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Virus-induced gene silencing for phenylalanine ammonia-lyase affects pepper adaption to low temperature

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Abstract

Reverse genetics approaches in plants rely on post-transcriptional gene silencing to study the function of genes. In particular, virus-induced gene silencing (VIGS) has been successfully applied to identify gene function in some crops. To date, it is unclear whether phenylalanine ammonia-lyase (PAL) is involved in low temperature tolerance in the pepper. Here, we used an agroinfiltration protocol with tobacco rattle virus (TRV) constructs containing partial sequences from *CaPAL* for VIGS to test its role in anthocyanin biosynthesis and response to low temperature in the pepper (*Capsicum annuum*). We found that accumulation of anthocyanins in the leaves of pepper plants transformed with the TRV2:CaPAL vector was significantly reduced compared with peppers transformed with the empty TRV2 vector (TRV2:00). A significant reduction in expression of genes related to anthocyanins synthesis was also detected in peppers transformed with TRV2:CaPAL. When silenced pepper plants were exposed to a low temperature, we found decreased antioxidant system, PAL activity, and photosynthesis in plants transformed with TRV2:CaPAL compared with peppers transformed with TRV2:00. Low transcriptions of cold stress-response genes demonstrated that pepper tolerance to low temperature decreased. Future studies focused on the interaction between *CaPAL* and other abiotic and biotic stressors will shed further light into the role of *CaPAL* in stress response.

Additional key words: anthocyanins, *Capsicum annuum*, photosynthesis, transgenic plants, TR2:CaPAL vector.

Introduction

A major challenge for biology is to integrate research approaches to address how organisms adapt to environmental stress (Conde *et al.* 2011). To achieve this, understanding the function of genes is indispensable. To date, most reverse genetics approaches in plants have relied on post-transcriptional gene silencing to study the function of genes (Zhai *et al.* 2017). This is especially suitable for non-model species (Tavakol 2018), which do not have a catalogue of well-characterized mutants, but it is also a useful tool for model species (Jupin 2013). Virus-induced gene silencing (VIGS) suppresses gene transcripts, and has been successfully used to characterize the function of plant genes in *Glycine max* (Jeong *et al.* 2005). The most widely used VIGS vectors are based on the tobacco rattle virus (TRV), and TRV-mediated VIGS is used to silence genes in a number of crops, including rice (Purkayastha *et al.* 2010) and tomato (Fantini and Giuliano 2016).

Anthocyanins are plant pigments derived from the phenylpropanoid pathway (Van Tunen and Mol 1991, Oren-Shamir 2009), and the anthocyanin biosynthetic pathway is an important branch of flavonoid pathway (Ubi 2007). The content and stability of anthocyanins are affected by environmental factors (Jenshi Roobha *et al.* 2011, Kovačević *et al.* 2015). In general, plants can have higher tolerance for abiotic or biotic stressors by accumulating anthocyanins (Filiz *et al.* 2010, Schulz *et al.* 2015).

Phenylalanine ammonia-lyase, encoded by the *PAL* gene, is the first key enzyme involved in the phenylpropanoid pathway, and it is important for anthocyanin biosynthesis, and thus plant defence against environment stresses (Pombo *et al.* 2011, Aza-González *et al.* 2013, Zhu *et al.* 2015). The PAL has been successfully isolated from rice and pepper (Kim and Hwang 2014,

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Abbreviations: APX - ascorbate peroxidase; c_i - intercellular CO_2 concentration; g_s - stomatal conductance; MDA - malondialdehyde; PBS - phosphate buffer solution; PAL - phenylalanine ammonia-lyase; P_N - net photosynthetic rate; POD - peroxidase; qPCR - quantitative PCR; ROS - reactive oxygen species; SOD - superoxide dismutase; E - transpiration rate; TRV - tobacco rattle virus; TRV2:00 - empty TRV vector; TRV2:CaPAL - TRV2 vector with *CaPAL* gene; TRV2:CaPDS - TRV2 vector with *CaPDS* gene; VIGS - virus-induced gene silencing.
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Tonnessen *et al.* 2014). Environmental conditions can regulate transcription of *PAL* gene and activate respective protein function (Christopoulos and Tsantili 2015). *Phenylalanine ammonia-lyase* knock-out plants show damage induced by environmental stresses (Rohde 2004).

Pepper is cultivated widely throughout the world (Pimenta *et al.* 2016). However, cold weather often results in significant reduction of its yield and quality. Although PAL (GenBank: KF279696) in the pepper is a rate-limiting enzyme in the plant defence system, studies on its biological function have been restricted to its role in anthocyanins biosynthesis. The *pal1* knockout plants are deficient in anthocyanin pigments suggesting that PAL1 has an important role in flavonoid biosynthesis (Huang *et al.* 2010). Here, we employed VIGS to knock down the *PAL* gene in a color-leafed pepper cultivar Z1 to investigate the effect of *PAL* expression on anthocyanins biosynthesis and response to low temperature. Our work will provide insights into the mechanism of pepper anthocyanin biosynthesis and offer a potential for improving breeding in the future.

Materials and methods

Plants and cultivation: The pepper (*Capsicum annuum* L.) purple-leafed cultivar Z1 was used for all experiments (Fig. 1 Suppl.). Pre-germinated seeds were sown in 0.2-dm^3 nutritional bowls with 85 g of matrix and germinated at a temperature of 28 °C. Seedlings at the cotyledon stage were transferred to growth cabinets with a 16-h photoperiod, an irradiance of 500 µmol m^{-2} s^{-1}, day/night temperatures of 22/18 °C, and a relative humidity of 75 - 80 %.

Construction of TRV plasmids and generation of *CaPAL*-silenced plants: The tobacco rattle virus (TRV)-based VIGS system was used to silence the *CaPAL* gene in the Z1 pepper cultivar. The empty vector (TRV2:00) was used as a negative control. Following previous studies, the *CaPDS* gene (GenBank: X68058.1), encoding a phytoene desaturase in biosynthesis pathway of carotenoids, was used as a positive visual marker, whose silencing produces a photobleaching phenotype in plant newly-growing tissues/organs (Cunningham and Gantt 1998). A fragment of the *CaPDS* coding region was amplified using gene-specific primers forward VCaPDS with an EcoRI restriction site, and reverse VCaPDS with a BamHI restriction site (Table 1 Suppl.). The resulting product was inserted into the TRV2 vector to generate the TRV2:CaPDS vector as a positive control (Fig. 2 Suppl.).

The full length of *CaPAL* mRNA sequence (GenBank: KF279696) contains 2 318 base pairs (bp) with an open reading frame, and a 488 bp fragment (location: 1179 - 2266 bp) from the 3' end of the gene was cloned in the TRV2 vector using gene-specific primers and generated the TRV2:CaPAL (Table 1 Suppl., Fig. 2 Suppl.). To ensure the specificity of *CaPAL* gene and avoid a product of other homologous genes, the primer should be designed in the nonconservative domain of the gene. The TRV1, TRV2:00 (negative control), TRV2:CaPDS, and TRV2:CaPAL vectors were transformed into the *Agrobacterium tumefaciens* strain GV3101. The GV3101 cells carrying TRV1 were mixed with TRV2:00, TRV2:CaPDS, and TRV2:CaPAL at a 1:1:1 ratio. The suspensions of *Agrobacterium* inoculation containing TRV1, TRV2:00, TRV2:CaPDS, and TRV2:CaPAL (with absorbance at 600 nm equal to 1.0) were infiltrated into the fully expanded cotyledons of wild type pepper using a sterilized syringe without the needle. The *Agrobacterium*-inoculated pepper plants were grown in a growth chamber at 18 °C and a 45 % relative humidity in darkness for 2 d, and then transferred into a growth chamber at 22 °C, a 60 % relative humidity, a 16-h photoperiod, and an irradiance of 500 µmol m^{-2} s^{-1}. When the photobleaching symptom appeared on the leaves of pepper seedlings inoculated with TRV2:CaPDS (representing the success of VIGS), the young leaves of TRV2:CaPAL and TRV2:00 were sampled for testing silencing efficiency and stress treatments.

The selected TRV2:CaPAL and TRV2:00 plants were placed at 4 °C for 16 h for low temperature treatment. Young leaves were sampled for measuring metabolites as described below. Three biological replicates were conducted for each treatment, and each replicate contained three pepper seedlings.

Measurement of anthocyanin content and PAL activity: Anthocyanin content was quantified following methods of Christie *et al.* (1994) with slight modifications. After one month of injection, fresh leaves (1.0 g) were grinded in liquid nitrogen, dissolved with a homogenization solution HCl (conc.) : methanol (100 %) 1:100, v/v, and incubated at 4 °C for 4 h. The mixture was centrifuged at 24 170 *g* for 20 min and the supernatant was measured spectrophotometrically at 530 nm.

Activity of PAL was determined by the production of cinnamate at 30 °C during 1 h, as measured by the change in absorbance at 290 nm following published protocol (Zhou *et al.* 1990) with slight modifications. After one month of injection, fresh leaves (1.0 g) were grinded in liquid nitrogen and dissolved in 0.1 M sodium borate buffer (pH 8.8) containing 5 mM mercaptoethanol and 5 % (m/v) tetrapyrrolidone. A 0.1 cm^3 of supernatant was added to an assay mixture with 0.02 M phenylalaninein to a total volume of 3.1 cm^3 after the buffer was centrifuged (2 832 *g*) for 15 min. Assays were performed in triplicate. Under the experimental conditions, an increase in absorbance at 290 nm was linear for up to 1 h. A unit (U) of PAL was defined as a 1 % loss in PAL activity per min.

Extraction of RNA and real time quantitative PCR: Quantitative PCR (qPCR) was conducted to test the silencing efficiency after the positive control showed symptoms of photobleaching. Total RNA was isolated following the method of Guo *et al.* (2012), and cDNA was synthesized using a *PrimeScript* kit (*Takara*, Dalian, China) according to the manufacturer's instructions. Primers were designed to generate 150 - 250 base pair fragments using the *Primer5* software, and are listed in

Table 1 Suppl. The PCR and detection were performed as described previously (Feng *et al.* 2012). The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative gene expressions (means from three replicates). The qPCR was performed using *iQ5* (*Bio-Rad*, Hercules, USA), and the ubiquitin-conjugating gene *CaUbi3* (AY486137) was used as a reference gene.

Photosynthetic parameters measurement: Photosynthetic rate (P_N), stomatal conductance (g_s), intercellular CO_2 concentration (c_i) and transpiration rate (E) were determined by a portable photosynthesis system (*LI-6400, LI-COR*, Lincoln, NE, USA) mounted with a red/blue LED diode (*6400-02B*, *LI-COR*) on the youngest fully developed leaves (the 4th leaf from the top). All measurements were conducted at 22 ± 2 °C and an ambient humidity of 75 ±5 %. Photosynthetic photon flux density at the leaf surface was set at 500 μmol m^{-2} s^{-1}.

Measurement of antioxidant enzymes and malondialdehyde content: To measure superoxide dismutase (SOD) activity, 0.5 g of fresh samples were grinded in a mortar and pestle with 5 cm^3 of phosphate buffer solution (PBS) containing 50 mM PBS, 25 mM nitrotetrazolium blue chloride, 0.003 mM riboflavin, and 0.1 mM ethylene diamine tetraacetic acid at pH 7.8. Homogenates were centrifuged at 24 170 *g* and 4 °C for 15 min. The supernatants were exposed to an irradiance of 500 μmol m^{-2} s^{-1} for 20 min.

Activity of SOD was quantified spectrophotometrically at 560 nm (A_{560} of the control containing water instead of the supernatant was determined in darkness). The SOD activity was calculated based on Dionisio-Sese and Tobita (1998). One unit (U) of SOD activity was defned as the amount of the enzyme which caused a 50 % inhibition of initial rate of the reaction in the absence of the enzyme.

To measure peroxidase (POD) activity, 0.1 g of fresh samples were grinded in a mortar and pestle with 5 cm^3 of PBS (20 mM, pH 6.0). Homogenates were centrifuged at 24 170 *g* and 4 °C for 10 min. The supernatants were exposed to an irradiance of 500 μmol m^{-2} s^{-1} for 20 min. Activity of POD was quantified spectrophotometrically at 470 nm, and absorbances were recorded every 30 s. The POD activity was calculated by the method of Dionisio-Sese and Tobita (1998). One unit (U) of POD activity was defined as a change in absorbance per minute.

For ascorbate peroxidase (APX) activity, 1.0 g of samples were frozen in liquid nitrogen, ground using a mortar and pestle, and immediately homogenized with 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer solution (pH 7.2) containing 1.0 mM Na_2EDTA, 1 mM sodium ascorbate, 3 % (m/v) insoluble polyvinylpyrrolidone, and 0.3 % (v/v) polyethylene glycol *tert*-octylphenyl ether at 4 °C for 15 min. The homogenates wwere filtered through nylon cloth and centrifuged at 24 710 *g* for 10 min. Ascorbate peroxidase activity was determined following the method from Nakano and Asada (1987). One unit (U) of APX activity was defined as a decrease of absorbance at 290 nm per minute.

To measure malondialdehyde (MDA) content, 0.2 g of samples were ground in liquid nitrogen using a pestle and mortar into which 5 cm^3 of ice-cold 10 % (m/v) trichloroacetic acid was added. Content of MDA was measured following Dionisio-Sese and Tobita (1998) with modifications. Briefly, the homogenates were centrifuged at 24 170 *g* for15 min and the supernatants were added to the same volume of a 10 % (m/v) thiobarbituric acid solution containing 0.6 % (m/v) trichloroacetic acid. The mixtures were heated at 100 °C for 20 min and the reaction was rapidly halted by placing the mixtures into an ice bath. The cooled reaction solutions were then centrifuged at 24 170 *g* for 10 min, and the absorbance of the supernatants was measured at 450, 532, and 600 nm.

Data analysis: The experiment was designed to be completely randomized within the growth chamber with three replicates per treatment, and the entire experiment was performed in duplicate. The data were analyzed using one-way analysis of variance using the *SPSS 11.0* software. Statistical significance was inferred at $P < 0.05$. The data were presented as means ± SDs of the three replicates for all measured parameters.

Results

To understand the role of *CaPAL* in the pepper tolerance to low temperature, we generated plants to knock down its expression through VIGS. *Agrobacterium* stains harboring TRV2:00, TRV2:CaPAL, and TRV2:CaPDS were injected to the cotyledons of purple-leafed pepper line Z1 (Fig. 1 Suppl.). A month after injection, plants treated with the positive control, TRV2:CaPDS, showed photobleaching, and bleaching the new leaves was maintained until the seedlings died. Furthermore, *CaPDS*-silenced peppers grew at a slower rate compared to peppers treated with the empty vector TRV2:00 (the negative control). We did not observe obvious symptoms on pepper seedlings treated with TRV2:00 (Fig. 1*A*,*B*). Plants transformed with TRV2:CaPAL showed changes in growth and color compared to the peppers of TRV2:00. The *CaPAL*-silenced leaves were completely green (Fig. 1*C*). Interestingly, peppers transformed with TRV2:CaPAL had a significantly lower anthocyanin content and PAL activity compared to those transformed with TRV2:00 (Fig. 1*D*-*F*). These data support that VIGS successfully knocked down the expression of the target gene in pepper, and that *CaPAL* was silenced with a high efficiency one month after injection (Fig. 1*E*).

To understand the role of *CaPAL* in pepper tolerance to low temperature, *CaPAL*-silenced peppers were placed at 4 °C for 16 h, and plants transformed with TRV2:CaPAL showed more wilting compared to the negative control plants transformed with TRV2:00 (Fig. 2*A*). Comparing new leaves of the *CaPAL*-silenced peppers and the control, we found that the apexes and sides of the new leaves in the *CaPAL*-silenced seedlings were shrunk compared to those transformed with TRV2:00 under low temperature conditions.

We next investigated the effects of low temperature on *CaPAL*-silenced plants at the physiological level. Low temperature treatment increased the total anthocyanins content in both silenced peppers and the negative control; however, the negative control plants (TRV2:00) had higher anthocyanins content than the *CaPAL*-silenced plants (Fig. 2*B*). Under normal conditions, PAL activity in plants transformed with TRV2:CaPAL was lower compared to peppers of TRV2:00. Low temperatures led to a higher PAL activity in the negative controls transformed with TRV2:00 but to a lower PAL activity in TRV2:CaPAL plants (Fig 2*C*). Content of MDA increased in the leaves of TRV2:00 and TRV2:CaPAL under low temperature, and the rate of increase was higher in the *CaPAL*-silenced plants (Fig. 3*A*). Interestingly, although peppers exposed to low temperature had higher anthocyanins content, anthocyanin amounts in CaPAL-silenced peppers were lower than those in peppers of TRV2:00 (Fig. 2) suggesting that silencing *CaPAL* led to a reduced anthocyanins syntheses and the accumulation of MDA in plants.

We tested the activities of antioxidant enzymes to examine whether *CaPAL*-silencing affected pepper tolerance to low temperature. Under normal conditions, enzyme activities were similar between silenced peppers and the negative control. Enzyme activities were higher in plants exposed to low temperature and the negative controls had significantly higher enzyme activities compared to the silenced peppers at low temperature conditions (Fig. 3*B*-*D*).

Furthermore, photosynthetic parameters decreased after low temperature treatment (Fig. 4). Under normal conditions, P_N, and E in the silenced peppers were higher than those in the negative control, and there was no difference in g_s between the negative control and the silenced peppers. However, after low temperature treatment, all parameters were lower in the silenced peppers compared the negative control. Even E in the negative control was two-fold higher than in the silenced peppers. These results demonstrate that the silenced peppers had a poor photosynthesis capacity.

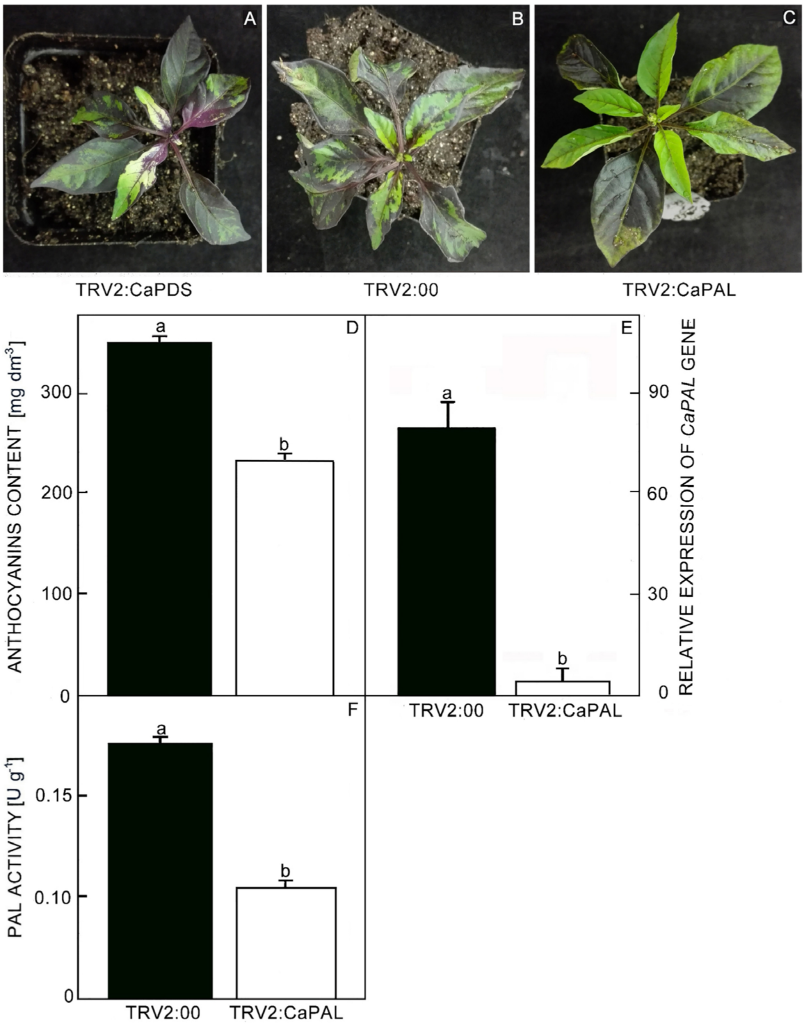

Fig. 1. Effect of *CaPAL*-silencing in pepper plants. Phenotypes of pepper seedlings transformed with TRV2:CaPDS as a positive control (*A*), TRV2:CaPAL (*B*), and the empty vector as a negative control (*C*). Anthocyanins content (*D*), expression of the *CaPAL* gene (*E*), and phenylalnine ammonia-lyase (PAL) activity (*F*) in silenced pepper TRV2:CaPAL and negative control TRV2:00. The experiment was conducted with three biological replicates, and each replicate contained three pepper seedlings. Means ± SDs, different lowercase letters mark significant differences at $P < 0.05$.

To further explore the effect of low temperature on *CaPAL*-silenced peppers, expressions of genes involved in pigment metabolism were investigated by real-time qPCR. Under normal conditions, expressions of anthocyanins-related genes in the negative control was higher than the silenced peppers (Fig. 5). After low temperature treatment, the *CaPAL*-silenced peppers did not show a difference in the expression of anthocyanins-related genes, whereas the TRV2:00 plants had higher expressions of these genes (Fig. 5*A*). For example, the TRV2:00 seedlings had higher expressions of *CaMYC* (a *transcription factor from the bHLH_family*), a *transcription factor from the MYB family*, *UDP-glucose:flavonoid 3-glucosyltransferase*, *anthocyanin synthase*, and *chalcone synthase* under low temperature, whereas the TRV2:CaPAL seedlings had a similar *CaMYC* expression at low temperature and at normal conditions. The expressions of carotenoids-related and chlorophyll-related genes were also different between the negative control and the *CaPAL*-silenced peppers, but expressions of all genes decreased under low temperature (Fig. 5*B-C*).

Expressions of antioxidant-related genes *C-repeat binding factor 1*, *proline oxidoreductase 1* and *superoxide dismutase* were enhanced in both the silenced pepper TRV2:CaPAL seedlings and the control TRV2:00 seedlings, but much less in the former than in the latter (Fig. 6).

Discussion

Virus-induced gene silencing is considered to be an effective tool for analyzing gene function in plants. Over the last decade, VIGS has been used for both forward and reverse genetics to study gene function in various model plants (Ramegowda *et al.* 2014). Here, we demonstrate that VIGS could be effectively used to down-regulate a gene *CaPAL* involved in anthocyanin biosynthesis in peppers. The accumulation of anthocyanins allows plants to adapt to adverse environments (Landi *et al.* 2015), and PAL is an important enzyme in the anthocyanin biosynthetic pathway, catalyzing the non-oxidative deamination of phenylalanine to trans-cinnamate (Kim and Hwang 2014). Although the role of *PAL* genes in stress response has been reported in *Arabidopsis* (Olsen *et al.* 2008), the effect of *PAL* down-regulation on pepper anthocyanins has not been investigated. Hence, to test the involvement of PAL in anthocyanins biosynthesis in peppers, we took a VIGS approach to silence *PAL* gene in pepper.

The PAL catalyzes the first step of the phenylpropanoid pathway and so the synthesis of diverse natural products based on the phenylpropane skeleton (Mauch-Mani and Slusarenko 1996). We characterized the role of *CaPAL* in pepper anthocyanins biosynthesis using plants with knocked down expression of *CaPAL*. We found that *CaPAL* is a positive regulator of pepper anthocyanins

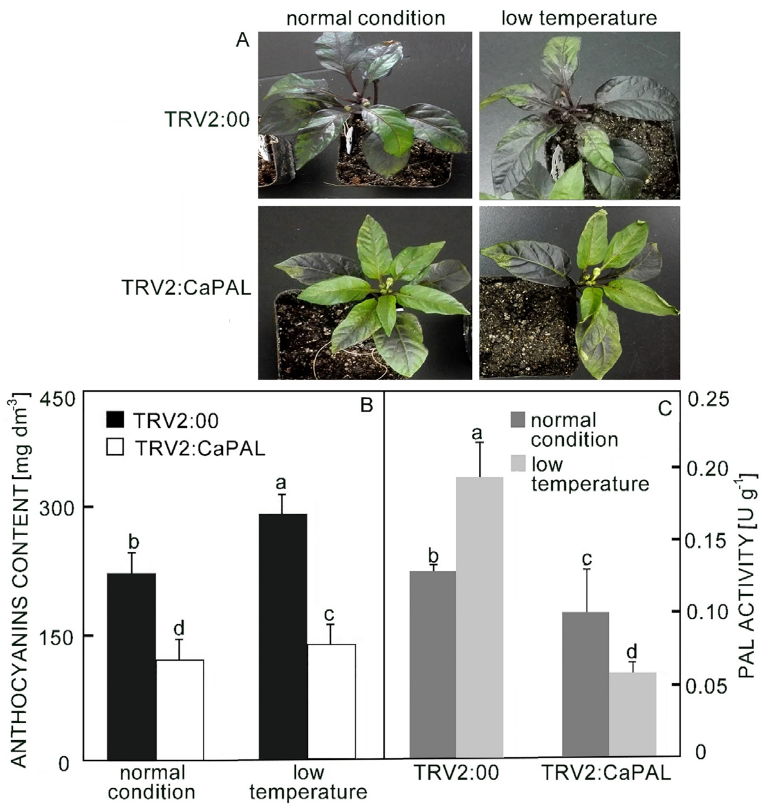

Fig. 2. Effect of low temperature (4 °C for 16 h) on phenotype and contributing parameters in silenced peppers. Phenotypes of silenced pepper TRV2:CaPAL and negative control TRV2:00 (*A*); their anthocyanins content (*B*); theirand PAL activity (*C*). The experiment was conducted with three biological replicates, and each replicate contained three pepper seedlings. Means ± SDs, different lowercase letters mark significant differences at $P < 0.05$.

biosynthesis, as knocking down *CaPAL* expression resulted in a significant decrease in anthocyanin content in pepper leaves. Silencing *PAL* may have prohibited flux in the flavonoid pathway, thereby resulting in a low yield of some secondary metabolites (Olsen *et al.* 2008, Cheng *et al.* 2018). Expression of *CaPAL* significantly increases PAL activity, triggering synthesis of anthocyanins in plants (Wang *et al.* 2000, Kim and Hwang 2014). The *CaPAL* positively regulates anthocyanin production in peppers (Cheng and Breen 1991). Furthermore, silencing *CaPAL* results in a reduced expression of anthocyanin-related genes (*e.g.*, *anthocyanin synthase* and *chalcone synthase*), further suggesting that PAL is involved in the anthocyanin biosynthetic pathway.

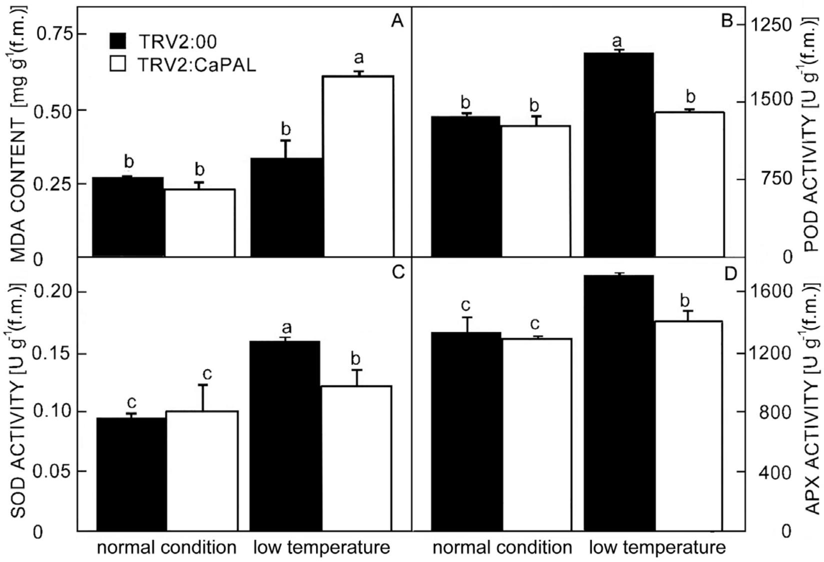

Fig. 3. Malondialdehyde (MDA) content and activities of antioxidant enzymes in peppers exposed to low temperature (4 °C for 16 h). *A* - MDA content; *B* - peroxidase (POD) activity; *C* - superoxide dismutase (SOD) activity; *D* - ascorbate peroxidase (APX) activity of silenced pepper TRV2:CaPAL and negative control TRV2:00. The experiment was conducted with three biological replicates, and each replicate contained three pepper seedlings. Means ± SDs, different lowercase letters mark significant differences at $P < 0.05$.

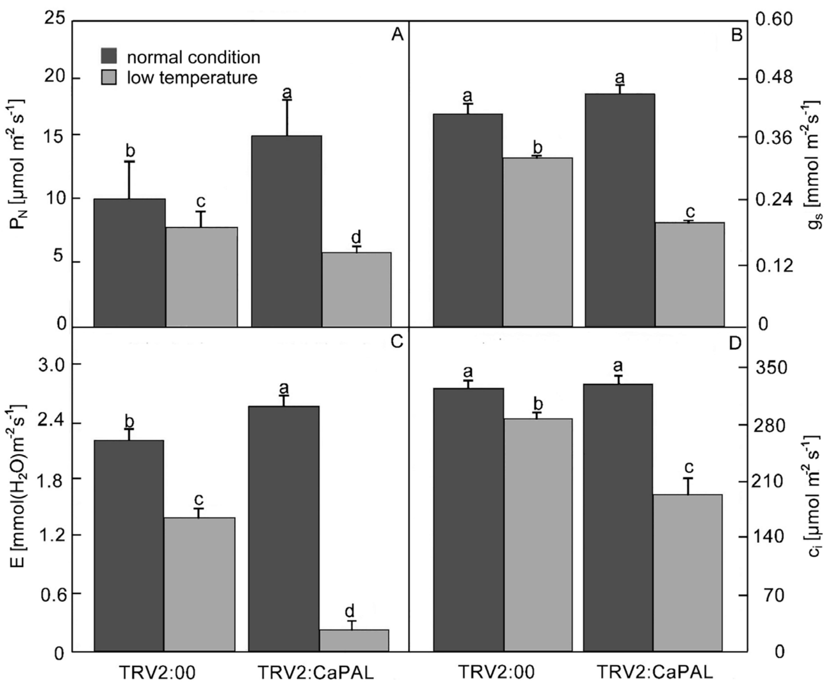

Fig. 4. Photosynthetic capacity in peppers exposed to low temperature (4 °C for 16 h). *A* - Photosynthetic rate (P_N); *B* - stomatal conductance (g_s); *C* - transpiration rate (E); *D* - intercellular carbon dioxide concentration (c_i) of silenced pepper TRV2:CaPAL and negative control TRV2:00. The experiment was conducted with three biological replicates, and each replicate contained three pepper seedlings. Means ± SDs, different lowercase letters mark significant differences at $P < 0.05$.

Similar to the studies on PAL in *Arabidopsis* (Leyva *et al.* 1995), the knocking down *CaPAL* expression rendered pepper seedlings more sensitive to low temperature. Under low temperature, the pepper seedlings transformed with TRV2:CaPAL had a higher MDA content (Fig. 3) and lower activities of antioxidant enzymes compared to the negative control plants transformed with the empty vector TRV2:00. These results suggest that silencing *CaPAL* causes a reduced ability to remove reactive oxidative species (ROS), leading to an increased membrane damage. Similarly, deficiency in anthocyanins resulting from knocking down genes involved in anthocyanin biosynthesis also made plants hypersensitive to ROS, resulting in the accumulation of insoluble protein aggregates under stressful conditions. Interestingly, our results are not in agreement with Kim and Hwang (2014), who reported that *PAL* overexpression can increase ROS burst and cell death. This may be explained by the fact that an optimal amount of ROS can improve plant growth under adverse conditions, whereas excess ROS are detrimental to plant

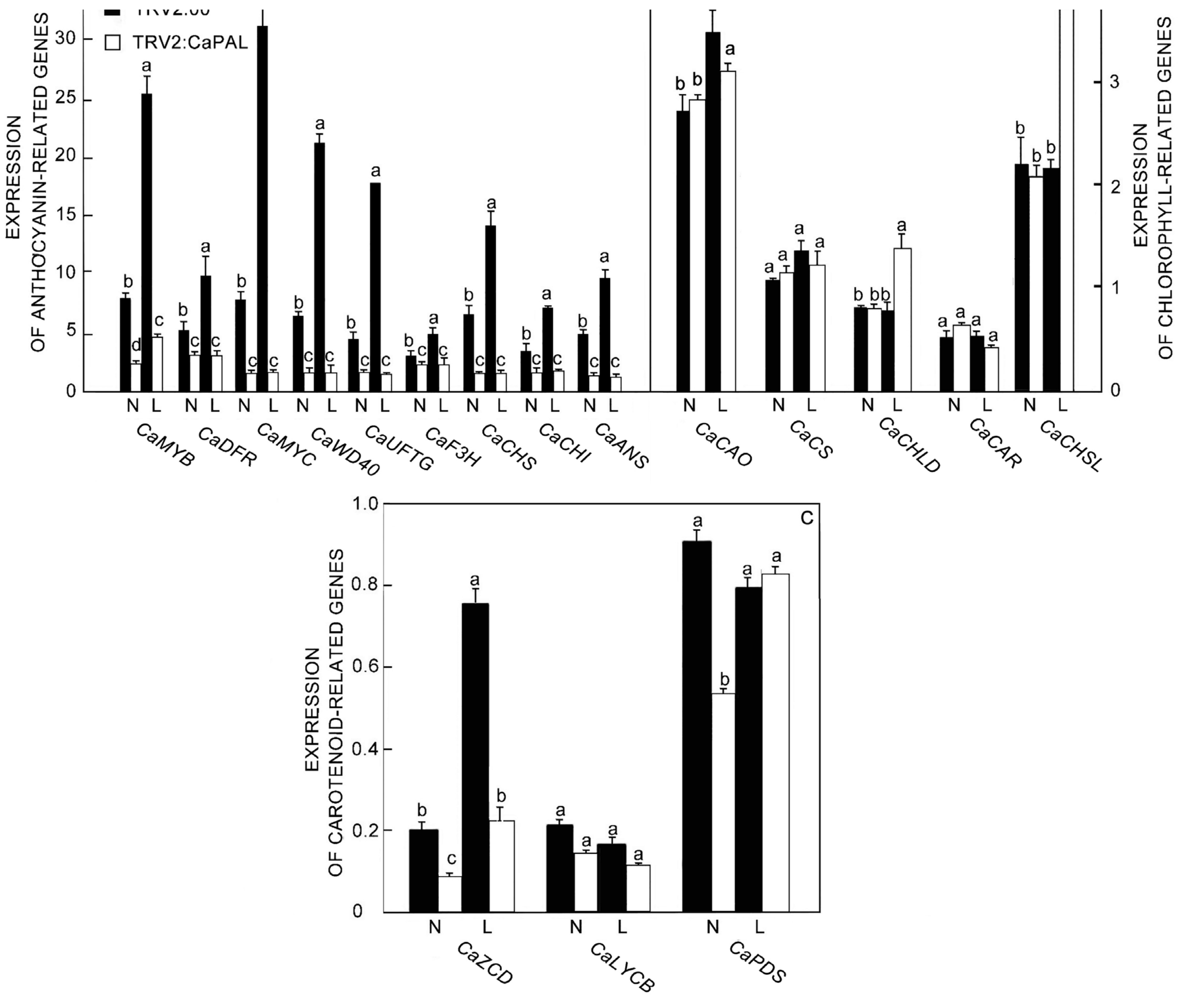

Fig. 5 Expressions of pigment-related genes in the leaves of silenced pepper TRV2-CaPAL and negative control plants TRV2:00 exposed to low temperature (4 °C for 16 h). *A* - Expressions of anthocyanin-related genes a *transcription factor* from the MYB family (*CaMYB*), *dihydroflavonol 4-reductase* (*CaDFR*), a *transcription factor* from the bHLH family (*CaMYC*), a *WD40repeat protein* (*CaWD40*), *UDP-glucose:flavonoid 3-O-glucosytransferase* (*CaUFGT*), *flavanone 3-hydroxylase* (*CaF3H*), *chalcone synthase* (*CaCHS*), *chalcone isomerase* (*CaCH*), *anthocyanin synthase* (*CaANS*); *B* - expressions of chlorophyll-related genes *chlorophyllide a osygenase* (*CaCAO*), *chlorophyll synthase* (*CaCS*), *D subunit of magnesium chelatase*(*CaCHLD*), *chlorophyll a reductase* (*CaCAR*), a gene resulting in inhibition of chlorophyll synthesis (*CaCHSL*); *C* - expressions of carotenoid-related genes *zeaxanthin cleavage dioxygenase* (*CaZCD*), *lycopene β-cyclase* (*CaLYCB*), *phytoene desaturase* (*CaPDS*). The expression of these genes was normalized to *CaUbi3*. N and L represent normal conditions and low temperature, respectively. The experiment was conducted with three biological replicates, and each replicate contained three pepper seedlings. Means ± SDs, different lowercase letters mark significant differences at $P < 0.05$.

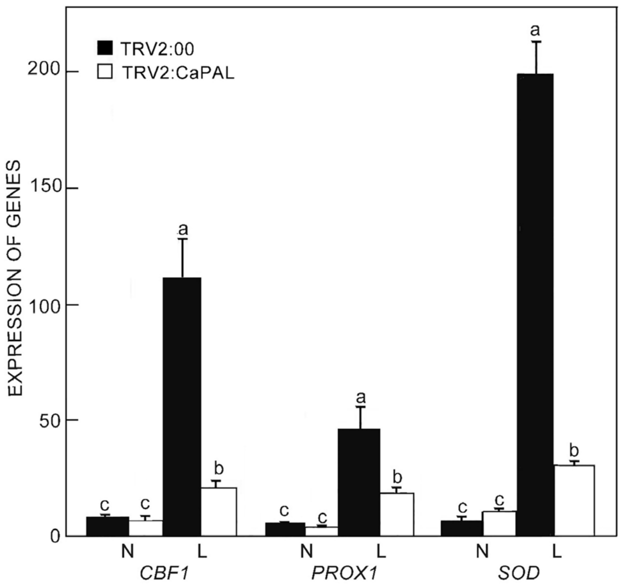

Fig. 6. Changes in antioxidant-related gene (*C-repeat binding factor 1* - *CBF1*, *proline oxidoreductase 1* - *PROX1*, and *superoxide dismutase* - *SOD*) expressions of *CaPAL*-silenced peppers TRV2-CaPAL and negative control plants TRV2:00 exposed to low temperature (4 °C for 16 h). Gene expressions were normalized to *CaUbi3* expression. The experiment was conducted with three biological replicates, and each replicate contained three pepper seedlings. Means ± SDs, different lowercase letters mark significant differences at $P < 0.05$.

growth and development (Zhao *et al.* 2016). Plants possess an extensive spectrum of antioxidants and antioxidative enzymes that allow survival under stress conditions, ensure optimal cellular ROS content to avoid damage, and allow signaling to proceed. This system is highly dynamic and involves both generation and scavenging ROS to retain balanced levels in plant cells (Mittler *et al.* 2004). Here, under low temperature conditions, enhanced activities of antioxidant enzymes in both the negative control and the *CaPAL*-silenced pepper plants may have been induced to scavenge a higher ROS production. This is also supported by the higher expression of antioxidant genes under low temperature (Fig. 6).

In order to further confirm the contribution of *CaPAL* to the pepper plant tolerance to low temperature, photosynthetic parameters were investigated under low temperature conditions. It is known that anthocyanins can improve photosynthetic capacity under low temperature. At low temperatures, plant can maintain growth and development by accumulating anthocyanins (Zhou *et al.* 2017). Anthocyanins are located in the outer palisade cells, protecting the photosynthetic tissues from excessive radiation. An increase of anthocyanin content is positively correlated with photosynthesis capacity (Devacht *et al.* 2009). Anthocyanins can protect the photosynthesis apparatus in stressful environments most likely through absorbing visible radiation and shielding UV radiation in plants exposed to low temperature (Chen *et al.* 2007). On the contrary, anthocyanin deficiency in the *CaPAL*-silenced peppers inhibited photosynthetic rate under low temperature probably due to loss of photoprotection. These data suggest the positive role of anthocyanins in photosynthetic capacity.

Conclusions

We found that inhibiting *CaPAL* expression in the pepper by VIGS disrupted biosynthesis of anthocyanins and caused abnormal development. The *CaPAL*-silenced peppers were more sensitive to low temperature showed a reduced ability to remove ROS, and had a lower photosynthetic capacity. At the molecular level, low transcriptions of genes involved in pigment biosynthesis and antioxidant system support the result. Our work provides an insight into the role of PAL in anthocyanin biosynthesis and tolerance to low temperature stress.

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