balance. Our research showed that the activities of enzymatic antioxidants and proline content were significantly higher in *ntrav4* mutant plants than in WT plants under drought stress. Accordingly, the increased expression levels of *NtSOD*, *NtPOD*, and *NtCAT* in mutant plants resulted in a stronger ROS-scavenging capacity under drought stress. Coincidently, *NtP5CS* and *NtOAT* genes encoding vital enzymes involved in proline synthesis were upregulated after drought treatment. In addition, the expression level increased significantly in *ntrav4* mutant plants than in WT and OE plants, regulating osmotic balance and protecting the plasma membrane (Bartels and Sunkar, 2005). In addition, *NtLEA5* encodes a group of late embryogenesis abundant proteins, which maintains the proteins and membranes stability (Amara et al. 2012). The lipid transfer protein *LTP1* is induced by ABA, drought, low temperature, and salt treatment (Hu et al. 2013). Under drought stress, the expression levels of the above genes in mutant plants were all upregulated, which indicated that tobacco drought tolerance was enhanced by inhibiting the expression of *NtRAV4*.

Under internal and external environmental pressures, plants may adopt a complicated mechanism to preferentially remove excess ROS (Dat et al. 2000). The ascorbic-glutathione cycle (AsA-GSH), an antioxidant defence system of plants, enables plants to scavenge active oxygen. The APX enzyme is involved in the H_2O_2 decomposition reaction in the AsA-GSH cycle, and the improvement of its activity indicates the progress of the active oxygen degradation rate (Shigeoka. et al. 2002). GR is the last rate-limiting enzyme in the AsA-GSH circulation pathway, necessary for AsA regeneration and antioxidant stress response (Yao et al. 2021). In our study, the activities of AsA, GSH, APX, and GR in vivo increased significantly to remove ROS in mutant plants after drought stress. These results indicate that mutant plants activate the ROS-scavenging system to reduce oxidative damage and enhance drought tolerance.

The present study found that *ntrav4* mutant plants enhanced drought tolerance in transgenic tobacco by ABA-mediated stomatal closure to reduce water loss. In this study, we proposed a model of how *NtRAV4* negatively regulates tobacco growth and development under drought stress (Fig. 10). (1) Silencing of *NtRAV4* expression altered

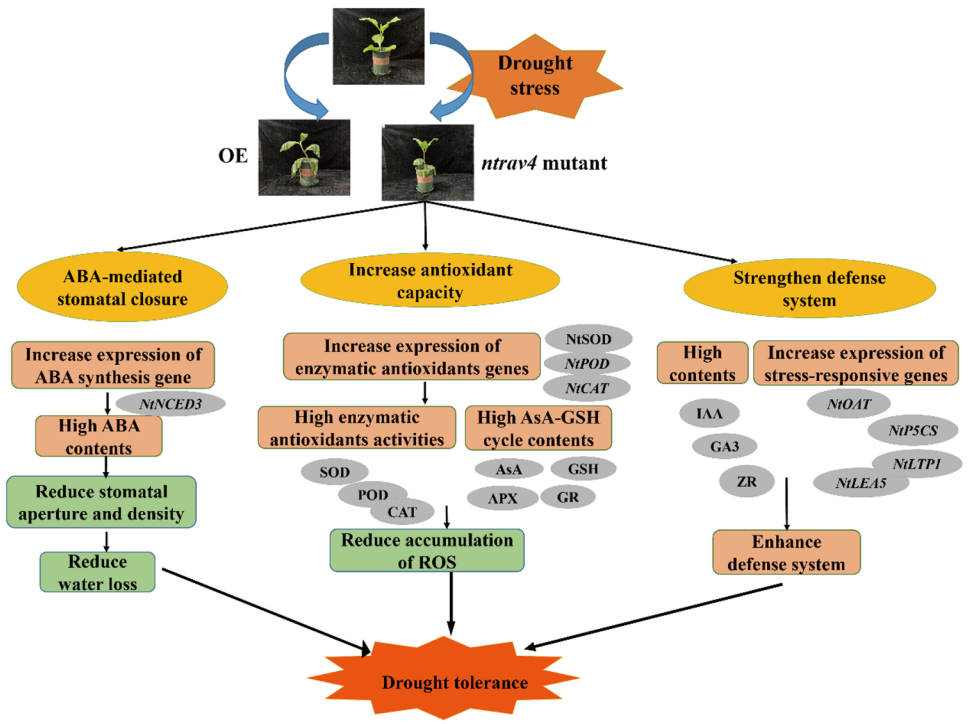

Fig. 10 A mechanism diagram of *NtRAV4* negatively regulating drought tolerance

morpho physiology, regulating stomatal movement to prevent water loss. (2) *ntrav4* mutant plants enhanced antioxidant enzyme activity to scavenge ROS accumulation to maintain the steady state of the ROS system. (3) NtRAV4 TF mediated the hormone levels and the drought-related genes expression and then conferred drought tolerance in plants. However, further research is needed to determine which downstream genes *NtRAV4* directly regulates.

Conclusions

Our results suggested that NtRAV4 is a nucleus-localised protein, and transactivational and binding activity analysis results showed no self-activation effect. The promoter of *NtRAV4* contains many abiotic stress-responsive *cis*-regulatory elements, and the gene expression of CRISPR/Cas9-mediated knockout of the *NtRAV4* increases in response to drought, ABA, and MeJA in tobacco. Silencing of *NtRAV4* reduced water loss, increased antioxidant capacity, and enhanced drought tolerance by regulating ABA-mediated stomatal closure, increasing antioxidant enzyme activity, and upregulating stress-related genes.

Author contribution statement

YG, YY and JX designed the experiments. YG, XM and LQ performed the experiments. YG, JY, WD and ZX analyzed the data. YG and YY writing the original draft. YY and JX review and editing the paper. All authors have read and agreed to the published version of the manuscript.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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