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Research Article

Function analysis of anthocyanidin synthase from *Morus alba* L. by expression in bacteria and tobacco

Jun Li [a,b,*], Aichun Zhao [c], Maode Yu [c], Yaofeng Li [a], Xiaoqing Liu [a], Xiangyun Chen [a]

[a] Guiyang University of Chinese Medicine, 550025 Guiyang, PR China
[b] National Engineering Research Center of Miao's Medicines, 550025 Guiyang, PR China
[c] State Key Laboratory of Silkworm Genome Biology, College of Biotechnology, Southwest University, 400715 Chongqing, PR China

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ABSTRACT

Background: Flavonoids are a kind of important secondary metabolite and are commonly considered to provide protection to plants against stress and UV-B for a long time. Anthocyanidin synthase (ANS), which encodes a dioxygenase in the flavonoid pathway, catalyzes the conversion of leucoanthocyanidins to anthocyanidins, but there is no direct evidence indicating that it provides tolerance to stress in plants.
Results: To investigate whether ANS can increase tolerance to abiotic stress, *MaANS* was isolated from mulberry fruits and transformed into tobacco. Our results suggested that the bacterially expressed MaANS protein can convert dihydroquercetin to quercetin. Overexpression of *MaANS* remarkably increased the accumulation of total flavonoids in transgenic lines and anthocyanins in corollas of flowers. Transgenic lines showed higher tolerance to NaCl and mannitol stress.
Conclusions: These results indicated that MaANS participates in various dioxygenase activities, and it can protect plants against abiotic stress by improving the ROS-scavenging ability. Thus, this alternative approach in crop breeding can be considered in the improvement of stress tolerance by enriching flavonoid production in plants.
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1. Introduction

Anthocyanins, flavonols, flavones, and proanthocyanidins belong to the flavonoid class of secondary metabolites and are found in different tissues of plants. These pigments are involved in several important functions such as providing protection to plants against pathogen (bacteria and fungi) attack, UV-B protection, photo-perception, and hormone responses [1,2,3]. It is reported that flavonoids have been used for applications owing to their anticancer, antimutagenic, antimicrobial, anti-inflammatory, and antiatherosclerotic properties [4,5,6,7,8].

The flavonoid biosynthetic pathway shares a common feature with the anthocyanin biosynthetic pathway with phenylalanine as the first substrate, and it may be one of the most important pathways for secondary metabolism in plants [9,10]. Molecular engineering of genes in the flavonoid biosynthetic pathway was considered as an approach in agricultural and biomedicine industries for obtaining plants enriched with a high level of flavonoids and antioxidant activity. Genes of some key enzymes involved in the flavonoid pathway isolated from model plants were used to develop transgenic technologies [11]. Overexpression of *chalcone isomerase* (CHI) in transgenic tomato increased the flavonol level by approximately 78-fold [12]. A significant increase in flavonoid and anthocyanin levels can also be induced by monogenic overexpression or multigene co-expression of CHI, *chalcone synthase* (CHS), and *dihydroflavonol reductase* (DFR) [13].

Anthocyanidin synthase (ANS) is one of the key enzymes that occur late in the flavonoid pathway, and it can catalyze the conversion of leucoanthocyanidins to anthocyanidins [14]. Numbers of ANS genes have been cloned from a variety of plants, but no further function of this enzyme was determined until its activity was expanded with trans-dihydroquercetin (trans-DHQ) as substrate [15]. ANS belongs to dioxygenase gene family in the flavonoid pathway and catalyzes a number of two-electron oxidations such as hydroxylations, desaturations, and oxidative ring closures [16]. A previous study also indicated that the *ANS* gene can improve the content of flavonoids, thus leading to an increase in the antioxidant potential [17]. Therefore, if *ANS* in plants can increase dioxygenase activities and a mixture of flavonoids, then its overexpression may provide higher tolerance to salt and osmotic stress in transgenic plants. For this

* Corresponding author.
E-mail address: speker@163.com (J. Li).
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Table 1
Primers for inverse PCR.

Gene	Sequence (5′–3′)
Left-F1	GTGTTGCCCGTCTCACTGGTGA
Left-R1	CAGCTGATTGCCCTTCACCGCCT
Left-F2	CGTCCGCAATGTGTTATTAAGTT
Left-R2	CTGAGAGAGTTGCAGCAAGCGGT

purpose, the MaANS gene was cloned [18,19] and transformed into tobacco for producing plants enriched with flavonoids by single-gene overexpression of a dioxygenase from mulberry.

In view of all these facts, we isolated the *MaANS* gene and investigated the functionality through the *in vitro* enzyme activity of the protein expressed in *Escherichia coli* and its ectopic expression in tobacco (*Nicotiana tabacum*). The flavonoid content and potential antioxidant activities of transgenic tobacco plants were analyzed. The plants were further evaluated for protective abilities against stress in transgenic lines of *MaANS* overexpression. This study may help to explain the mechanism responsible for hydroxylation in the flavonoid pathway of plants.

2. Material and method

2.1. Quantification of anthocyanins in mulberry fruits

Morus atropurpurea cv. *Jialing* No. 40 is a new cultivated variety and grown in the *Morus* garden at Southwest University, Chongqing, China. The fruits (20 for each cultivar) were picked at 35 d after full bloom (DAFB). The fruits were frozen in liquid N_2 and stored at −80°C for RNA extraction and other analyses.

2.2. Isolation of total RNA and reverse transcription-PCR

Total RNA of mulberry fruits was extracted using the RNA Extraction Kit TransZol Plant (TransGen Biotech, China). Contaminating DNA was removed using DNase I (Takara, Japan). The first-strand cDNA was synthesized from total RNA by using M-MLV reverse transcriptase (Takara, Tokyo, Japan) with random primers by standard methods. cDNA was diluted by tenfold and used for real-time PCR.

2.3. Prokaryotic expression of MaANS in E. coli, purification, and enzyme assay

The coding sequence of *MaANS* (gi: 1074044840) was amplified from the cDNA of mulberry fruits by using the Taq DNA polymerase with the specific forward primer (5′-CGCGGATCCATGGTGATCTCTGTGGCTCC-3′) and reverse primer (5′-CGAGCTCAATAAACCTTTAAGACAACG-3′). The amplified fragment was purified and cloned into the pMD19-T simple vector. The fragment of *MaANS* was obtained from the TA vector by double digestion of *BamH*I/*Sac* and subcloned into the multiple cloning sites of pET28a (+) with an N-terminus His-tag. The expression vector of pET-*MaANS* was confirmed and transformed into the *E. coli* strain BL-21. The *E. coli* strains carrying the recombinant plasmid were grown in LB medium and induced by 1-mM isopropyl β-D-thiogalactoside (IPTG) at 37°C for 2 h when the OD600 value reached 0.6. Cells were harvested by centrifugation and disrupted by sonication; the supernatant was collected and analyzed by SDS-PAGE with a 12% gel. High-affinity Ni-NTA Resin (GenScript, NJ, USA) was used for protein purification.

The MaANS enzymatic activity was assayed by a method as described previously [14]. The enzyme activity analysis was performed in a 0.5-mL reaction volume containing 0.3 mg of the MaANS protein, 200-mM NaCl, 20-mM potassium phosphate buffer (pH = 7.0), 10-mM maltose, 4-mM sodium ascorbate, 5-mM DTT, 0.4-mM $FeSO_4$, and 1-mM 2-oxoglutaric acid. Total protein extracted from strains harboring empty pET28a (+) was represented as a control. The enzymatic reaction was evaporated to dryness and redissolved in methanol. The product was confirmed by HPLC using a Waters 1525 system (Waters, MA, USA). An Elite® C18 column (250 mm × 4.6 mm, 5 μm; Amersham Biosciences, Buckinghamshire, UK) and an auto-injector were used. The samples were separated and eluted by using methanol as the only mobile phase at a flow rate of 1 mL min^{-1}.

2.4. Expression vector construction, plant transformation, and confirmation

The isolated cDNA fragment of *MaANS* from *M. atropurpurea* cv. *Jialing* No. 40 was cloned into the pBI121 plasmid with double digestion of *Nco*I/*Bgl*II. The vector pBI121-*MaANS* was constructed and introduced into the *Agrobacterium tumefaciens* strain LBA4404 by triparental mating. *A. tumefaciens* strains carrying the pBI121-*MaANS* vector and the empty vector pBI121 were used for leaf disc transformation according to a

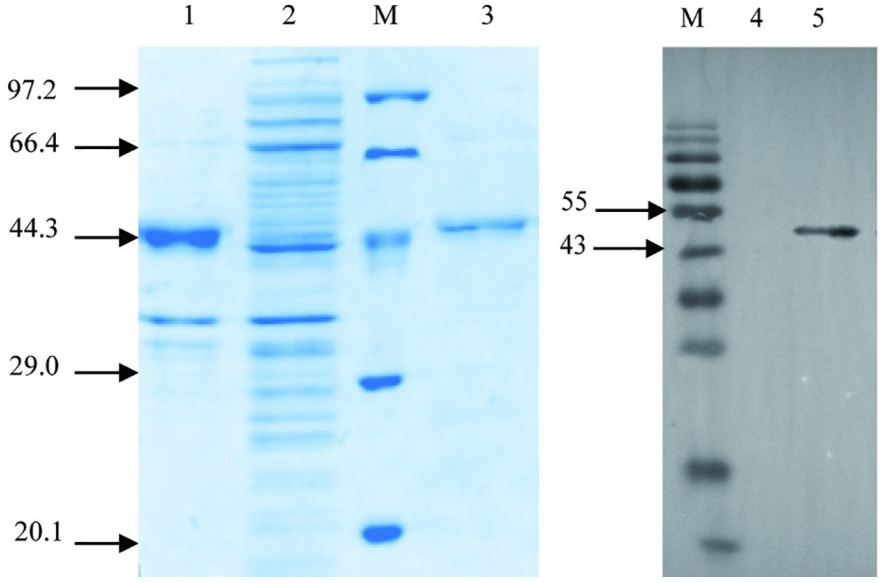

Fig. 1. Bacterial expression, purification, and western blot analysis of MaANS. M: Marker; 1: Inclusions of the induced bacteria; 2: Supernatant of the induced bacteria; 3: Eluted using 250 mmol L^{-1} of imidazole; 4: Total protein of bacterial cultures with the empty vector pET28; 5: Purified protein.

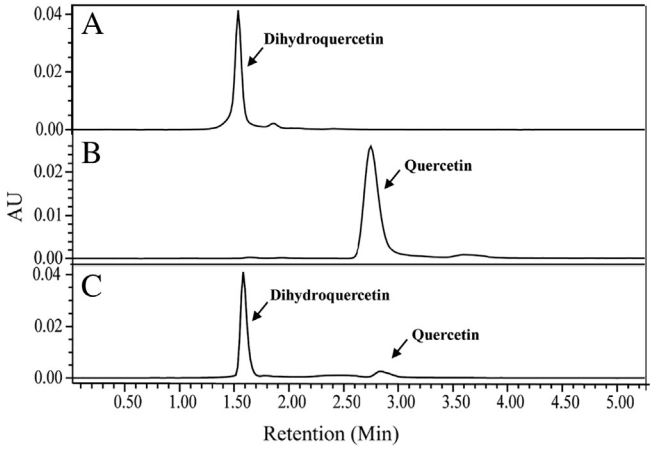

Fig. 2. HPLC analysis of ANS enzyme assay. (A) Trans-DHQ (1.5893 min); (B) Quercetin; (C) Enzyme reaction product.

standard protocol [20]; transgenic putative plants rooted on kanamycin selection (100 mg mL^{-1}) were identified for by PCR with specific primers (*MaANS* forward primer 5′-ATGGTGATCTCTGTGGCTCC-3′ and reverse primer 5′-AATAAACCTTTAAGACAACG-3′; *Npt*II forward primer 5′-GGTGCCCTGAATGAACTCCA-3′ and reverse primer 5′-GGTAGCCAACG CTATGTCCT-3′). The reactions were performed as follows: 94°C for 4 min and then 35 cycles of 94°C for 30 s, 58°C for 30 s (for *MaANS*), 50°C for 30 s (for *Npt*II), and 72°C for 2 min. Amplification products were visualized in a 1% TAE agarose gel and stained with ethidium bromide. The seeds obtained from T1 transformants by self-pollination were selected on half strength Murashige and Skoog medium with 100 mg mL^{-1} kanamycin. One month old plants were transplanted into pots, and they grew in a greenhouse environment for further research.

To determine the expression levels of *MaANS* in T2 transformants of tobacco, total RNA extraction and real-time PCR analysis of *MaANS* of transgenic lines were performed with specific forward primer (5′-CTATGAAGGCAAATGGGTGA-3′) and reverse primer (5′-CACCTTCTCCT TGTTGACGA-3′). The *ubi* gene (gi: NTU66264) was used as an internal reference; the forward and reverse primers for amplification were *ubi*-F (5′-TCCAGGACAAGGAGGGTATC-3′) and *ubi*-R (5′-CGGGTTGAC TCTTTCTGGAT-3′). The insertion site was determined by inverse PCR; primer details are shown in Table 1.

2.5. Estimation of flavonoid content in transgenic tobacco

Total flavonoid content of transgenic lines was analyzed by a colorimetric method [7]. Leaves of transgenic tobacco (0.5 g) were mixed with 30 mL of 75% (v/v) ethanol and incubated at approximately 65°C for 2 h. A sample of this solution (5 mL) was mixed with 10 mL of 95% ethanol, 1 mL of 10% (weight/volume) $AlCl_3 \cdot 6\ H_2O$, 1 mL of 1-M potassium acetate, and 33 mL of deionized water. After the reaction mixture was incubated at room temperature for 30 min, its absorbance was measured at 420 nm on a spectrophotometer (model 100-20; Hitachi, Tokyo, Japan), and rutin was used as the standard. The total flavonoid content in the fruit was determined by using at least three replicates and expressed as milligrams of rutin equivalents per wet mass unit.

2.6. Content of H_2O_2 and estimation of DPPH radical activity in transgenic tobacco

Content of H_2O_2 was assessed by using commercial kits (Nanjing Jiancheng Bio-Engineering Institute, China). Spectrophotometry-based and ESR-based methods were used to evaluate the free radical scavenging activity of methanol extracts from transgenic lines, with 2,2-diphenyl-1-picrylhydrazyl (DPPH) as the substrate [21]. Five hundred microliters of methanol extracts from transgenic plants and an equal volume of 0.1-mM DPPH in methanol were mixed and incubated in the dark for 30 min. The absorbance of the mixture was detected by spectrophotometry at 517 nm.

2.7. Stress Tolerance Analyses of Transgenic Tobacco

Forty-day-old plants of transgenic lines and CK plants (carrying the pBI121 empty vector) were treated with 0, 200, and 400 mM of NaCl as well as 0, 200, and 400 mM of mannitol daily for 2 weeks and recovered with distilled water for 1 week.

3. Results

3.1. MaANS enzyme activity analysis

To determine enzyme activity of the MaANS protein, the CDS of *MaANS* was cloned into the pET28a vector and expressed in the *E. coli* strain BL-21 (DE3) induced using IPTG. The 43-kDa MaANS protein with His-Tag was observed to have low abundance in the supernatant

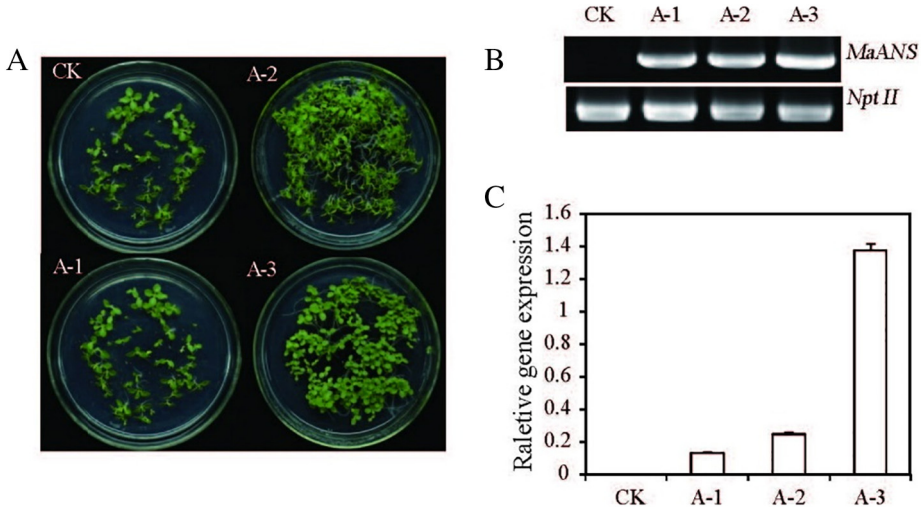

Fig. 3. Generation of *MaANS* transgenic tobacco. (A) Resistance selection of transgenic lines; (B) Genomic DNA PCR; (C) The transcript expression levels of MaANS in transgenic tobacco lines (CK, A-1, A-2, A-3). The *ubi* gene was used as the internal control, and experiments were repeated at least three times.

Table 2
The insertion sites of *MaANS* in transgenic tobaccos.

Transgenic plants	Scanford	Start	End	Strand
ANS-1	Niben.v0.4.2.Scf418 14.4	20632	20722	−
ANS-2	Niben.v0.4.2.Scf24152 15.3	16207	16604	+
ANS-3	Niben.v0.4.2.Scf14661 13.9	53786	53958	−

and high abundance in inclusion samples (Fig. 1). The expressed MaANS protein was purified from the supernatant using Ni-NTA resin. Anti-His-tag antibodies were used to determine the fusion protein, and a strong immunoblot signal was detected in the purified protein fraction.

The activity of MaANS *in vitro* was analyzed with dihydroquercetin (DHQ) as the substrate, and products were determined by HPLC. Quercetin can be detected in reaction with the MaANS protein at a retention time of 2.8472 min (Fig. 2C). This result indicated that the MaANS protein is a 2-oxoglutarate-dependent enzyme, and it can convert DHQ to quercetin.

3.2. Transformation and confirmation of transgenic tobacco overexpressing MaANS

To explain the function of *MaANS in vivo*, *MaANS* was transformed into tobacco by *Agrobacterium*-mediated technology. The seeds of T1-generation transgenic lines were selected on MS plates using kanamycin (Fig. 3A). The integration of *MaANS* ORF in the genome of transgenic lines was confirmed by PCR, and three putative lines (A-1, A-2, and A-3) were selected (Fig. 3B). The insertion sites were analyzed by inverse PCR, and the primer details are shown in Fig. S1. The insertion sites of *MaANS* are shown in Table 2. *MaANS* is expressed at different levels (Fig. 3C) in the three putative lines.

3.3. Overexpression of MaANS increased flavonoids, anthocyanidin accumulation, and antioxidant potential in transgenic tobacco

The content of total flavonoids in transgenic lines of pBI121 empty vector (CK) was 143.3-mg quercetin equivalent (QE) 100 g^{-1} FW, whereas the total flavonoid content in *MaANS*-overexpressing transgenic lines ranged from 165.9 to 181.4 mg QE 100 g^{-1} FW (Fig. 4A). Methanolic extract from leaves of *MaANS* transgenic lines showed higher DPPH scavenging activity than those from CK plants (Fig. 4B). The color of corollas of *MaANS* transgenic lines had deepened, and anthocyanin content of these lines was significantly increased compared to that of CK lines (Fig. 4C). The H_2O_2 content was significantly reduced in *MaANS* transgenic lines (Fig. 4D).

3.4. Overexpression of MaANS enhanced tolerance to salt and osmotic stress

CK lines and three *MaANS* transgenic lines were used to evaluate tolerance to salt and drought stress under soil conditions (Fig. 5). The *MaANS* transgenic lines showed higher tolerance to 200 mM NaCl and 400 mM mannitol treatment. These results suggested that overexpression of *MaANS* in plants can maintain turgidity under osmotic stress and decrease the toxicity of salt.

4. Discussion

Four kinds of dioxygenases, namely, F3H, FLS, ANS, and FS I, have been reported in plants, and they use 2-oxoglutarate and oxygen as co-substrates, Vc as the reducing agent, and Fe^{2+} as the cofactor [16,22,23]. ANS is one of the important dioxygenases in plants, and it can catalyze leucoanthocyanidins to anthocyanidins and convert dihydroquercetin to quercetin [24]. In our study, we provided evidence *in vivo* and *in vitro* that the *MaANS* can also catalyze conversion of flavonoid metabolites.

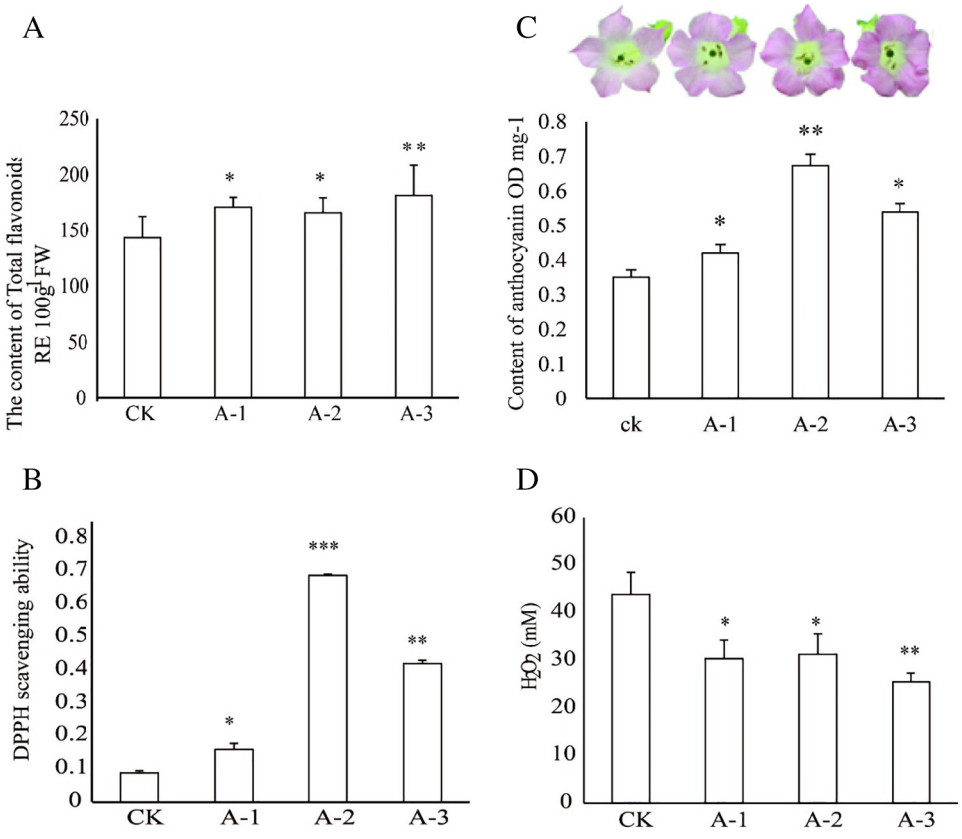

Fig. 4. *MaANS*-overexpressing transgenic lines show higher contents of total flavonoids, free radical-scavenging activity, and anthocyanin accumulation in transgenic tobacco flowers. (A) Total flavonoids in transgenic tobaccos; (B) The scavenging activity of methanol extracts from transgenic lines; (C) Anthocyanins in flowers of transgenic tobacco; (D) H_2O_2 contents in the leaves of transgenic plants. Data are represented as the mean of three replications, with error bars indicating ±SD. Statistical significance is indicated as (*) for $P < 0.05$.

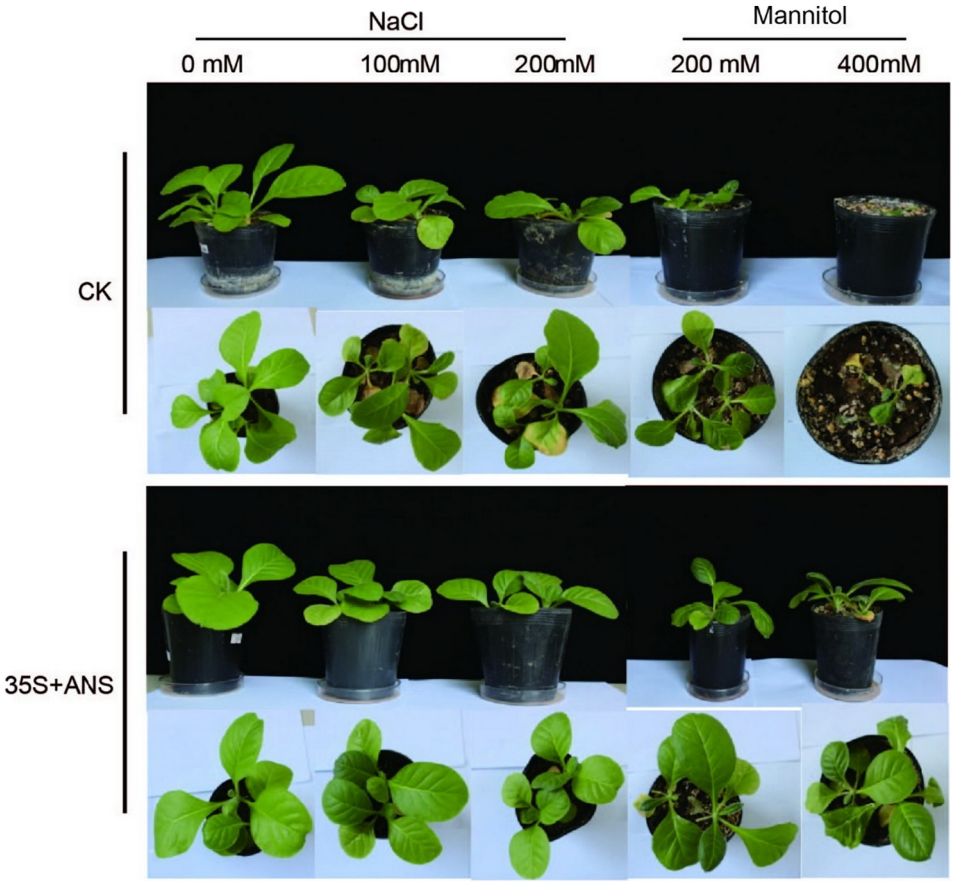

Fig. 5. Effects of NaCl and mannitol on tobacco plants. Forty day old transgenic lines were treated with 0, 200, and 400 mM of NaCl as well as 0, 200, and 400 mM of mannitol every day for 2 weeks.

Overexpression of *MaANS* in tobacco can deepen the color of corollas by increasing anthocyanin accumulation (Fig. 4A and B). Similar results were reported in *Medicago truncatula* and cranberry [25,26,27].

Major environmental stresses (drought and salinity) have a strong impact on the development of plants. Many putative responses involving salt and drought stress have been isolated and identified [28,29], such as morphological, biochemical, and physiological features. Reactive oxygen species (ROS) also increased, which was induced by oxidative stress. Excessive ROS involved in environmental factor might induce programmed cell death [30,31]. Several published works demonstrate that flavonoids have a high antioxidant activity against deleterious free radicals [32,33,34,35,36]. In our work, we confirmed that overexpression of *MaANS* can increase total flavonoids (Fig. 4A) and antioxidant activity in transgenic lines (Fig. 4B). These results suggested that overexpression of *MaANS* can induce the redirection of metabolism and offer a feasible approach for metabolic engineering in plants.

In conclusion, this study determined the biological function *in vivo* and *in vitro* of the *MaANS* gene in flavonoid biosynthesis. MaANS may participate in various dioxygenase activities, and it can protect plants against stress by improving the ROS-scavenging capacity. This gene can be used to produce transgenic plants with higher tolerance to drought and salt stress.

Supplementary material

https://doi.org/10.1016/j.ejbt.2018.09.001

Conflicts of interest

The authors declare no conflict of interest.

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References

[1] Madhuri G, Reddy AR. Plant biotechnology of flavonoids. Plant Biotechnol 1999;16(3):179–99. https://doi.org/10.5511/plantbiotechnology.16.179.
[2] Zhang Y, Vareed SK, Nair MG. Human tumor cell growth inhibition by nontoxic anthocyanidins, the pigments in fruits and vegetables. Life Sci 2005;76(13):1465–72. https://doi.org/10.1016/j.lfs.2004.08.025. [PMID: 15680311].
[3] Li YS, Liu XB, Henson JF. Advances in crop responses to enhanced UV-B radiation. Appl Ecol Environ Res 2016;14(3):339–67. http://dx.doi.org/10.15666/aeer/1403_339367.
[4] Padmavati M, Reddy AR. Flavonoid biosynthetic pathway and cereal defence response: an emerging trend in crop biotechnology. J Plant Biochem Biotechnol 1999;8(1):15–20. https://doi.org/10.1007/BF03263051.
[5] Nijveldt RJ, van Nood E, van Hoorn DE, et al. Flavonoids: a review of probable mechanisms of action and potential applications. Am J Clin Nutr 2001;74(4):418–25. https://doi.org/10.1093/ajcn/74.4.418.. [PMID: 11566638].
[6] Kong JM, Chia LS, Goh NK, et al. Analysis and biological activities of anthocyanins. Phytochemistry 2003;64(5):923–33. https://doi.org/10.1016/S0031-9422(03)00438-2.
[7] Proestos C, Boziaris IS, Nychas GJE, et al. Analysis of flavonoids and phenolic acids in Greek aromatic plants: Investigation of their antioxidant capacity and antimicrobial activity. Food Chem 2006;95(4):664–71. https://doi.org/10.1016/j.foodchem.2005.01.049.
[8] Schijlen EG, de Vos CHR, van Tunen AJ, et al. Modification of flavonoid biosynthesis in crop plants. Phytochemistry 2004;65(19):2631. https://doi.org/10.1016/j.phytochem.2004.07.028.
[9] Mol J, Grotewold E, Koes R. How genes paint flowers and seeds. Trends Plant Sci 1998;3(6):212–7. https://doi.org/10.1016/S1360-1385(98)01242-4.

[10] Xie DY, Sharma SB, Paiva NL, et al. Role of anthocyanidin reductase, encoded by *BANYULUS* in plant flavonoid biosynthesis. Science 2003;299(5605):396–9. https://doi.org/10.1126/science.1078540. [PMID: 12532018].
[11] Martens S, Knott J, Seitz CA, et al. Impact of biochemical pre-studies on specific metabolic engineering strategies of flavonoid biosynthesis in plant tissues. Biochem Eng J 2003;14(3):227–35. https://doi.org/10.1016/S1369-703X(02)00224-3.
[12] O'Neill EC, Kelly S. Engineering biosynthesis of high-value compounds in photosynthetic organisms. Crit Rev Biotechnol 2017;37(6):779–802. https://doi.org/10.1080/07388551.2016.1237467.
[13] Lukaszewicz M, Matysiak-Kata I, Skala J, et al. Antioxidant capacity manipulation in transgenic potato tuber by changes in phenolic compounds content. J Agric Food Chem 2004;52(6):1526–33. https://doi.org/10.1021/jf034482k. [PMID: 15030206].
[14] Saito K, Kobayashi M, Gong Z, et al. Direct evidence for anthocyanidin synthase as a 2-oxoglutaratedependant oxygenase: molecular cloning and functional expression of cDNA from a red forma of *Perilla frutescens*. Plant J 1999;17:181–9. https://doi.org/10.1046/j.1365-313X.1999.00365.x. [PMID: 10074715].
[15] Nakajima J, Tanaka Y, Yamazaki M, et al. Reaction mechanism from leucoanthocyanidin to anthocyanidin 3-glucoside, a key reaction for coloring in anthocyanin biosynthesis. J Biol Chem 2001;276:25797–803. https://doi.org/10.1074/jbc.M100744200. [PMID: 11316805].
[16] Schofield CJ, Zhang ZH. Structural and mechanistic studies on 2-oxoglutarate dependent oxygenases and related enzymes. Curr Opin Struct Biol 1999;9(6):722–31. https://doi.org/10.1016/S0959-440X(99)00036-6. [PMID: 10607676].
[17] Reddy AM, Reddy VS, Scheffler BE, et al. Novel transgenic rice overexpressing anthocyanidin synthase accumulates a mixture of flavonoids leading to an increased antioxidant potential. Metab Eng 2007;9(1):95–111. https://doi.org/10.1016/j.ymben.2006.09.003. [PMID: 17157544].
[18] Li J, Lü RH, Zhao AC, et al. Isolation and expression analysis of anthocyanin biosynthetic genes in *Morus alba* L. Biol Plant 2014;58(4):618–26. https://doi.org/10.1007/s10535-014-0450-5.
[19] Qi XW, Shuai Q, Chen H, et al. Cloning and expression analyses of the anthocyanin biosynthetic genes in mulberry plants. Mol Genet Genomics 2014;289(5):783–93. https://doi.org/10.1007/s00438-014-0851-3. [PMID: 24748075].
[20] Zhang Z, Wang Y, Chang L, et al. *MsZEP*, a novel zeaxanthin epoxidase gene from alfalfa (*Medicago sativa*), confers drought and salt tolerance in transgenic tobacco. Plant Cell Rep 2016;35(2):439–53. https://doi.org/10.1007/s00299-015-1895-5. [PMID: 26573680].
[21] Jiang L, Li X, Wang D. Development of a rapid method for the evaluation of DPPH radical scavenging activity of ginger (*Zingiber officinale*) foods based on cyclic voltammetry. Food Anal Methods 2016;10(5):1419–29. https://doi.org/10.1007/s12161-016-0702-4.
[22] Prescott AG, John P. DIOXYGENASES: molecular structure and role in plant metabolism. Annu Rev Plant Mol Biol 1996;47:245–71. https://doi.org/10.1146/annurev.arplant.47.1.245. [PMID: 15012289].
[23] Prescott AG, Stamford NP, Wheeler G, et al. *In vitro* properties of a recombinant flavonol synthase from *Arabidopsis thaliana*. Phytochemistry 2002;60(6):589–93. https://doi.org/10.1016/S0031-9422(02)00155-3. [PMID: 12126705].
[24] Gebhardt Y, Witte S, Forkmann G, et al. Molecular evolution of flavonoid dioxygenases in the family Apiaceae. Phytochemistry 2005;66(11):1273–84. https://doi.org/10.1016/j.phytochem.2005.03.030. [PMID: 15913674].
[25] Aida R, Yoshida K, Kondo T, et al. Copigmentation gives bluer flowers on transgenic torenia plants with the antisense dihydroflavonol-4-reductase gene. Plant Sci 2000;160(1):49–56. https://doi.org/10.1016/S0168-9452(00)00364-2. [PMID: 11164576].
[26] Xie DY, Jackson LA, Cooper JD, et al. Molecular and biochemical analysis of two cDNA clones encoding dihydroflavonol-4-reductase from *Medicago truncatula*. Plant Physiol 2004;134(3):979–94. https://doi.org/10.1104/pp.103.030221. [PMID: 14976232].
[27] Li Y, Liu X, Cai X, et al. Dihydroflavonol 4-reductase genes from *Freesia hybrida* play important and partially overlapping roles in the biosynthesis of flavonoids. Front Plant Sci 2017;8:428. https://doi.org/10.3389/fpls.2017.00428. [PMID: 28400785].
[28] Rocha FR, Papini-Terzi FS, Nishiyama Jr MY, et al. Signal transduction-related responses to phytohormones and environmental challenges in sugarcane. BMC Genomics 2007;8:71. https://doi.org/10.1186/1471-2164-8-71. [PMID: 17355627].
[29] Rodrigues FA, Da Graca JP, Laia ML, et al. Sugarcane genes differentially expressed during water deficit. Biol Plant 2011;55(1):43–53. https://doi.org/10.1007/s10535-011-0006-x.
[30] Chaves MM, Flexas J, Pinheiro C. Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Ann Bot 2009;103(4):551–60. https://doi.org/10.1093/aob/mcn125.. [PMID: 18662937].
[31] Miller G, Suzuki N, Ciftci-Yilmaz S, et al. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant Cell Environ 2010;33:453–67. https://doi.org/10.1111/j.1365-3040.2009.02041.x.. [PMID: 19712065].
[32] Tsuda T, Watanabe M, Ohshima K, et al. Antioxidative activity of the anthocyanin pigments cyanidin3-O-beta-D-glucoside and cyanidin. J Agric Food Chem 1994;42 (11):2407–10. https://doi.org/10.1021/jf00047a009.
[33] Cao G, Sofic E, Prior RL. Antioxidant capacity of tea and common vegetables. J Agric Food Chem 1996;44(11):3426–31. https://doi.org/10.1021/jf9602535.
[34] Dixon RA, Xie DY, Sharma SB. Proanthocyanidins–a final frontier in flavonoid research? New Phytol 2005;165(1):9–28. https://doi.org/10.1111/j.1469-8137.2004.01217.x. [PMID: 15720617].
[35] Pietta PG. Flavonoids as antioxidants. J Nat Prod 2000;63(7):1035–42. https://doi.org/10.1021/np9904509.
[36] Ali HM, Almagribi W, Al-Rashidi MN. Antiradical and reductant activities of anthocyanidins and anthocyanins, structure-activity relationship and synthesis. Food Chem 2016;194:1275–82. https://doi.org/10.1016/j.foodchem.2015.09.003.