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Overexpression of R2R3-MYB gene leads to accumulation of anthocyanin and enhanced resistance to chilling and oxidative stress

X. MENG, B. YIN, H.-L. FENG, S. ZHANG, X.-Q. LIANG, and Q.-W. MENG*

College of Life Science, State Key Laboratory of Crop Biology, Shandong Agricultural University, Tai'an, Shandong 271018, P.R. China

Abstract

LeAN2 encoding an anthocyanin-associated R2R3-MYB transcription factor was isolated from tomato. The expression of *LeAN2* in tomato was induced by low temperature and oxidative stress. Green fluorescent protein was fused to *LeAN2* and the complex was expressed transiently in onion epidermal cells. Green fluorescence was observed only in the nucleus. Overexpression of *LeAN2* under the control of the CaMV35S promoter in tobacco induced expression of several anthocyanin biosynthetic genes and the content of anthocyanin was markedly higher in transgenic tobacco compared with wild type plants. Transgenic tobaccos conferred tolerance to chilling stress by maintaining a higher chlorophyll content, net photosynthetic rate, and maximal photochemical efficiency of PS II compared to the wild type plants. Furthermore, the transgenic plants showed lower ion leakage, a lower content of reactive oxygen species, and a higher content of non-enzymatic antioxidans under the chilling stress. They also showed an enhanced resistance to the oxidative stress induced by methyl viologen based on a decreased chlorophyll content loss, lower ion leakage, and an enhanced maximal photochemical efficiency of PS II. These results indicate that overexpression of *LeAN2* resulted in an increased anthocyanin accumulation and enhanced resistance to the chilling and oxidative stresses in transgenic tobacco.

Additional key words: *Allium cepa*, chlorophyll, fluorescence, *Lycopersicum esculentum,* net photosynthetic rate, *Nicotiana tabacum*, onion, tomato, transcription factor, transgenic tobacco

Introduction

Anthocyanins, which are colored products of the flavonoid biosynthetic pathway, have received particular attention in recent years. Their accumulation is induced by a range of developmental and environmental signals (Leyva *et al.* 1995, Oren and Levi 1997, Solfanelli *et al.* 2006, Allan *et al.* 2008, Shan *et al.* 2009). Anthocyanins function as an antioxidant and photoprotective screens and play important roles in many physiological processes, including photoprotection, stress resistance, pollination, and seed dispersal (Chalker-Scott 1990, Zhang *et al.* 2010, Alcalde-Eon *et al.* 2013). In addition, anthocyanins are potentially health-protecting components in the human diet (Bovy *et al.* 2010).

Under abiotic stresses, imbalance in metabolic processes may lead to an increased accumulation of reactive oxygen species (ROS; Foyer *et al.* 2011). The excessive accumulation of ROS causes a variety of serious injuries when stress is severe (Singh and Singhal 1999). Fortunately, plants have developed enzymatic and non-enzymatic antioxidant defense systems to detoxify ROS. The non-enzymatic antioxidants, such as carotenoids, tocopherols, ascorbic acid, glutathione, and flavonoids scavenge ROS to protect plant cells. Anthocyanins may serve as antioxidants due to the hydroxyl groups

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Abbreviations: *Aft* - *anthocyanin fruit* genotype; *ANS* - *anthocyanidin synthase* gene; *CHS* - *chalcone synthase* gene; DAB - diaminobenzidin; *DFR* - *dihydroflavonol 4-reductase* gene; DPPH - α,α-diphenyl-β-picrylhydrazyl; F_v/F_m - the variable to maximum chlorophyll fluorescence ratio; GFP - green fluorescence protein; H_2O_2 - hydrogen peroxide; MV - methyl viologen; NBT - nitroblue tetrazolium; $O_2^{\bullet-}$ - superoxide anion radical; PFD - photon flux density; P_N - net photosynthetic rate; REL - relative electrolyte leakage; ROS - reactive oxygen species; TFs - transcription factors; TTG1 - WDR-type proteins; WDR - WD40 repeats; WT - wild type.

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* Corresponding author; fax: (+86) 538 8226399, e-mail: qwmeng@sdau.edu.cn

present in the glycon (Yokozawa *et al.* 1998).

The structural genes, such as *chalcone synthase* (*CHS*), *anthocyanidin synthase* (*ANS*), and *dihydroflavonol 4-reductase* (*DFR*) encode enzymes that directly participate in anthocyanin formation (Quattrocchio *et al.* 1993, Winkel-Shirley 2001) and the MYB and bHLH transcription factors (TFs) and WDR-type proteins (TTG1) activate the structural genes (Gonzalez *et al.* 2008). MYB/bHLH/TTG1 transcription complexes regulate anthocyanin biosynthesis in a spatio-temporal manner. Recent research indicates that there are MYBs which are involved in anthocyanin biosynthesis but independently of TTG1, such as MYB11, MYB12, and MYB111, and PAP1 homologs, such as MYB113 and MYB114 (Mehrtens *et al.* 2005, Gonzalez *et al.* 2008). Contrary to the positive TFs, negative TFs also exist, such as the R3-MYB protein MYBL2 which is a negative regulator of flavonoid biosynthesis in *Arabidopsis thaliana* (Dubos *et al.* 2008). Hence, the anthocyanin accumulation in a specific cell type may be regulated by a balance between positive transcription complexes and negative transcription complexes (Dubos *et al.* 2008, Matsui *et al.* 2008).

LeANT1 and *LeAN2* share high homology and encode two MYB TFs involved in anthocyanin regulation in tomato (Mathews *et al.* 2003, Boches 2009). The *anthocyanin fruit* (*Aft*) genotype, originating from the wild tomato species *Lycopersicon chilense*, is characterized by anthocyanin accumulation in its mature fruits, resulting in tomato fruits with purple skin colour which is modulated by radiation (Mes *et al.* 2008). Jones *et al.* (2003) considered that the anthocyanin accumulation in fruit peels of the *Aft* genotype is regulated by a single dominant gene. Boches (2009) insisted that *AN2* is a more likely candidate for *Aft* than *ANT1*. But later, Schreiber *et al.* (2011) provided further support to the hypothesis that *ANT1* is the gene responsible for anthocyanin accumulation in fruits of the *Aft* genotype. It is still unclear which gene is responsible for *Aft* phenotype. *LeANT1* has been previously characterized (Mathews *et al.* 2003, Sapir *et al.* 2008, Schreiber *et al.* 2011). By contrast, *LeAN2* has been studied much less than *LeANT1*. Whether *LeAN2* could function in other *Solanaceae* species such as tobacco is unclear.

Ectopic expression of *LeAN2* remains an attractive area to investigate its significance in regulating anthocyanin accumulation and protecting plants against chilling stress and methyl viologen (MV) stress. In this study, we isolated the *LeAN2* from tomato and developed transgenic tobacco plants.

Materials and methods

Plants and treatments: Seeds of wild tobacco (*Nicotiana tabacum* L. cv. NC89), transgenic tobacco lines (T-3, T-7 and T-20), and wild tomato [*Lycopersicum esculentum* Mill. (syn. *Solanum lycopersicum*), cv. Zhongshu 6] were germinated on moistened filter paper at 25 °C for 3 d. Sprouted seedlings were transplanted into plastic pots (one plant per pot) filled with sterilized soil and grown in a greenhouse at day/night temperatures of 25/20 °C, relative humidity of 50 - 60 %, a 16-h photoperiod, and photon flux density (PFD) of 300 - 400 μmol m^{-2} s^{-1}. When the sixth leaf was fully expanded, the plants were exposed to abiotic stresses. For a chilling treatment, plants were incubated at 4 °C for 3, 6, 9, 12, and 24 h at PFD of 100 μmol m^{-2} s^{-1}. After the chilling stress, the plants recovered for 12 h at 25 °C under PFD of 200 μmol m^{-2} s^{-1}. For an oxidative stress treatment, plants were sprayed either with 100 μM methyl viologen (MV) or with water as a control for one time only at the beginning and were exposed to a PFD of 100 μmol m^{-2} s^{-1} for 24 h. The stressed leaves were immediately frozen in liquid nitrogen and stored at -80 °C until use. In another experiment, fifteen leaf discs (0.8 cm^2 each) from the WT and transgenic lines were floated on a MV solution (0, 50, or 100 μM) in a Petri dish under continuous PFD of 100 μmol m^{-2} s^{-1} for 48 h. The phenotypic changes and chlorophyll content were detected. Living onion epidermal cells (*Allium cepa* cv. Jinguan 1) were used to conduct subcellular localization analysis.

Isolation of *LeAN2*: Total RNA was extracted from young leaves with the RNA simple kits (*Tiangen Biotech*, Beijing, China), and DNase was used to clean out DNA before reverse-transcription using *MMLV* reverse transcriptase (*Tiangen*). A RT-PCR (*TaKaRa*, Shiga, Japan) with specific primers 5'-ATGAATACT CCTATGTGTGCAT-3'and 5'-TTTAAACTCAGTCGG ACTCT-3' according to Boches (2009) was used to amplify the full-length cDNA of tomato *LeAN2*. Amplified products were isolated, gel-purified, and cloned into a pMD18-T vector (*TaKaRa*) to generate pTAN2 for further sequencing.

Subcellular localization of LeAN2 fusion protein: The competed coding region of *LeAN2* in the pMD18-T simple vector was digested with *XbalI* and *KpnI* and inserted into the pBINmGFP5-ER vector upstream and in frame with the green fluorescence protein (GFP) coding region. The recombinant plasmid was transformed into living onion epidermal cells by plasmid bombardment as described by Kinkema *et al.* (2000). The subcellular location of the LeAN2 was detected by monitoring the transient expression of GFP in onion epidermal cells. The transformed cells were incubated in a Murashige and Skoog (MS) medium at 25 °C for 24 - 48 h and then observed with a fluorescence microscope (*BX51*, model

7.3, *Olympus*, Tokyo, Japan) and the images obtained were recorded automatically.

Real-time RT-PCR analysis was performed on the a *Bio-Rad* (Hercules, CA, USA) *CFX96TM* real-time PCR system using *SYBRReal Master Mix* (*Tiangen*) according to the supplier's protocols. *LeEF1a* (acc. No. X144491) and *NtUbiquitin* (acc. No. U66264) were used as internal reference genes for calculating relative transcript levels. Primer sequences were as follows: EF-F: 5'-GGAACTTGAAGGAGCCTAAG-3'; EF-R: 5'-CAACACCAACAGCAACAGTCT-3'; ubiquitin-F: 5'-TCCAGGACAAGGAGGGTAT-3'; ubiquitin-R: 5'-CATCAACAACAGGCAACCTAG-3'; AN2-F: 5'-CAGGAAGGACAGCAAACGATGT-3'; AN2-R: 5'-GAGAATGAGGATGTAGATGAGGAGC-3'; ANS1-F: 5'-AAGCCCCTACCTGAGACTATAACT-3'; ANS1-R: 5'-CAGCAGCAGCATCCTGATCAT-3'; ANS2-F: 5'-TCGACTCAGAGGACAAGGAAATT-3'; ANS2-R: 5'-TCAATTAGCTGATCTGATATGCCAT-3'; CHS-F: 5'-GTATCACTAATAGCGAGCATAAGGTT-3'; and CHS-R: 5'-AAGATTTCCTCTGTTAAG TGCATGT-3'.

Vector construction and plant transformation: The full-length *LeAN2* cDNA was inserted into expression vector pBI121 between BamHI and SacI downstream of the CaMV35S promoter. The 35S-CaMV-LeAN2 constructs were introduced into *Agrobacterium tumefaciens* strain LBA4404 by the freezing transformation method and verified by PCR and sequencing. Tobacco transformation was performed by co-cultivation of leaf discs with *A. tumefaciens* for 15 min. Transformed leaf discs were incubated on a MS medium to induce shoots. The MS media contained benzylaminopurine (3 mg dm^{-3}), naphthalene acetic acid (0.2 mg dm^{-3}), cefotaxime (250 mg dm^{-3}), and kanamycin (100 mg dm^{-3}). Regenerated shoots were rooted on the MS basal medium. The profusely rooted plantlets were transferred into pots and maintained 2 weeks in a greenhouse for acclimatization.

Measurement of the anthocyanin content: The anthocyanins were extracted with a methanol-HCl method according to Rabino and Mancinelli (1986) with slight modifications. Samples (0.2 g) were incubated in 1 cm^3 of an acidic methanol (MeOH, HPLC quality) solution, consisting of 80 % (v/v) MeOH, 0.16 % (m/v) ascorbic acid, 0.16 % (m/v) *t*-butyl hydroquinone, and 0.1 % (m/m) HCl at room temperature with gentle shaking for 18 h. After centrifugation at 12 000 *g* for 2 min, 0.4 cm^3 of the supernatant was added to 0.6 cm^3 of acidic methanol and then filtered through a 0.2 μm *Teflon* filter before analysis. The absorbances of the extracts were measured at 530 and 657 nm. The anthocyanin content (Q) was determined as: $Q = (A_{530} - 0.25 \times A_{657})/M$, where A_{530} and A_{657} are the absorbances at the indicated wavelengths and M [g] is the mass of the plant material used for extraction.

Detection of reactive oxygen species: H_2O_2 and $O_2^{\bullet-}$ were visually detected by treating leaves with 3,3-diaminobenzidine (DAB) as described by Giacomelli *et al.* (2007), and with nitroblue tetrazolium (NBT), as described by Hückelhoven *et al.* (2000) with little modification. Briefly, to detect H_2O_2, detached leaves were placed in a solution of 1 mg cm^{-3} DAB, pH 3.8, in the dark overnight. For NBT staining, detached leaves were placed in a solution of 0.5 mg cm^{-3} NBT, pH 7.8. Subsequently, these stained samples were decolorized by boiling in an acetic acid : glycerol : ethanol (1:1:3, v/v/v) solution. After cooling, the samples were placed into fresh ethanol at room temperature and photographed.

H_2O_2 content was measured spectrophotometrically according to Sui *et al.* (2007) with slight modifications. Leaf samples (0.5 g) were homogenized with 3 cm^3 of cold acetone. The homogenate was centrifuged at 2 000 *g* for 10 min. The supernatant (1 cm^3) was mixed with 0.1 cm^3 of 5 % (m/v) titanium sulfate and 0.2 cm^3 of 25 % NH_4OH and the mixture was then centrifuged at 10 000 *g* and 10 °C for 10 min. After washing five times with 1 cm^3 of acetone and subsequent centrifugation under the same conditions as above, the precipitate was dissolved in 5 cm^3 of 2 M H_2SO_4 followed by addition of acetone to a final volume of 10 cm^3. The absorbance of the sample was measured at 410 nm.

The assay for $O_2^{\bullet-}$ was performed as described by Yang *et al.* (2011). Frozen leaf samples (0.5 g) were homogenized with 5 cm^3 of a grinding medium containing 0.05 M phosphate buffer (pH 7.8) in an ice bath. The homogenate was centrifuged at 12 000 *g* and 4 °C for 20 min. The reaction mixture contained 0.5 cm^3 of supernatant with 0.5 cm^3 of 0.05 M phosphate buffer (pH 7.8) and 1 cm^3 of 1 mM hydroxylammonium chloride. After incubation at 25 °C for 1 h, 1 cm^3 of 17 mM *p*-aminobenzene sulfonic acid and 1 cm^3 of 7 mM α-naphthylamine were added, and the mixture was incubated at 25 °C for 20 min. Finally, an equal volume of ethyl ether was added into the mixture that was centrifuged at 1 500 g for 5 min. The water phase was used to determine the absorbance at 530 nm.

Measurement of net photosynthetic rate and chlorophyll *a* fluorescence: Net photosynthetic rate (P_N) was measured with a portable photosynthetic system (*CIRAS-2*, *PP Systems*, Herts, UK) under ambient temperature of 25 °C, relative humidity of 80 %, CO_2 concentration of 360 μmol mol^{-1}, and PFD of 800 μmol m^{-2} s^{-1}. Before P_N measurement, plants were kept at PFD of 100 μmol m^{-2} s^{-1} for 30 min to induce stomata opening.

Chlorophyll *a* fluorescence was measured with a portable fluorometer (*FMS2*, *Hansatech*, Norfolk, UK) according to the protocol described by Duan *et al.* (2012). Plants were left to acclimate in darkness for 15 min.

Minimal fluorescence (F_0) was determined under a weak modulated irradiance and the maximal fluorescence (F_m) was determined after 0.8 s saturating radiation pulse of 7 000 μmol m^{-2} s^{-1}. F_v is the difference between F_o and F_m. The ratio F_v/F_m is considered as a measure of the maximal photochemical efficiency of photosystem (PS) II.

The chlorophyll content was determined in leaf discs homogenized in 1 cm^3 of 80 % (v/v) acetone. The homogenate was centrifuged at 3 500 *g* for 5 min and the absorbances of the supernatant were recorded at 663 and 646 nm.

Measurement of electrolyte leakage: Fifteen leaf discs (0.8 cm^2 each) were put into a cuvette containing 15 cm^3 of distilled water and shaked for 3 h before measurement of the initial electric conductance (S_1). Then the cuvette was heated in boiling water for 30 min and cooled to room temperature to determine the final electric conductance (S_2). The distilled water was used as the blank (S_0). The relative electrolyte leakage (REL) was evaluated as follows: REL [%] = $(S_1 - S_0)/(S_2 - S_0) \times 100$.

Antioxidant capacity was determined according to Zhang *et al.* (2010) with a few modifications. Frozen leaf samples (0.3 g) were extracted in 10 cm^3 of 70 % (v/v) ethanol, centrifuged at 1 000 *g* and 4 °C for 15 min, and the supernatant was used for the assay of total antioxidative capability. The α,α-diphenyl-β-picryl-hydrazyl (DPPH) stock solution was prepared by stirring 79 mg of DPPH in 1 dm^3 of 70 % ethanol overnight. For the assay, three different mixtures, consisting of 0.1 cm^3 of the extract + 2.9 cm^3 of the DPPH solution + 1.0 cm^3 of 70 % ethanol (A), 0.1 cm^3 of the extract + 3.9 cm^3 of 70 % ethanol (B), and 1.1 cm^3 of 70 % ethanol + 2.9 cm^3 of the DPPH solution (C) were prepared. After 30 min in darkness, the absorbances of A, B, and C solutions were determined at 517 nm. Antioxidant activity [%] was calculated as: $[1 - (A - B)/C] \times 100$.

Statistical analysis: Statistical analysis was conducted using *DPS* (date processing system) procedures (Zhejiang University, China). Differences between the means were compared using Duncan's multiple range tests at 0.05 probability level.

Results

RT-PCR with the specific primers 5'-ATGAATACTCCT ATGTGTGCAT-3' and 5'-TTTAAACTCAGTCGG ACTCT-3' was used to amplify the full-length cDNA of *LeAN2*. The *LeAN2* transcript amounts under low temperature and MV treatments were detected by qRT-PCR. The chilling stress caused a gradual increase in the transcription, peaking at 9 h when transcription was approximately 10-fold. Under the 50 μM MV treatment,

Table 1. The *LeAN2* mRNA content relative to control (set to 1) in tomato under the chilling (4 °C) or oxidative (50 μM MV) stresses. Means ± SD of four independent experiments. Different letters indicate statistically significant differences at $P \leq 0.05$.

mRNA content	0 h	3 h	6 h	9 h	12 h	24 h
4 °C	1.00 ± 0.03f	2.86 ± 0.20e	3.64 ± 0.20d	10.5 ± 0.57a	8.8 ± 0.44b	7.51 ± 0.46c
50 μM MV	1.00 ± 0.17e	3.11 ± 0.52c	8.67 ± 0.50b	9.4 ± 1.10b	19.8 ± 0.53a	2.17 ± 0.15d

Table 2. The *LeAN2* mRNA content relative to control (set to 1) and anthocyanin content $[(A_{530} - 0.25 \times A_{657})\ g^{-1}$(f.m.)] in WT and different lines of transgenic tobacco plants. Means ± SD of four independent experiments. Different letters indicate statistically significant differences at $P \leq 0.05$.

	WT	T-3	T-7	T-10	T-11	T-20
LeAN2 expression	0.01 ± 0.00f	2.45 ± 0.25c	3.70 ± 0.33a	1.00 ± 0.17e	1.88 ± 0.36d	2.84 ± 0.11b
Anthocyanin content	0.04±0.02d	2.56±0.19b	3.86±0.38a	1.37±0.06c	2.22±0.25b	3.59±0.30a

Table 3. Relative expression of anthocyanin biosynthetic genes in WT (set to 1) and transgenic plants determined by qRT-PCR. Means ± SD of four independent experiments. Different letters indicate statistically significant differences at $P \leq 0.05$.

	WT	T-3	T-7	T-20
*LeANS1*expression	1.00 ± 0.58c	117.17 ± 51.50c	4889.49 ± 293.20a	1358.67 ± 620.07b
LeANS2 expression	1.00 ± 0.50c	1.45 ± 0.04c	15.07 ± 0.85b	17.03 ± 1.00a
LeCHS expression	1.00 ± 0.27d	32.23 ± 1.77c	119.40 ± 7.26b	128.26 ± 8.47a

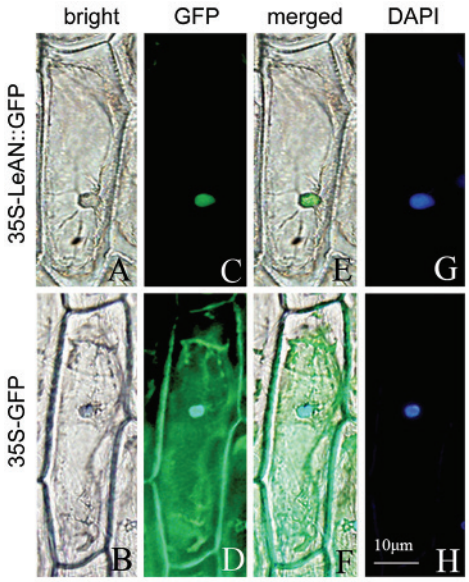

Fig. 1. Subcellular localization of *LeAN2* in onion epidermal cells. *A*, *B* - Onion epidermal cells in bright field. *C*, *D* - Green fluorescence of the LeAN2-GFP fusion protein and green fluorescent protein (GFP), respectively. *E*, *F* - Merged images of *A*, *C* and *B*, *D*, respectively. *G*, *H* - The location of nucleus by DAPI staining. The experiment was repeated four times with similar results.

the transcription of *LeAN2* increased gradually from 0 to 12 h, peaking at 12 h (Table 1).

To investigate the subcellular localization of *LeAN2*, a reporter gene encoding GFP in the binary vector pBINmGFP5-ER was fused to *LeAN2* and expressed transiently in onion epidermal cells. The onion epidermal cells with the transferred plasmid p35S-LeAN2-GFP expressing the LeAN2-GFP fusion protein showed green fluorescence only in the nucleus (Fig. 1*C*,*E*). In contrast, in the individual cells with the transferred control plasmid p35S-GFP (expressing the GFP coding sequence alone), green fluorescence was distributed in the whole cell (Fig. 1*D*,*F*). Thus, LeAN2 is a nuclear protein.

After transformation, twenty five kanamycin resistant tobacco lines were obtained. These initial kanamycin resistant plants were named T_0 and the progeny obtained from T_0 were named T_1. Kanamycin tolerant T_1 plants were checked by PCR and an upstream primer of pBI121 and a 3' primer of *LeAN2* were used in the amplification. Five lines named T-3, T-7, T-10, T-11, and T-20, and a WT plant were selected for qRT-PCR analyses. The T-3, T-7, and T-20 transgenic lines showed relatively high expressions of *LeAN2* (Table 2). The transgenic plants had purple roots, stems, leaves, and flowers. Except for the colour, the transgenic plants were apparently not different from the WT plants.

The anthocyanin content was quantified in five transgenic lines T-3, T-7, T-10, T-11, T-20, and in the WT plants. All the lines were grown and sampled in triplicate. Anthocyanin accumulation was much higher in the transgenic plants than in the WT plants (Table 2). The T-3, T-7, and T-20 transgenic lines showed the highest anthocyanin accumulation. The anthocyanin accumulation detected in these lines correlated well with the *LeAN2* transcription in these lines (Table 2). Thus, these transgenic lines were selected for further experiments.

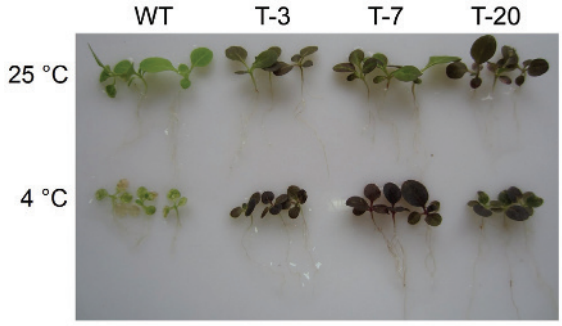

Fig. 2. The phenotype of WT and transgenic tobacco plants at 25 °C and 4 °C. The experiment was repeated four times with similar results.

The transcription of several structural genes involved in anthocyanin biosynthetic pathway were measured by qRT-PCR. Overexpression of *LeAN2* resulted in upregulation of several anthocyanin biosynthetic genes, including *NtANS1*, *NtANS2*, and *NtCHS* (Table 3). These results indicate that overexpression of *LeAN2* could affect anthocyanin biosynthesis in the transgenic tobacco plants.

No differences were observed between the growth of the transgenic plants (T-3, T-7, and T-20) and the WT plants at 25 °C for 15 d. However, after the treatment at 4 °C for 7 d, the growth of all the plants was suppressed but the suppression in the WT plants was much more serious than in the transgenic plants (Fig. 2). The fresh

Table 4. Fresh mass and chlorophyll content of WT and transgenic tobacco plants under temperature of 25 or 4 °C for 7 d. Means ± SD of four independent experiments. Different letters indicate statistically significant differences at $P \leq 0.05$.

	Temperature	WT	T-3	T-7	T-20
Fresh mass	25 °C	13.23 ± 0.35a	13.41 ± 0.72a	14.06 ± 0.35a	13.24 ± 0.44a
[mg plant^{-1}]	4 °C	6.79 ± 0.33c	8.20 ± 0.42b	10.42 ± 0.38a	8.38 ± 0.33b
Chlorophyll content	25 °C	0.87 ± 0.025a	0.86 ± .051a	0.85 ± 0.024a	0.87 ± 0.0087a
[mg g^{-1}(f.m.)]	4 °C	0.63 ± 0.031b	0.75 ± 0.016a	0.87 ± 0.029a	0.77 ± 0.091a

Table 5. Effect of chilling stress on some physiological parameters in WT and transgenic plants. During chilling stress, plants were adapted in darkness for 15 min before F_v/F_m measurement and F_v/F_m was measured at 4 °C. The abbreviations "r3" and "r6" mean a 3-h recovery treatment and a 6-h recovery treatment, respectively. Plants were treated at 4 °C for 24 h and P_N was measured at 25 °C under ambient CO_2 and PFD of 800 μmol m^{-2} s^{-1}. Plants were exposed to 4 °C for 24 h before measurement of REL and DPPH activity. Plants were exposed to 4 °C for 12 h before measurement of ROS. Means ± SD of four independent experiments. Different letters indicate statistically significant differences at $P \leq 0.05$.

Parameter	Time [h]	WT	T-3	T-7	T-20
F_v/F_m	0	0.84 ± 0.01a	0.85 ± 0.01a	0.86 ± 0.01	0.86 ± 0.01
	3	0.82 ± 0.01a	0.84 ± 0.00a	0.85 ± 0.02a	0.85 ± 0.01a
	6	0.82 ± 0.01a	0.83 ± 0.02a	0.82 ± 0.01a	0.83 ± 0.02a
	9	0.78 ± 0.01b	0.79 ± 0.01ab	0.82 ± 0.00ab	0.81 ± 0.01a
	12	0.77 ± 0.02b	0.79 ± 0.01b	0.80 ± 0.02b	0.80 ± 0.01a
	24	0.73 ± 0.01c	0.76 ± 0.02bc	0.79 ± 0.04a	0.79 ± 0.02ab
	r3	0.79 ± 0.02a	0.82 ± 0.01a	0.84 ± 0.01a	0.83 ± 0.00a
	r6	0.80 ± 0.01b	0.84 ± 0.01a	0.84 ± 0.01a	0.84 ± 0.00a
P_N	0	9.63 ± 0.20a	9.66 ± 0.25a	9.93 ± 0.39a	9.53 ± 0.30a
[μmol m^{-2} s^{-1}]	24	1.24 ± 0.17c	2.72 ± 0.18ab	2.60 ± 0.20b	2.95 ± 0.17a
REL	0	17.43 ± 1.26a	16.65 ± 1.12a	17.63 ± 1.42a	18.15 ± 1.57a
[%]	24	53.28 ± 2.28a	36.56 ± 2.37b	38.90 ± 2.48b	40.24 ± 2.78b
DPPH activity	0	31.30 ± 1.11c	53.29 ± 0.33a	51.52 ± 1.03a	46.80 ± 1.55b
[%]	24	25.46 ± 1.66b	53.20 ± 0.65a	50.24 ± 0.25a	50.40 ± 4.29a
H_2O_2	0	0.27 ± 0.02a	0.26 ± 0.02a	0.27 ± 0.02a	0.26 ± 0.02a
[μmol g^{-1}(f.m.)]	12	0.43 ± 0.02a	0.31 ± 0.02b	0.34 ± 0.02b	0.34 ± 0.02b
$O_2^{\bullet-}$	0	3.30 ± 0.13a	3.02 ± 0.15a	3.14 ± 0.12a	3.08 ± 0.16a
[μmol g^{-1}(f.m.) min^{-1}]	12	7.07 ± 0.25a	5.20 ± 0.24b	5.05 ± 0.25b	5.47 ± 0.26b

Table 6. Effect of oxidative stress on physiological parameters in WT and transgenic plants. Chlorophyll content in leaf discs of transgenic lines and WT plants after 48 h treatment with different concentrations of MV (0, 50 and 100 μM). Plants were sprayed with 100 μM MV once for measurement of F_v/F_m and REL. Means ± SD of four independent experiments. Different letters indicate statistically significant differences at $P \leq 0.05$.

Parameter	Treatment	WT	T-3	T-7	T-20
Chlorophyll content	0 μM MV 48 h	1.91 ± 0.13bc	2.20 ± 0.07a	2.05 ± 0.11b	1.84 ± 0.03c
[mg g^{-1} (f.m.)]	50 μM MV 48 h	0.84 ± 0.03c	1.52 ± 0.07a	1.32 ± 0.06b	1.18 ± 0.05b
	100 μM MV 48 h	0.10 ± 0.01d	0.73 ± 0.04a	0.33 ± 0.03b	0.20 ± 0.02c
F_v/F_m	100 μM MV 0 h	0.84 ± 0.01a	0.86 ± 0.01a	0.86 ± 0.01a	0.85 ± 0.01a
	100 μM MV 12 h	0.50 ± 0.04b	0.66 ± 0.03a	0.65 ± 0.01a	0.65 ± 0.01a
	100 μM MV 24 h	0.40 ± 0.04b	0.60 ± 0.04a	0.63 ± 0.03a	0.59 ± 0.02a
REL [%]	100 μM MV 0 h	14.41 ± 1.22a	14.60 ± 1.00a	14.66 ± 1.44a	14.72 ± 0.76a
	100 μM MV 12 h	31.09 ± 1.77a	20.82 ± 1.42b	20.59 ± 2.35b	20.90 ± 0.35b
	100 μM MV 24 h	39.68 ± 2.93a	31.01 ± 1.40b	33.22 ± 1.46b	29.74 ± 0.67b

mass of all plants under the chilling stress decreased obviously compared with those grown under 25 °C, and the decrease was much higher in the WT plants (Table 4). Also the chlorophyll content decreased under the chilling stress more in the WT than in the transgenic plants (Table 4). The F_v/F_m was reduced in all the plants after the 4 °C treatment with a greater reduction in the WT plants, and the recovery of F_v/F_m in the WT plants was slower than that in the transgenic plants (Table 5). After the 24 h chilling stress, P_N of WT, T-3, T-7, and T-20 decreased to 12.9, 28.1, 26.2 and 30.9 %, respectively (Table 5). After the 12 h chilling stress, REL increased in both the WT and transgenic plants but less in the transgenic plants (Table 5). DPPH activity was significantly higher in the transgenic plants both before and after the stress than in the WT ones (Table 5).

Intracellular localization of H_2O_2 and $O_2^{\bullet-}$ in the leaves of the WT and transgenic plants was analyzed by diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining. At 25 °C, no significant difference in accumulations of H_2O_2 and $O_2^{\bullet-}$ were detected between WT plants and the transgenic lines (Fig. 3*A*,*C*). After the 4 °C treatment, the H_2O_2 and $O_2^{\bullet-}$ accumulations increased. The colour was deeper in the WT plants than

in the transgenic lines (Fig. 3*B*,*D*). Quantitative analysis of H_2O_2 and $O_2^{\bullet-}$ showed similar results (Table 5).

After floating leaf discs on MV solutions (0, 50, or 100 μM) for 48 h, the necrosis was observed in all the plants and the situation was more serious in the WT plants (Fig. 4). As concern the chlorophyll content, no significant difference was observed in all the plants without MV treatment. For the 50 μM MV treatment, the chlorophyll content in the WT plants decreased by 56.0 %, whereas decreases by 30.9, 35.6, and 35.9 % were noted in the T-3, T-7, and T-20 plants. Under the 100 μM MV treatment, the chlorophyll content in the WT plants decreased by 94.8 %, whereas decreases by 66.8, 83.9, and 89.1 % were observed in the T-3, T-7, and T-20 (Table 6).

After spraying for 12 and 24 h with the solution containing 100 μM MV, F_v/F_m in the WT and transgenic lines decreased, but the reduction was more serious in the WT plants (Table 6). After the MV treatment, REL increased significantly in both the WT and transgenic plants but less in the transgenic plants (Table 6).

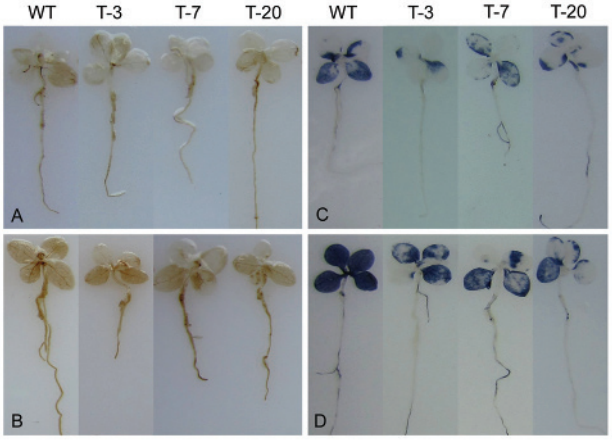

Fig. 3. Detection of ROS in WT and transgenic tobacco plants. *A* - DAB staining for H_2O_2 of WT and transgenic tobacco plants under normal conditions. *B* - DAB staining for H_2O_2 of cold-treated leaves of WT and transgenic tobacco plants. *C* - NBT staining for $O_2^{\bullet-}$ of WT and transgenic tobacco plants under normal conditions. *D* - NBT staining for $O_2^{\bullet-}$ in cold-treated leaves of WT and transgenic tobacco plants. The experiment was repeated four times with similar results.

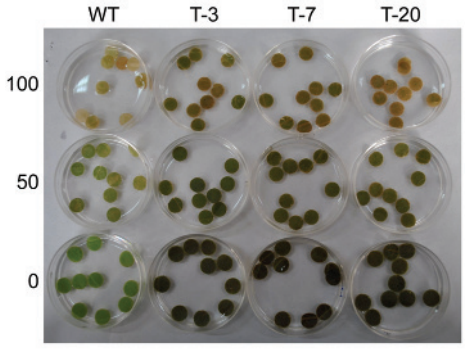

Fig. 4. The phenotypic differences in the leaf discs from transgenic and WT plants after 48 h treatment with different concentrations of MV (0, 50 and 100 μM) under continuous irradiance of 100 μmol m^{-2} s^{-1} and temperature of 25 °C. The experiment was repeated four times with similar results.

Discussion

LeAN2 and its homolog, *LeANT1*, are mapped to a tomato chromosome 10 as candidate genes that control anthocyanin accumulation in fruit peels of the *Aft* genotype (Sapir *et al.* 2008, Boches 2009). *LeANT1* alone can generate the characteristic anthocyanin accumulation in *Aft* fruits (Schreiber *et al.* 2011). However, the knowledge about *LeAN2* expression and function is much more limited than that of *LeANT1*. In this study, *LeAN2* was isolated from tomato. The subcellular localization analysis indicates that LeAN2 was targeted to the nucleus (Fig. 1). To further understand the function of LeAN2, we examined the expression patterns of *LeAN2* in relation to a chilling stress, and an oxidative stress induced by MV. *LeAN2* in tomato was significantly induced by the chilling stress (4 °C) and by the treatment with 50 μM MV (Table 1).

Many anthocyanin-associated MYB TFs are well characterized at the molecular level and can induce anthocyanin biosynthesis in other species (Ban *et al.* 2007, Geekiyanage *et al.* 2007, Butelli *et al.* 2008). The overexpression of *LeANT1* in tobacco generates a range of phenotypes, from green to slightly or intensely purple plants (Mathews *et al.* 2003). We generated transgenic tobacco plants overexpressing *LeAN2* which also showed a purple phenotype. The anthocyanin content was significantly increased in transgenic lines compared to WT plants (Table 2), and the transcription of *NtANS1*, *NtANS2*, and *NtCHS* genes involved in anthocyanin biosynthesis were significantly up-regulated in the transgenic lines (Table 3). These results show that *LeAN2* induced anthocyanin accumulation by up-regulating genes that encode proteins participating in anthocyanin biosynthesis.

ROS are generated during normal metabolic processes, but especially under environmental stresses (Asada 1999). ROS accumulation can aggravate photoinhibition and induce damage to the biological membrane system (Yang *et al.* 2007). Therefore, ROS generated in excess must be effectively scavenged. Anthocyanins are effective antioxidants (Gonzali *et al.* 2009). Previous study using an anthocyanin-deficient mutant indicated that anthocyanin contributes to plant resistance to high temperature (Shao *et al.* 2007). Chilling is one of the most serious environmental stresses which

disrupts the metabolic balance of cells resulting in an enhanced production of ROS. Anthocyanin mitigates chilling injury by efficiently scavenging ROS (Gould 2004). In our experiments, the content of ROS during chilling was much lower in the transgenic plants than in the WT plants (Fig. 3 and Table 5). The transgenic lines had a greater total antioxidative capability according to the DPPH analysis (Table 5). After the 4 °C treatment, F_v/F_m and P_N decreased significantly both in the transgenic and WT plants, but the decrease in the WT plants was faster (Table 5). The REC analysis demonstrated that membrane damage was less serious in the transgenic plants than in the WT plants (Table 5). These results suggest that the high content of anthocyanins in transgenic tobacco led to a greater resistance to the chilling stress.

MV catalyzes the photoreduction of O_2 at PS I, thereby accelerating the productions of $O_2^{\bullet-}$ and H_2O_2, and simultaneously inhibits the photoreduction of ferredoxin (Mano *et al.* 2001, Li *et al.* 2010). Visible injury of leaf discs by MV treatment is often used to assay the tolerance of plants to oxidative stress (Yoshimura *et al*. 2004). Severe necrosis was observed in the leaf discs of the WT plants whereas only partial necrosis was observed at the boundaries of the leaf discs of the transgenic plants (Fig. 4). The chlorophyll content was higher in the transgenic plants compared with the WT (Table 6). These results imply that oxidative damage of leaf discs in the transgenic lines was lower than in the WT plants. Accumulation of H_2O_2 may cause significant damage to cellular components such as membrane lipids (Kwon *et al.* 2002, Yabuta *et al.* 2002). After the 100 μM MV treatment, the ion leakage was lower in the transgenic plants than in the WT plants (Table 6), and F_v/F_m was higher in the transgenic lines (Table 6) indicating that the extent of photoinhibition of PS II and membrane damage in the transgenic plants was not so serious as in the WT plants. These results suggest that the transgenic tobacco plants exhibit an enhanced resistance against oxidative stress resulting from an enhanced anthocyanin content by overexpressing *LeAN2*.

In conclusion, the increase in anthocyanin accumulation due to overexpression of MYB TF, *LeAN2*, enhanced tolerance of the transgenic tobacco plants to the chilling and oxidative stresses. The high content of anthocyanins played an important role in sustaining a relatively low accumulation of ROS and thus protected the plants from these stresses. Our findings provide a new insight into the mechanism by which anthocyanin-associated MYB TFs enhance tolerance to abiotic stresses in transgenic plants.

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