The Ethylene Response factor SmERF1 Improves Salinity Tolerance and Impacts Seed Size in Tobacco

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Research article

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Abstract

**Background** Ethylene response factor (ERF) proteins play vital roles in plant resistance and plant development. However, little is known about the ERF transcription factors of *Salvia miltiorrhiza*, which is a famous Chinese herb with good resistance to stress.

**Result** Screened from our previous transcriptome data, *SmERF1*, an ERF transcript factor, was isolated from *S. miltiorrhiza*. *SmERF1* had a single AP domain and was classified in the ERF E3 subfamily. *SmERF1*-expressing tobacco plants showed slower growth, less biomass and a decrease in chlorophyll only at the seedling stage, and there was no significant difference in other growth stages. In addition, seeds of tobacco plants with *SmERF1* expression were smaller and lighter than those of wild plants, similar to some AP2 TFs. Under NaCl treatment, transgenic tobacco lines showed better tolerance to salinity, and the proline content, SOD and POD activities of transgenic lines were higher than those of wild-type plants, while MDA content was lower than that of wild-type plants. Furthermore, we determined the phytohormones related to plant resistance and showed that transgenic tobacco plants had higher ABA levels and lower GA levels than wild tobacco. The expression of *SmERF1* regulated the expression of key enzyme genes related to plant hormone biosynthesis, such as *NtSDR*, *NtGA20ox*, *NtACO* and *NtACS*.

**Conclusions** The *SmERF1*-expression in tobacco affect plant growth at seedling stage, and increase plant tolerance to salt. And the expression of *SmERF1* cause tobacco seeds smaller and lighter. Our study suggested that *SmERF1* enhanced tobacco tolerance to salt and impacted seed size through an ABA-dependent pathway.

**Background**

The ethylene-responsive factor (ERF) proteins are a large family of transcription factors known to play significant roles in various plant biological processes, such as plant growth, development, metabolism and biotic and abiotic stress responses[1–3]. ERF members contain one conserved AP2/ERF domain consisting of approximately 60 to 70 amino acids in the DNA-binding region, which can bind to CC-box containing DNA fragments and activate expression of the defense-related genes [4]. An increasing number of studies have shown that ERFs modulate multiple responses to abiotic and biotic stresses. The overexpression of *ERF1* in Arabidopsis can increase resistance to both *Botrytis cinerea* and *Pseudomonas syringae* [5]. Transgenic plants overexpressing *OsDERF1* (a member of the ERF family from rice) exhibited reduced tolerance to drought stress in rice at the seedling stage, while knockdown of *OsDERF1* expression conferred enhanced tolerance at the seedling and tillering stages[6]. The tomato ERF TSRF1 (or TERF1) positively regulates pathogen resistance in tomato and tobacco [7]. AtORA59 and AtERF1 in Arabidopsis could activate the expression of the *PDF1.2* gene and further activate the expression of resistance genes in response to stress[8].

Evidence suggests that ERF transcription factors are involved in regulating plant hormone synthesis and integrating hormone signaling pathways. For example, the expression of the cotton ERF gene *GbERF*, the
tobacco ERF gene \textit{NtCEF1} and the tomato ERF gene \textit{JERF1/3} has been shown to be induced by abscisic acid (ABA)\cite{9}. \textit{JERF1} was inducible by ethylene (ET) and ABA\cite{7}. The soybean ERF gene \textit{GmERF135} participates in ABA and ET signaling pathways to regulate the expression levels of stress-related genes\cite{10}. \textit{CrERF5} from \textit{Catharanthus roseus} responds to both ethylene and jasmonate (JA) signals\cite{11}. Moreover, some ERFs have been shown to regulate plant hormone biosynthesis. Rice TSRF1, peach PpERF3 and Arabidopsis ORA47 were shown to regulate ABA level\cite{12–14}. Apple \textit{MdERF2} and \textit{AtERF11} can affect ET or gibberellic acid (GA) biosynthesis \cite{15, 16}. ABA, GA and ET, are well known to play major roles in mediating plant defense responses against biotic and abiotic stresses\cite{17}.

\textit{Salvia miltiorrhiza} Bunge is a well-known medicinal plant, the dry roots of which are widely used in the treatment of cardiovascular, cerebrovascular, hyperlipidemia, and acute ischemic stroke diseases. Due to its good curative effect on cardiovascular and cerebrovascular diseases, the demand for \textit{S. miltiorrhiza} has increased. Over 10\% of traditional Chinese patent medicines and simple preparations contain \textit{S. miltiorrhiza}. The annual consumption of \textit{S. miltiorrhiza} has exceeded 16 million kilograms in China\cite{18}. With the increased demand for \textit{S. miltiorrhiza}, wild resources are gradually being exhausted and the cultivation of new resources has become a research hotspot. The contents of active substances and plant active resistance are the main evaluation indexes of \textit{S. miltiorrhiza} quality.

Based on the genome data of \textit{S. miltiorrhiza}, 79 ERFs were identified\cite{19}. \textit{SmERF6}, \textit{SmERF1L1} and \textit{SmERF128} can positively regulate the biosynthesis of tanshinones \cite{20–22}, while \textit{SmERF115} can positively regulate the phenolic acid biosynthesis by affected the expression of key enzymes in the biosynthetic pathway of phenolic acids\cite{23}. Additionally, \textit{SmERF115}, \textit{SmERF1L1} and \textit{SmERF128} ERFS were responsive to methyl jasmonate (MeJA) or yeast extraction (YE) treatment, which are thought to be involved in active ingredient biosynthesis and plant resistance\cite{24–27}. However, functions of most other ERFs in \textit{S. miltiorrhiza} are still unknown. In this study, we isolated one novel ERF gene (named \textit{SmERF1}) by screening our transcriptome library\cite{28}, which was also responsive to methyl jasmonate MeJA or YE treatment. Our study showed that the expression of \textit{SmERF1} affected the expression of ABA and GA biosynthesis, affected seed size, and enhanced the tolerance of transgenic tobacco to drought, revealing the important roles of \textit{SmERF1} in improving crop agricultural characteristics.

\section*{Results}

\section*{isolation and characterization of \textit{SmERF1}}

The isolated full-length \textit{SmERF1} (GenBank Accession No. KC405081.1) contained an ORF comprising 549 bp, encoding a polypeptide of 182 amino acids with a molecular weight of 19.6 kDa and a pI of 9.64. A single conserved AP2 DNA-binding domain with 63 amino acids was identified in \textit{SmERF1} by multiple sequence alignment (Fig. 1A). \textit{SmERF1} was predicted to be a nuclear-localized protein. \textit{SmERF1} showed high identities (\geq 74\%) with ERF proteins from other plants, such as tobacco (\textit{NtERF5}, AAU81956), \textit{Lithospermum erythrorhizon} (LeERF-1, GQ246186.1) and \textit{Artemisia annua} (AaERF1, AEQ93554.1).
Phylogenetic analysis revealed that SmERF1 was classified in the B3 subfamily, together with NtERF5 and AaERF1 (Fig. 1B).

**Expressing SmERF1 in tobacco resulted in smaller tobacco seed size**

Young leaves from transgenic plants selected with Basta were used to extract DNA and RNA. After detecting the CaMV35S promoter sequence in transgenic plants, 15 transgenic tobacco lines were confirmed (Fig S1A). Through qPCR verification, four lines (OE-3, OE-4, OE-7 and OE-10) with high expression levels of SmERF1 were obtained and used for follow-up experiments (Fig S1B). There were some differences only at the seedling stage, and there was no significant difference in other growth stages between transgenic and wild plants (see results in 3.4).

Furthermore, we harvested T2 generation seeds from four tobacco transgenic lines of SmERF1. We found that the seed size of each tobacco transgenic line was smaller than that of the wild-type plants (WT) (Fig. 2A). According to the statistical analysis, the thousand kernel seed weights of OE-3, OE-4, OE-6, OE-7 and OE-10 were 59%, 63%, 55%, 56% and 58% of the WT, respectively (Fig. 2B).

**Expression of SmERF1 in tobacco affected young tobacco seedling growth**

During the germination stage, white buds appeared on the WT seeds of tobacco at 7 days after sowing, while the buds on tobacco transgenic seeds (T1) appeared later at 9 days. Two weeks after sowing, the growth rate of transgenic tobacco was significantly slower than that of wild plants (Fig. 3A). The biomass of transgenic tobacco seedlings was 48% ~ 0.53% (Fig. 3B). In addition, we observed that the leaves of transgenic tobacco seedlings were yellower than those of wild-type seedlings (Fig. 3A). The chlorophyll content of transgenic seedlings was 82%-84% of that of the WT (Fig. 3D); however, there was no significant difference in the germination rates between transgenic and WT seeds under normal conditions (Fig. 3C).

**Expressing SmERF1 in tobacco enhances salt tolerance**

To detect tolerance to salt, two-month-old plantlets of T2 generation transgenic and WT plants were treated with 400 mM NaCl. Leaves of all wild-type plants wilted and rolled up, and some old leaves turned yellow, while leaves of transgenic lines showed better status than that of WT, and only old leaves rolled up (Fig. 4A).

The physiological parameters, including SOD, POD, MDA content, proline content, and transgenic and WT tobacco plants, were compared under both normal and 400 mM NaCl conditions (Fig. 4B). (1) After NaCl treatment, the POD activity of transgenic tobacco plants was significantly higher than that of WT plants. Under normal conditions, the POD activities of transgenic tobacco plants were higher than those of WT plants, but there was no significant difference. (2) After NaCl treatment, the transgenic tobacco plants also showed markedly higher SOD activity than that of WT plants. However, under normal conditions, there were no significant differences. (3) The MDA content of the transgenic tobacco plants was significantly lower than that of WT under both conditions. (4) There was no significant difference in
proline content between transgenic tobacco plants and WT under normal conditions, while proline content was 1.35–1.48 times higher in transgenic tobacco plants than WT after NaCl treatment.

**Expressing SmERF1 in tobacco regulates ABA and GA biosynthesis**

Abscisic acid (ABA) and gibberellins (GA) are a pair of classic phytohormones that antagonistically mediate several plant developmental processes, including seed maturation, seed dormancy and germination, primary root growth, and flowering time control (Shu et al., 2018). We found that the ABA contents of transgenic tobacco lines were 1.53–2.51 times higher than those of WT (Fig. 4-1c), and the GA contents of transgenic tobacco lines were 0.60–0.74 times lower than those of WT (Fig. 4-1d). The results from qRT-PCR showed that the expression of *NtSDR* (short-chain dehydrogenase/reductase, GenBank Accession No. AJ223177.1) was activated in *SmERF1*-overexpressing tobacco, which encodes a key enzyme of ABA biosynthesis. *NtGA20ox* (GenBank Accession No. JQ413251), a key enzyme-encoding gene of GA biosynthesis, was repressed in *SmERF1*-overexpressing tobacco. The study showed that the *SmERF1*-overexpressing seedlings had higher ABA content and lower GA content than WT plants (Fig. 4b), indicating that SmERF1 can regulate the plant hormone response of transgenic tobacco seedlings to drought through the ABA pathway.

Moreover, we also tested the expression of *NtACS* (GenBank Accession No. NM_001326220.1) and *NtACO* (GenBank Accession No. NM_001325967), two key enzyme-encoding genes in plant hormone ethylene biosynthesis. Our qRT-PCR analysis showed that the transcription of both *NtACS* and *NtACO* increased in transgenic tobacco lines, which suggested acceleration of ET production.

**Discussion**

Over 10% of traditional Chinese patent medicines and simple preparations contain *S. miltiorrhiza*. The annual consumption of *S. miltiorrhiza* has exceeded 16 million kilograms in China[18]. More and more genes involved in *S. miltiorrhiza* resistance have been studied. For example, genes of three multigene universal stress proteins (SmUSPs) were cloned, and their expression enhanced *Escherichia coli* tolerance to salt and heat stress[29]. Overexpression of *SmSnRK2.6* (sucrose non-fermenting-1-related protein kinase 2) improved *S. miltiorrhiza* tolerance to abiotic stresses [30]. Overexpression of *SmLEA* (late embryogenesis abundant proteins) improves drought and salinity tolerance in *S. miltiorrhiza* [31]. Ectopic expression of tomato *prosystemin* (*LePS*), Arabidopsis *DREB1A/CBF3* and *AtDREB1A* was also used to improve *S. miltiorrhiza* resistance against biotic and abiotic stress separately[32–34].

ERF proteins play vital roles in a variety of stress responses in plants. Some ERF genes, such as *NtERF5*, *TaERF3*[35], *GmERF3*[36], *GmERF113*[37] and tomato *JERF1*[12], were thought to be candidates to improve crop-plant resistance because their overexpression enhances resistance to multiple diseases and improves tolerance to drought, salt, and freezing in transgenic plants [1]. To date, 79 ERFs have been identified from the *S. miltiorrhiza* genome [19] and the functions of four SmERFs in regulation of active ingredient biosynthesis have been investigated[20, 21, 23]. Few reports have described the resistance functions of *S. miltiorrhiza* ERFs in plants. In our study, the growth of transgenic tobacco lines with
Some ERFs were shown to regulate hormone levels in plants. For instance, apple MdERF2 can negatively affect ethylene biosynthesis by suppressing MdACS1 transcription [15]. TSRF1 activates the expression of a putative rice abscisic acid (ABA) synthesis gene, SDR, resulting in enhanced ABA levels [12]. Peach PpERF3 regulates ABA biosynthesis by activating PpNCED2/3 transcription [13]. ORA47 of Arabidopsis thaliana can activate ABA biosynthesis genes (NCED3 and NCED9) [14]. Overexpression of AtERF11 resulted in elevated bioactive GA levels by upregulating the expression of GA3ox1 and GA20ox genes [16]. ABA, GA and ET, are well known to play major roles in mediating plant defense responses against biotic and abiotic stresses [17]. Our qPCR results show that the expression levels of NtSDR, a key gene of ABA biosynthesis, was upregulated in SmERF1-expressing tobacco, while the expression of NtGA20ox, a key gene of GA biosynthesis was downregulated. In the SmERF1-expressing tobacco seedlings, the expression of NtACS and NtACO, two key enzyme-encoding genes in the ethylene biosynthesis, were upregulated compared with wild-type plants. It was also implied that the expression of SmERF1 many also cause an increase of ethylene. Our further study also indicated that SmERF1-expressing tobacco seedlings had higher ABA content and lower GA content than wild tobacco. There was antagonism between ABA and GA [38], and ABA could also induce ET biosynthesis[39]. Therefore, we inferred that SmERF1 can regulate the plant hormones of transgenic tobacco seedlings in response to drought through the ABA pathway. ABA is believed to be a general inhibitor of plant growth during the early development of seedlings [40]. It is indicated that the higher ABA content resulted in slower growth and decreased biomass in SmERF1-expressing tobacco seedlings.

Seed size is the most important agronomic traits in crop domestication[41], and it is desirable to increase seed yield because grains represent significant sources of food. Many transcription factors have been identified as regulators on controlling seed formation. AtMYB56, encoding an R2R3 MYB transcription factor, positively regulates Arabidopsis seed size by coordinately controlling the expansion of endothelium layer and proliferation[42]. ARF2 (AUXIN RESPONSE FACTOR 2) and NGAL2 can negative regulate seed size by suppressing cell proliferation[43, 44]. The AP2-type transcription factors Aechmea fasciata AfAP2-2, rice SMALL ORGAN SIZE1 (SMOS1) and Larix kaempferi LkAP2L2 also affect seed size[45–47]. Furthermore, SMOS1 may integrate auxin and BR signaling to control rice grain size[46]. In the current research, SmERF1 only contains one AP2 DNA binding domain belonging to the ERF subfamily, differing from members of the AP2 subfamily containing two AP2 DNA binding domains. However, expressing SmERF1 in tobacco also causes seeds to be smaller and lighter than wild-type tobacco, similar to AfAP2-2 [45].

Plant hormones also respond during seed development. ABA play a negative role in seed morphogenesis. ABA suppresses seed development by negatively regulating ABI5 (ABA response factor), while ABA-deficient mutants (abi5) can produce larger and heavier seeds for the increase of embryo cell number and
endosperm proliferation[48]. GAs also play important roles in the developing embryo and endosperm in seeds [49]. Loss-of-function mutation in OsGA20ox2 (a key GA biosynthesis gene) resulted in lower GA levels, and delayed seed morphogenesis and maturation in rice[50]. Overexpression of GASA4 (a member of gibberellic acid-stimulated Arabidopsis family), which is expressed in response to GAs, result in increase of seed size and total seed weight, while the gasa4 mutant has smaller seeds[51]. Our study also indicated that SmERF1-expressing seedlings had higher ABA content and lower GA content than WT plants. We inferred that SmERF1 may regulate the plant hormones of transgenic tobacco seedlings, resulting in smaller seed sizes. Crosstalk exists between ABA and GA, and these two phytohormones antagonistically mediate plant developmental processes [52]. Decreasing the gibberellin/abscisic acid (GA/ABA) ratio can weak seed repression of soybean seed germination [53]. The delayed germination of seeds, slower growth and loss of mass of SmERF1-expressing tobacco seedlings all verified the change in GA/ABA ratio. Additionally, ET participates in integration with ABA and Gas [49]. Overexpression of ACC deaminase resulted in reduced levels of ET, GAs and IAAs, and smaller seeds in Brassica napus [54]. In the SmERF1-expressing seedlings, the increase in ET may also contribute to sharp seed shape.

**Conclusion**

*S. miltiorrhiza* is a famous medicinal plant in Chian. We isolated one ERF transcription factor, SmERF1, from *S. miltiorrhiza*, and expressed SmERF1 in tobacco. The SmERF1-expression in tobacco affect plant growth at seedling stage, and increase plant tolerance to salt. And the expression of SmERF1 cause tobacco seeds smaller and lighter. The ABA level were increased and GA levels were decreased in SmERF1-expression tobacco plant. Our study suggested that SmERF1 enhanced tobacco tolerance to salt and impacted seed size through an ABA-dependent pathway.

**Methods**

**Clone and analysis of SmERF1**

According to the unigene (unigene-25151) sequence from our transcriptome data[28], we isolated the cDNA sequences of SmERF1 from *S. miltiorrhiza* with accession number: KC405081.1. Multiple sequence alignments were performed using ClustalW2 (https://www.ebi.ac.uk/Tools/msa/clustalw2/), and the results were rendered using the Boxshade Server. The conserved domain of SmERF1 was searched using the Conserved Domain Search Service (https://www.ncbi.nlm.nih.gov/guide/domains-structures/). The subcellular location of SmERF1 protein was predicted with WoLF PSORT (https://wolfpsort.hgc.jp/). The phylogenetic tree was constructed with MEGA X software using a Maximum Likelihood (ML) method based on an JTT model, and 500 bootstrap test replicates were used during the construction with other parameters as default[55].

**Plant Materials**
Mature seeds of *S. miltiorrhiza* Bunge were surface sterilized as described previously (Yan and Wang, 2007) and cultured on MS basal media (Murashige and Skoog, 1962) for germination. One-month-old seedlings were used for RNA isolation.

Tobacco (*Nicotiana tabacum*) plants for agroinfiltration were grown in a growth chamber at 22 °C under 16 h of light for 6 weeks. One-month-old tobacco plants were used for transient transformation. Seeds (T2 generation) collected from *SmERF1*-expressing tobacco plants were surface-sterilized with 25% bleach and planted on Murashige–Skoog (MS) medium for germination, then transplanted into soil and maintained at 25 °C with a 16 h light/8 h dark cycle. Seedlings after transplant were used for treatments and various tests.

**Extraction Of RNA, CDNA Synthesis**

Genomic DNA was isolated from young leaves of 2-month-old *S. miltiorrhiza* seedlings or tobacco seedlings with the Biospin Plant Genomic DNA Extraction Kit (BioFlux, China). Total RNA was extracted from transgenic tobacco and wild type plants, using BIOZOL reagent (BIOER, Hangzhou) according to the manufacturer’s instructions. The quality and concentrations of genomic DNA and RNAs were determined by 1.0% agarose gel electrophoresis and analysis on a spectrophotometer (SHIMADZU UV-2450, Japan). cDNA was synthesized with Revert Aid First Strand CDNA Synthesis Kit (Takara, Dalian).

**Plant Transformation And Transgenic Plant Verification**

We cloned the *SmERF1* sequence with the attB recombination sites and inserted into pDONOR221 via the Gateway BP clonase (Invitrogen Corporation) reaction to create entry clones. Then, the sequence was moved into the destination vector pEarleyGate100 using Gateway LR clonase (Invitrogen Corporation) to create the expression vector (pO-ERF). The new constructs of the pO-ERF vector were transformed into *Agrobacterium tumefaciens* strain *GV3101* via a freeze-thaw method[56]. Then, the transient transformation of tobacco leaves was accomplished according to Sparkes et al. [57]. The expression vector pO-ERF contained a Basta resistance selection marker; therefore, transformants were selected on 1/2 strength MS medium supplemented with IBA 0.5 mg/L and Basta 10 mg·mL⁻¹.

To determine whether the recombinant vector was inserted into the plant, we detected the *CaMV35S* promoter sequence in all transgenic tobacco lines (T0, T1 generation) by qPCR, primers were shown in Table 1.
### Table 1
Primers used in this study

| Genes (GenBank Accession) | Sequence |
|---------------------------|----------|
| **SmERF1 (for clone and vector construction)** | 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTC GCCTAGCTCACATAATGACGTTCGA-3′ (attB sites was underlined)  
5′-GGGGACCACTTTTGTAACAAGAAAGCTGGGTC CGACGTTAATTACTGTAAGCCGACTTC-3′ (attB site was underlined) |
| **SmERF1 (for qPCR)** | 5′-GCCTAGCTCACATAATGACGTTCGA-3′  
5′-CGACGTTAATTACTGTAAGCCGACTTC-3′ |
| **CAMV-35** | 5′-AATCTTTCGTCAACATGGTGACGT-3′  
5′-GCTGTCGTCGCGGAGAATA-3′ |
| **NtActin (U60495.1)** | 5′-CGTTATGGTTGGAATGGGACAGAA-3′  
5′-AAGAACAGGGTGCTCCTCGTG-3′ |
| **NtsDR (AJ223177.1)** | 5′-GAAGAGGAGGTCGCAAGGGCA-3′  
5′-CACTGATGGGTATTTTCGGATGAGA-3′ |
| **NtGA2Oox (JQ413251)** | 5′-GAAACCAGACCTCAGTTCAGGGAC-3′  
5′-TGAAAGAGCCATAAATGTATCGCCT-3′ |
| **NtACS (NM_001326220.1)** | 5′-TCGGGCTCGTTTCAACACAGA-3′  
5′-GAACATCCGTGTCTTTTCCCTA-3′ |
| **NtACO (NM_001325967)** | 5′-TCCAAGATGACAAAGTAAGCGGC-3′  
5′-TGTTTGCTCTCCGCTGCCTC-3′ |

Transgenic and wild-type tobacco lines with good growth for approximately 6 weeks in tissue culture were transplanted to the artificial culture room after seedling refining. The culture temperature was 24 °C, the humidity was 60%, and the light/darkness was 14/10 h.

**Expression analyses of SmERF1 in transgenic tobacco**

After selective culture, the transgenic plants were transferred to MS culture. Approximately 6 weeks after subculture, young seedlings (T0 generation) were transplanted into humus soil. Seeds (T1 and T2 generation) were collected when they were mature. The sizes of T2 seeds were observed with a stereomicroscope. The thousand seed weights of tobacco transgenic lines were also evaluated.

**Detection of ABA and GA levels in transgenic plants of tobacco (T2)**
Whole plant samples were collected from each transgenic plant. The contents of ABA and GA were detected according to the manuals of ELISA kits from ShaanXi Maiyuan Biotechnology Co., Ltd.

Detection of chlorophyll content of transgenic plants of tobacco (T2)

Two-week seedlings after germination of T2 generation seeds were used to calculate biomass and detect the chlorophyll content. Chlorophyll (Chl) content was measured according to the method described by Frank et al. with slight modification[58]. Briefly, 200 mg of fresh seedlings was collected and ground with sterile mortar and pestle. The samples were transferred into 10 mL tubes, and then 10.0 mL of 80% acetone was added. After mixing well, the tubes were stratified at 4 °C for 24 h. The samples were centrifuged at 12000 g at 4 °C for 3 min, and then the OD value of the supernatant was assayed with a spectrophotometer (SHIMADZU UV-2450, Japan) at 665 nm and 649 nm wavelengths. Chlorophyll content (a & b) was calculated using the following formulas where A is the absorbance[59]: Chl (a) = 13.95 × A_{665} − 6.88 × A_{645}; Chl (b) = 24.96 × A_{649} − 7.32 × A_{665}.

Resistance Analyses Of Transgenic Tobacco

The seeds of the T1 generation of transgenic and wild-type tobacco were germinated on filter paper. When the seedlings grew to approximately 1 cm in size, they were transplanted into the sowing and seedling substrate, one in each pot, cultivated in the artificial culture room, and watered once every 3 days. Two months after transplantation into the soil, the tobacco seedlings were watered with 400 mmol/L NaCl solution every other day. After 7 days of treatment, the growth states of the tobacco lines were observed, and the aerial parts of these seedlings were quickly frozen with liquid nitrogen for the detection of physiological indexes as follows.

The activities of catalase (CAT, EC 1.11.1.6), peroxidase (POD, EC 1.11.1.7) and superoxide dismutase (SOD, EC 1.15.1.1) were assayed using the CAT Assay Kit (A007–1, Nanjing Jiancheng, Nanjing, China), Plant POD Assay Kit (A084–3, Nanjing Jiancheng, Nanjing, China) and Total SOD Assay Kit (S0102, Nanjing Jiancheng, Nanjing, China), respectively, as described by Shi et al.[60, 61]. The MDA content was extracted using thiobarbituric acid reagent, and quantified by determining the absorbance of the supernatant at 450 nm, 532 nm and 600 nm as previously described[61].

Quantitative Real-time Pcr (qrt-pcr)

The quantitative reactions were performed on an IQ5 real-time PCR detection system (Bio-Rad), using SYBR® Premix Ex Taq™ (Perfect Real Time) (TaKaRa). PCR amplifications included the following conditions: 50 °C for 2 min and 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Gene-specific primers for qPCR are listed in Table 1. *Nicotiana tabacum* actin (Accession No. U60495.1) was used as an internal control. Their relative expression was calculated via the $2^{-\Delta\Delta Ct}$ method [62]. Each
sample was collected from five independent plants. Three independent biological replicates were used for each plant sample. Each data point represents the average of three experiments.

**Data analysis**

Data were analyzed using one-way analysis of variance (ANOVA) followed by the Turkey-Kramer test ($P < 0.05$) using SPSS software (version 19.0). Levels of statistical significance were marked with $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

**Abbreviations**

ABA: abscisic acid; ERF: ethylene response factor; GA: gibberellic acid; MDA: malondialdehyde; MeJA: methyl jasmonate; POD: peroxidase; SOD: superoxide dismutase; YE: yeast extraction

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and material**

All data generated or analyzed during this study are included in this published article and its supplementary information files.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' contributions**

H WP, K WW, and W ZZ conceived and designed the experiments, H WP and C C wrote the manuscript. H WP, K WW carried out the experiments. C C and W ZZ helped to design the experiments and analyzed
data.

All authors read and approved the final manuscript.

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