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Effects of low temperature and low irradiance on the physiological characteristics and related gene expression of different pepper species

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Abstract

Pepper is a thermophilous and heliophilic vegetable. In China, pepper is grown in greenhouse during winter and spring under lower temperature and irradiation. In this study, we investigated the effects of low temperature and low irradiance (LTLI) on the physiological characteristics and the expression of related genes in five pepper species, *Capsicum annuum* L. (CA), *C. baccatum* L. (CB), *C. chinense* Jacquin (CC), *C. frutescens* L. (CF), and *C. pubescens* Ruiz & Pavon (CP) in order to screen for greenhouse species that is resistant to such adverse conditions. We observed significant reductions not only in photosynthetic pigments and stomatal conductance but also in proline, total soluble sugar, enzyme activity, and root activity; disordered arrangements of leaf palisade and spongy tissues; and first rising and then falling expression of C-repeat binding factor (*CBF3*) and cold-regulated genes (*CORc410*). These results indicate that pepper is not resistant to LTLI. We also found that CP showed significantly higher photosynthetic activity, more proline and total soluble sugar, higher enzyme activity, higher root activity, higher *CBF3* and *CORc410* expression levels, more tightly packed leaf palisade and spongy tissues, and thicker bundle sheath than the other four species did under LTLI, while CF exhibited the lowest values for these indicators. It demonstrated significant differences in the ability to resist to LTLI among different species, with CP showing the strongest resistance, followed by CB. Therefore, we recommend the introduction of CP and CB to greenhouse cultivation to further screen for low temperature and low light-resistant pepper varieties to increase pepper production by strengthening intervariety hybridization.

Additional key words: adventitious root; catalase; chlorophyll content; osmotic substances; photosynthesis; superoxide dismutase.

Introduction

Low temperature and irradiance are adverse conditions for many, especially thermophilous plants. Under these conditions, plants have reduced capacity of light energy conversion, which severely affects their growth, development, survival, and distribution. These stresses are important factors that limit geographic distribution of crops and reduce their yield and quality (Thomashow 1999). Low temperature-induced photoinhibition may lead to photooxidation damages (Hodgson *et al*. 1989). Under low temperature and low irradiance, plants usually show decreased photosynthetic rate (P_N) (Fan *et al*. 2010), elevated water-use efficiency (WUE) (Tsuneo *et al*. 2012), changed antioxidant enzyme activity (Aya and Keńichi 2012), and damaged PSI and PSII functions (Liu *et al*. 2009, An *et al*. 2010, Liang *et al*. 2010). Different varieties of the same species can tolerate low temperature and low irradiance differently (Xu *et al*. 2010, Deng *et al*. 2011). In order to adapt and resist the low temperature stress, plants have developed during evolution "low temperature acclimation" response and protection mechanism (Thomashow 1990). After sensing low temperature, plants initiate defense mechanisms that include physical structure adaptation (changes in plasma membrane composition and rearrangement of cytoskeleton), enhance-ment of intracellular osmoregulation substances (fatty acid, betaine and free amino acids), rise in antioxidant synthesis, and

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Abbreviations: CA – *Capsicum annuum* L.; Car – carotenoids; CAT – catalase; CB – *Capsicum baccatum* L.; *CBF3* – C-repeat binding factor; CC – *Capsicum chinense* Jacquin; CF – *Capsicum frutescens* L.; Chl – chlorophyll; *CORc410* – cold-regulated gene; CP – *Capsicum pubescens* Ruiz & Pavon; *E* – transpiration rate; F_m – maximal fluorescence; F_m' – maximal fluorescence under light; FM – fresh mass; F_0 – minimal initial fluorescence; F_0' – minimal fluorescence; g_s – stomatal conductance; GR – glutathione reductase; LTLI – low temperature and low irradiance; q_N – nonphotochemical quenching; q_P – photochemical quenching; P_N – net photosynthetic rate; SOD – superoxide dismutase; POD – peroxidase; WUE – water-use efficiency; Φ_{PSII} – actual photochemical efficiency of PSII in the light.
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in low-temperature stress protein expression and hormone contents. These changes help plants to create a new balance of materials and energy metabolism to survive colder environments (Guy *et al*. 1990, Thomashow 1999, Xin *et al*. 2000, Lin *et al*. 2012, Onyango *et al*. 2012).

Pepper is a widely accepted thermophilous vegetable that originates in the tropical regions of Central and South America. It is a major crop cultivated in the winter and spring, when low temperature and low irradiance can severely affect the growth and development of pepper plants. As a result, the pepper production can be significantly reduced. Therefore, a major goal in pepper breeding is to screen for pepper varieties that are resistant to LTLI and suitable for facility cultivation, especially for that with energy-saving solar greenhouses in China. International Plant Genetic Resources Board has classified pepper into five species: *Capsicum annuum* L., *C. baccatum* L., *C. chinense* Jacquin, *C. frutescens* L., and *C. pubescens* Ruiz & Pavon. Studies on pepper physiological changes and related gene expression under LTLI have been focused on the *Capsicum annuum* L. (Xie *et al*. 2009, 2011; Chai *et al*. 2010, Zhang *et al*. 2010). Although they are used less, the other four species possess some useful traits, such as resistance to blights and verticillium wilt in *C. frutescens* L., resistance to verticillium wilt in *C. chinense* Jacquin, resistance to tobacco mosaic virus cytomegalovirus, and blights in *C. baccatum* L. These less cultivated species may be a good source of resistance genes that can improve the production of the more cultivated variety of *C. annuum* L. under LTLI. In this study, we investigated the changes in photosynthesis, proline and soluble sugar, enzyme activity, root system, and related gene expression in different pepper species under a simulated environment. We also analyzed the differences in the resistance to LTLI of these pepper species. Our study provided not only theoretical bases for screening for pepper species suitable for winter-spring cultivation, but it also laid the foundations for the wider pepper cultivation and parental sources for hybridization.

Materials and methods

Plant material: Five pepper species were used in this study: *Capsicum annuum* L. (CA), *C. baccatum* L. (CB), *C. chinense* Jacquin (CC), *C. frutescens* L. (CF), and *C. pubescens* Ruiz & Pavon (CP).

Experimental design: Pepper plants were planted in pots from April–May at the seedling stage. Each pot contained 5,750 g of soil with 110.9 mg kg^{-1} of available nitrogen, 4.1 mg kg^{-1} of available phosphorus, 207.5 mg kg^{-1} of available potassium, and 1.3 g kg^{-1} of organic matter. All pots were maintained in the Huaihua University germplasm nursery with regular watering and fertilizer management (watering once a day and fertilizing compound fertilizer of 1 g once a week) under the condition of temperature of 35 ± 2°C. Fifteen plants of each pepper species were randomly selected for treatment. The treatments in a climate chamber simulated the actual conditions in energy-saving solar greenhouses during the winter and spring. The temperature regime was set at 15/5°C during day/night (LT). The relative humidity was kept at 60%, irradiance was 100 μmol(photon) m^{-2} s^{-1} with a photoperiod of 12/12 h during day/night (LI). Fifteen plants of each pepper species were randomly selected for analysis. Measurements were performed 5, 10, 15, 20, and 25 days from the beginning of the treatment of low temperature and low irradianc (DAT). Several plants under normal growth conditions were randomly selected as controls at the beginning of treatments when plants were grown at temperature of 35 ± 2°C. Photosynthetic characteristics were determined at 25 DAT. The treatments were repeated three times and observed for three consecutive years.

Pigment analysis: Leaf samples were extracted in 95% acetone and measured by spectrophotometric method (*Ruili UV-2100*, Beijing, China) at 663, 645, and 470 nm. Contents of chlorophyll (Chl) *a*, *b*, and carotenoids (Car) were calculated using the equations of Arnon (1949):

$$\text{Chl } a = 12.21\ OD_{663} - 2.81\ OD_{645} \quad (1)$$

$$\text{Chl } b = 20.13\ OD_{645} - 5.03\ OD_{663} \quad (2)$$

$$\text{Car} = (1{,}000\ OD_{470} - 3.27\ \text{Chl } a - 104\ \text{Chl } b)/229 \quad (3)$$

Gas exchange: It was determined during 09:00−11:00 h (Beijing time) in mid-August (flowering stage) using *Licor-6400* portable photosynthesis system (*LICOR*, USA). The net photosynthetic rate (P_N), stomatal conductance (g_s), and transpiration rate (E) were measured at the irradiance of 1,000 μmol m^{-2} s^{-1}, temperature of 32 ± 0.5°C, and the natural CO_2 concentration. Water-use efficiency (WUE) was calculated as P_N/E.

Chl fluorescence kinetics: The minimal initial fluorescence (F_0) and maximal fluorescence (F_m) were determined after 20 min dark adaptation. Then minimal fluorescence (F_0') and maximal fluorescence (F_m') under light and fluorescence in steady state (F_s) were measured after a 1-h irradiation (Li *et al*. 2007). Other fluorescence parameters were calculated according to Genty *et al*. (1989): photochemical quenching (q_P) = $(F_m' - F_s)/(F_m' - F_0')$; nonphotochemical quenching (q_N) = $(F_m - F_m')/(F_m - F_0)$; actual photochemical efficiency of PSII in the light (Φ_{PSII}) = $(F_m' - F_s)/F_m'$. All these parameters were measured using *Licor-6400* portable photosynthesis system (*LICOR*, USA).

Proline determination: The fresh leaves were washed and dried with a paper towel. After the removal of rim and

veins, about 0.5 g of leaves were cut into pieces, mixed with 5 mL of 3% sulfosalicylic acid solution in a large tube, and boiled for 10 min with shaking. After cooling down, the extract was filtered with a funnel into a clean tube. The filtered extract (2 mL) was mixed with 2 mL of acetic acid and 2 mL of acid ninhydrin, sealed with plastic wrap, and boiled for 30 min. After cooling down, 4 mL of toluene was added into the tube and fully oscillated. The upper red solution was collected using toluene and its absorption at 520 nm was measured by spectrophotometer (*Ruili UV-2100*, Beijing, China). Proline content [μg mL^{-1}] in the 2 mL tested samples was obtained from the standard curve, and the proline concentration in fresh leaves was calculated based on the following formula: proline content [μg g^{-1}] = (x × 5/2)/ fresh leaf mass [g] (x was the proline content [μg mL^{-1}] from the standard curve).

Total soluble sugars: The content was measured with anthrone colorimetric method. In detail, 0.1 g of the sample was placed in a ground glass bottle, mixed with 20 mL of distilled water, and extracted twice in boiling water for 30 min; the extract was filtered into a 50 mL volumetric flask. The bottle and residues were washed repeatedly before being filled to a constant volume. The sample extract (0.5 mL) was mixed thoroughly with 1.5 mL of distilled water, 0.5 mL of anthrone ethyl acetate solution, and 5 mL of concentrated sulfuric acid before being immediately placed into boiling water bath for 1 min. The heated sample extract was cooled naturally to room temperature to determine the absorbance at 630 nm by spectrophotometer (*Ruili UV-2100*, Beijing, China). The final total soluble sugar contents were calculated from the standard curve: total soluble sugar [μg g^{-1}] = (corresponding sucrose content from standard curve [μg] × total extract volume [mL]/measurement volume [mL])/FM [g].

Enzyme activity: The activity of superoxide dismutase (SOD, EC 1.15.1.1) (hydroxylamine method), peroxidase (POD, EC 1.11.1.7) (colorimetric method), catalase (CAT, EC 1.11.1.6) (ammonium molybdate method), and glutathione reductase (GR, EC 1.6.4.2) (colorimetric method) was measured using commercial kits (*Nanjing Jiancheng Bioengineering Institute*, Nanjing, China) following the manufacturer's instructions. The absorbances were measured spectrophotometrically (*Ruili UV-2100*, Beijing, China).

Determination of adventitious root number and root activity: The number of adventitious roots was measured directly. Root activity was measured by triphenyl tetrazolium chloride (TTC) method. In detail, roots were collected and washed. They were dried carefully with absorbent paper. Root tips (0.5 g) were selected, cut into 1 cm pieces, placed in a small beaker, incubated with 10 mL of 1:1 (v/v) mixture of 1% TTC solution and 0.1 mol L^{-1} of phosphate buffer (pH 7.0) at 37°C for 1 h in dark and then mixed with 2 mL of 1 mol L^{-1} sulfuric acid. Control was performed similarly except adding sulfuric acid first, root sample second, and the mixture last. The roots were then collected and ground with 3–5 mL of ethyl acetate and quartz sand in a mortar after removal of moisture to extract trityl hydrazone (TTF). The remaining roots were further extracted with ethyl acetate three times. All the red extractions were collected, transferred into a new tube and diluted with ethyl acetate to 10 mL. Absorption of the diluted extracts was measured at 485 nm. The blank solution was obtained similarly without adding roots. Reduced TCC amount [mg] was obtained from the standard curve and its intensity in the root tip was calculated as follows: TTC reduction intensity [mg g^{-1} h^{-1}] = reduced TTC amount [mg] per unit of FM [g] during the incubation time [h].

Light microscopy: Leaves were fixed in formalin/alcohol acetate mixed solution (FAA solution) (37% formalin: glacial acetic acid:50% ethanol, 5:5:90). The samples were initially infiltrated under a vacuum at room temperature for 2 h and then preserved in FAA at room temperature. Samples were prestained with Ehrlich's haematoxylin, dehydrated in a graded ethanol series, embedded in paraffin, and then sectioned (8 mm in thickness) using a rotary microtome (*Leica RM-2016*, *Leica Microsystems*, Germany). All sections were photographed using an *Olympus BH60* microscope equipped with charge-coupled device (*Olympus Corporation*, Japan).

Semi-quantitative gene expression analysis: The leaves of CA from 0 to 25 DAT and other pepper species on 25 DAT were collected and their total mRNA was isolated using a RNA extraction kit (*Ambiogen Life Science Technology Ltd.*, Changsha, China). cDNA were synthesized using first strand cDNA synthesis kit (*Tiangen Biotech Co.*, *Ltd.*, Beijing, China) and used for semi-quantitative PCR analysis. C-repeat binding factor (*CBF3*) was according to Yang (2011): 5′-GGATCCATGAACAT CTTTAGAAGCTATTAT-3′ and 5′-GTCGACTACTTATA GGGAAGATCATAGC-3′, cold-regulated gene *CORc410* was according to He (2010): 5′-GCTAGGACTTGTCGT GGAGCTTG-3′ and 5′-GTGAAGAGTAGAAGAGAAA ATGG-3′. PCR reaction was conducted in a 50 mm^3 system containing 5.0 mm^3 10× PCR buffer, 10 pmol forward and reverse primers, 0.3 mm^3 10 mM dNTPs, 2 U Taq (*Ferments*, USA), and 60 ng template at the following conditions: 5 min at 94°C followed by 35 cycles of 60 s at 94°C, 50 s at 52°C (*CBF3*) or 60°C (*CORc410*), and 50 s at 72°C and 10 min at 72°C. PCR reactions were terminated before the reaction reached platform and the amplicons were examined with gel electrophoresis.

Statistical analysis: For parametric data, the analysis of variance (*ANOVA*) was used by *Excel* (2003). The *t*-test significance was set at $\alpha = 0.05$ for all tests.

Results

Photosynthetic characteristics: The contents of photosynthetic pigments displayed a downward trend under LTLI. Significant differences were observed among five pepper species with CP showing the smallest decline: Chl *a*, Chl *b*, and Car decreased by 8.8, 8.0, and 22.3%, respectively. CF exhibited the largest reduction: Chl *a*, Chl *b*, and Car decreased by 31.6, 27.3, and 26.4%, respecttively (Table 1). Overall, our results indicated significant decline in contents of photosynthetic pigments in peppers under LTLI.

Under control conditions, the five pepper species did not show significantly different photosynthetic rates. However, the P_N of all five pepper species was significantly affected by LTLI, with CP showing the smallest reduction of 70.2%, followed by CB with 84.7% (Table 2). Both E and g_s of all pepper species, but CP, were lowered significantly by LTLI treatments. However, only CP showed significantly enhanced WUE (Table 2).

Chl fluorescence analysis revealed that LTLI significantly affected PSII. The capture efficiency of PSII excitation energy (F_v'/F_m'), ϕ_{psII}, and q_P were significantly reduced, while q_N significantly increased. Only minor changes in fluorescence parameters were observed in CP, while the other four showed much larger changes (Table 3).

Structure: LTLI significantly affected the development of leaf palisade and spongy tissue by thinning the leaves but thickening the bundle sheaths. Among the five pepper species, CP showed clear morphology of tightly packed leaf palisade tissue and spongy tissue and obviously thickened bundle sheaths (Fig. 1*E2*), while CF and CA displayed obscure morphology of almost disintegrated palisade and spongy tissues and obviously thickened bundle sheaths (Fig. 1*A2,D2*).

Proline and total soluble sugar: Environmental stresses can cause substantial accumulation of proline, an important cytoplasmic osmotic adjustment substance in plants. Under LTLI, the contents of proline in CP kept increasing, while those of the other four pepper species showed first increasing and then decreasing trend; CF and CB showed the most and the least significant decreases, respectively (Fig. 2*A*).

The pepper leaves from different species displayed different trends of changes in the content of total soluble sugars after LTLI treatment. The content of total soluble sugars of CA, CC, and CF reached the maximum 15 DAT. CF showed the lowest content at 25 DAT. CB reached the maximum in total soluble sugar content at 20 DAT and then declined. CP kept increasing and reached the maximum in total soluble sugar content at 25 DAT, which was significantly higher than those of the other species (Fig. 2*C*).

Enzyme activity: Under LTLI, the activity of SOD, CAT, POD, and GR of all pepper species was first elevated, and then declined. The enzyme activity of all pepper species, except CP, reached the maximum at 15 or 20 DAT. The activity of SOD, POD, and GR of CP slightly increased after 25 DAT to 227.6%, 151.0% and 254.3%, respectively, while all the other pepper species showed reduced SOD, POD, CAT, and GR activity, with the reduction of CB being the smallest one (Fig. 3).

Root characteristics: Under LTLI, all five pepper species showed reduced root activity to different degree, with CF and CP showing the largest and smallest reductions, respectively (Fig. 2*B*). After 25 DAT, the number of adventitious roots increased in CA, CC and CP, while that of CB remained the same and that of CF was reduced (Fig. 2*D*). LTLI promoted the division of root phloem cells and thickened the xylem to withstand environmental adversity. Among the five pepper species, CP displayed the most significant acceleration in phloem cell division and thickening of the xylem, while both CA and CF showed reduced number of phloem cells, tapered xylem, and blocked development of both phloem and xylem (Fig. 1 *A4,D4*).

Table 1. Photosynthetic pigment content [g kg^{-1}(DM)] in different pepper species before (0 d) and after 25 d of low temperature and low irradiance treatment. Car – carotene; CA – *Capsicum annuum*; CB – *C. baccatum*; CC – *C. chinense*; CF – *C. frutescens*; Chl – chlorophyll; CP – *C. pubescens*. Means of five replications ± SE. *Lowercase letters* mean significance at 0.05 level.

Species	Chl *a*			Chl *b*			Car		
	0 d	25 d	ΔChl *a* [%]	0 d	25 d	ΔChl *b* [%]	0 d	25 d	ΔCar [%]
CA	9.83 ± 0.58	7.58 ± 0.25	–22.9^b	4.28 ± 0.12	3.64 ± 0.22	–15.0^c	1.51 ± 0.11	0.78 ± 0.11	–48.3^a
CB	9.31 ± 0.42	7.36 ± 0.36	–26.5^b	4.45 ± 0.21	3.55 ± 0.25	–20.2^b	1.19 ± 0.23	0.70 ± 0.21	–41.2^a
CC	8.05 ± 0.78	6.44 ± 0.50	–20.0^b	3.59 ± 0.25	2.83 ± 0.47	–21.2^b	1.09 ± 0.10	0.70 ± 0.31	–35.8^b
CF	11.15 ± 1.00	7.63 ± 0.64	–31.6^a	5.35 ± 0.53	3.98 ± 0.35	–27.3^a	1.06 ± 0.08	0.78 ± 0.25	–26.4^b
CP	7.92 ± 0.74	7.22 ± 0.25	–8.8^c	3.87 ± 0.14	3.56 ± 0.33	–8.0^d	1.12 ± 0.14	0.67 ± 0.12	–40.2^a

Table 2. Photosynthetic rate (P_N), transpiration rate (E), stomatal conductance (g_s), and water-use efficiency (WUE) of different pepper species before (0 d) and after 25 d of low temperature and low light treatment. *Lowercase letters* mean significance at 0.05 level. CA – *Capsicum annuum*; CB – *C. baccatum*; CC – *C. chinense*; CF – *C. frutescens*; CP – *C. pubescens*. Means of five replications ± SE.

Species	P_N [µmol m^{-2} s^{-1}]			E [mmol m^{-2} s^{-1}]			g_s [mol m^{-2} s^{-1}]			WUE [%]		
	0 d	25 d	ΔP_N [%]	0 d	25 d	ΔE [%]	0 d	25 d	Δg_s [%]	0 d	25 d	ΔWUE [%]
CA	17.73 ± 0.86	2.31 ± 0.21	–87.0^a	3.26 ± 0.45	0.45 ± 0.14	–86.2^a	0.47 ± 0.08	0.17 ± 0.02	–63.8^a	0.54 ± 0.75	0.51 ± 0.14	–5.7^c
CB	16.49 ± 2.53	2.53 ± 0.49	–84.7^a	3.79 ± 0.26	0.57 ± 0.17	–85.0^a	0.43 ± 0.08	0.21 ± 0.04	–51.2^a	0.44 ± 0.68	0.44 ± 0.36	2.1^b
CC	17.17 ± 2.37	2.37 ± 0.27	–86.2^a	3.36 ± 0.49	0.50 ± 0.14	–85.1^a	0.43 ± 0.08	0.18 ± 0.03	–58.1^a	0.51 ± 0.71	0.47 ± 0.33	–7.2^c
CF	21.77 ± 2.91	2.91 ± 0.14	–86.6^a	3.21 ± 0.47	0.46 ± 0.11	–85.7^a	0.55 ± 0.02	0.19 ± 0.03	–65.5^a	0.68 ± 1.21	0.63 ± 0.18	–6.6^c
CP	15.78 ± 2.71	4.71 ± 0.52	–70.2^b	4.05 ± 0.58	0.86 ± 0.12	–78.8^b	0.34 ± 0.02	0.25 ± 0.07	–26.5^b	0.39 ± 0.55	0.55 ± 0.13	40.9^a

Table 3. Chlorophyll fluorescence kinetics of different pepper species before (0 d) and after 25 d of low temperature and low light treatment. *Lowercase letters* mean significance at 0.05 level. CA – *Capsicum annuum*; CB – *C. baccatum*; CC – *C. chinense*; CF – *C. frutescens*; CP – *C. pubescens*. Means of five replications ± SE.

Species	F_v'/F_m'			q_P			q_N			Φ_{PSII}		
	0 d	25 d	$\Delta F_v'/F_m'$ [%]	0 d	25 d	Δq_P [%]	0 d	25 d	Δq_N [%]	0 d	25 d	$\Delta\Phi_{PSII}$ [%]
CA	0.55 ± 0.01	0.33 ± 0.03	–40.0^a	0.61 ± 0.01	0.36 ± 0.02	–41.0^a	0.60 ± 0.00	0.70 ± 0.00	16.7^a	0.44 ± 0.01	0.33 ± 0.06	–25.0^a
CB	0.51 ± 0.01	0.30 ± 0.05	–41.2^a	0.53 ± 0.02	0.33 ± 0.03	–37.7^a	0.66 ± 0.00	0.77 ± 0.00	16.7^a	0.41 ± 0.01	0.30 ± 0.06	–24.4^a
CC	0.52 ± 0.01	0.30 ± 0.08	–42.3^a	0.57 ± 0.01	0.30 ± 0.08	–47.4^a	0.63 ± 0.00	0.73 ± 0.01	15.9^a	0.45 ± 0.00	0.32 ± 0.05	–28.9^a
CF	0.63 ± 0.01	0.40 ± 0.08	–36.5^a	0.65 ± 0.02	0.39 ± 0.05	–40.0^a	0.55 ± 0.00	0.69 ± 0.00	25.5^a	0.50 ± 0.00	0.37 ± 0.04	–26.0^a
CP	0.50 ± 0.01	0.36 ± 0.10	–28.0^b	0.51 ± 0.01	0.36 ± 0.11	–29.4^b	0.68 ± 0.00	0.80 ± 0.01	17.7^a	0.37 ± 0.00	0.32 ± 0.03	–13.5^b

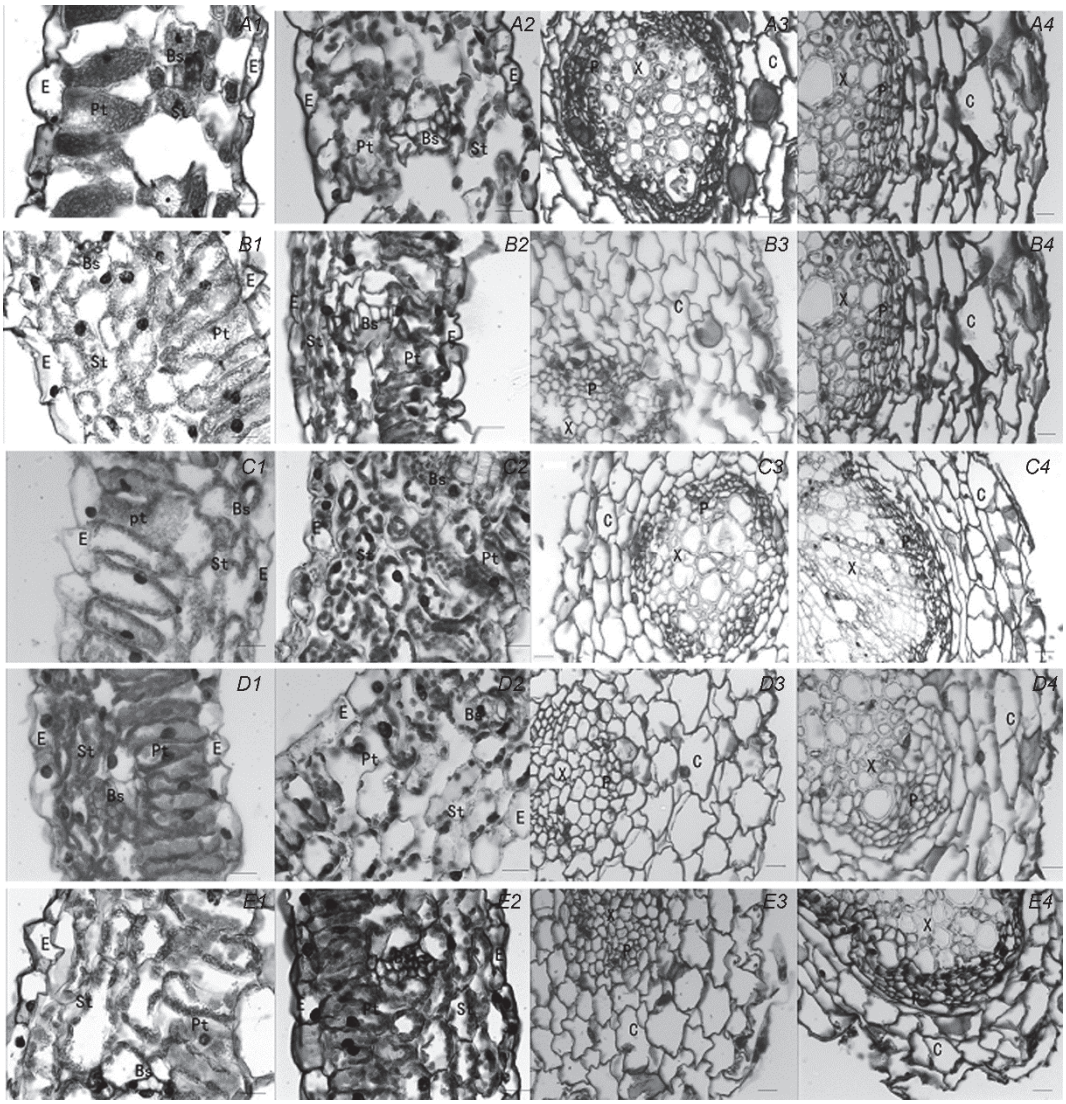

Fig. 1. Microscopic structure of leaf (*1,2*) and root (*3,4*) of *Capsicum annuum* (*A*), *C. baccatum* (*B*), *C. chinense* (*C*), *C. frutescens* (*D*), and *C. pubescens* (*E*) under control conditions (*1,3*) and after low temperature and low irradiance treatment (*2,4*). Pt – palisade tissue; St – spongy tissue; Bs – bundle sheath; E – epidermal cells; C – cortical cells; X – xylem; P – phloem. Scale bar 10 μm.

Gene expression: Along with the LTLI treatment, the expression of both *CBF3* and *CORc410* showed first an increasing and then decreasing pattern in CA. *CBF3* expression rapidly responded to LTLI, and it reached the maximum at 5 DAT and declined later. The increase in *CORc410* expression started from 0 to 15 DAT, decline was observed later (Fig. 4*A*).

The expression of both *CBF3* and *CORc410* was reduced in all five pepper species to different degree at 25 DAT. CP showed the highest expression levels of *CBF3* and *CORc410*, followed by CB, and the expression of both genes was the lowest in CF (Fig. 4*B*).

Discussion

The leaf is the main organ for plant photosynthesis and transpiration. Leaf morphology is closely correlated with surrounding environment and has great plasticity (Bai *et al*. 2010). Therefore, the structural characteristics of leaves reflect the impacts of environmental factors on plants or the adaptability of plants to the environment (Peng *et al*. 2003, Cai and Song 2001). LTLI can affect leaf structures by changing significantly the blade mesophyll

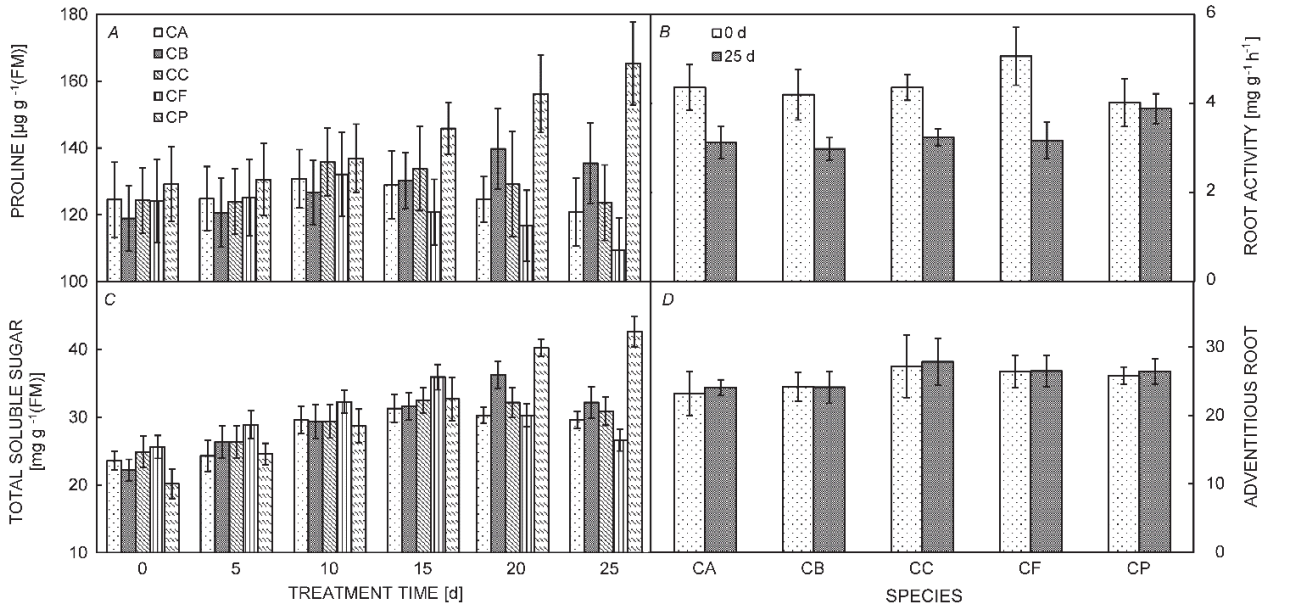

Fig. 2. Proline (*A*) and total soluble sugar content (*C*) of different pepper species during the low temperature and low irradiance treatment. Root activity (*B*) and number of adventitious roots (*D*) of different pepper species before and after 25 d of low temperature and low irradiance treatment. Means ± SE (n = 5). CA – *Capsicum annuum*; CB – *C. baccatum*; CC – *C. chinense*; CF – *C. frutescens*; CP – *C. pubescens*.

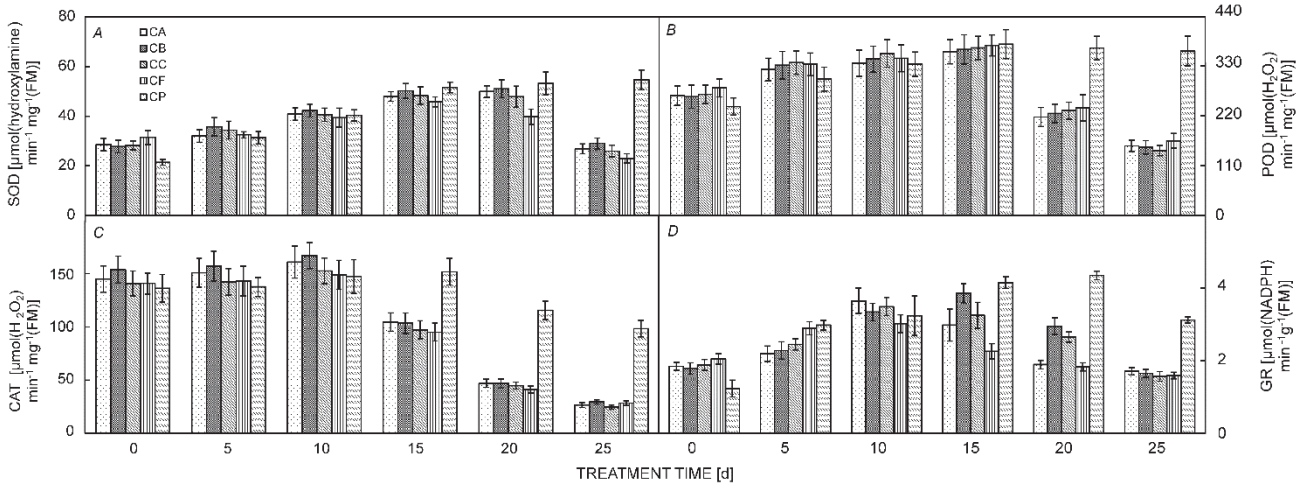

Fig. 3. Superoxide dismutase (SOD) (*A*), peroxidase (POD) (*B*), catalase (CAT) (*C*), and glutathione reductase (GR) (*D*) of different pepper species during the low temperature and low irradiance treatment. Means ± SE (n = 5). CA – *Capsicum annuum*; CB – *C. baccatum*; CC – *C. chinense*; CF – *C. frutescens*; CP – *C. pubescens*.

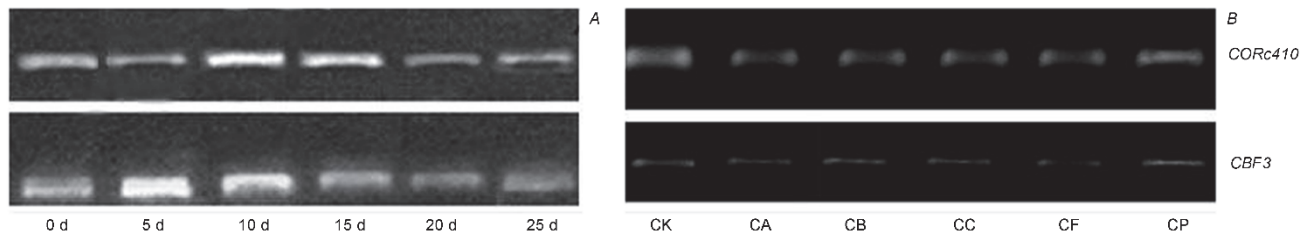

Fig. 4. Semi-quantitative RT-PCR analysis of *CBF3* and *CORc*410 of *Capsicum annuum* during the low temperature and low irradiance treatment (*A*) and of different pepper species after 25-d treatment of low temperature and low irradiance (*B*). CA – *Capsicum annuum*; CB – *C. baccatum*; CC – *C. chinense*; CF – *C. frutescens*; CP – *C. pubescens*. CK – CA control.

tissues (An *et al*. 2011, Yu *et al*. 2011). Chloroplasts are highly sensitive to low temperatures (Yang *et al*. 2013). The reduction in Chl contents at low temperatures may be caused by damaged Chl (Hetherington 1990) or by blocked Chl synthesis (Friend 1960). In this study, we found that LTLI caused disarranged leaf palisade and spongy tissues, thinned leaves, decreased contents of photosynthetic pigments, and reduced photosynthesis. These changes indicated that LTLI damaged pepper chloroplast structure and block Chl synthesis and then affectedthe photosynthetic characteristics. It has been shown that Car may protect the photosynthetic membrane from light damage by dissipating excessive excitation energy through the xanthophyll cycle. Car are also quenching agents for reactive oxygen and free radicals preventing photooxidation-induced destruction of chloroplasts (Bartley 1995, Britton 1995). However, another study reported that the species with lower content of Car were more resistant to LTLI (Xie *et al*. 2010). In this study, we found that the cultivar with the low Car content, CP, showed the highest resistance to LTLI, confirming the notion that lower Car contents render stronger resistance to LTLI in plants. It might occur because higher content of Car results in more energy absorption, which is more harmful to plants under these adverse conditions. Therefore, lower content of Car were observed in all five species after LTLI treatment, with the resistance to such adverse conditions being inversely correlated with Car content. This is consistent with a previous report that low temperatures significantly inhibited the enzymes in the dark reactions of photosynthesis and it seriously harmed photosynthetic apparatus (Liu *et al*. 2001b). In addition, LTLI promoted the growth of root phloem and stimulated xylem cell division and differentiation, which may prompt the formation of advanced vein system. At the same time, LTLI also made the cortex thinner to increase the root efficiency for nutrient absorption; it resists the deceleration in cell metabolism and the root absorption caused by these conditions.

Recent studies on the mechanisms of cold-resistance in plants have indicated that *CBF* is a "master switch" of gene expression in response to low temperature. Specifically, *CBF* activates the expression of downstream *COR* genes for the synthesis of cold-resistant materials and proteins, thereby enhancing the tolerance of plants to low temperature (Thomashow *et al*. 2001, Fowler and Thomashow 2002, Chinnusamy *et al*. 2007, Carvallo *et al*. 2011, Kendall *et al*. 2011, Yang *et al*. 2011). Subsequently, series of physiological and biochemical reactions occur, including the accumulation of soluble sugars and proline (Gilmour *et al*. 2000, Zongren 2000). In this study, we observed first increasing and then decreasing contents of soluble sugars and proline under LTLI. *CBF3* expression was rapidly elevated before declining; the response of *CORc410* expression was slower than that of *CBF3* but lasted longer, indicating that *CORc410* is the main target gene of *CBF3* to be activated in response to LTLI. Prolonged LTLI treatments resulted in reduced *CBF3* and *CORc410* expression levels, which caused damages to plants.

It has been previously reported that different varieties or species of the same plant may have significantly different resistance to environmental stresses, mainly due to the differences in P_N reduction, WUE increase, and related enzymatic activity increase (Liu *et al*. 2001a, He *et al*. 2011, Peng *et al*. 2011, Wang *et al*. 2011). Pepper breeding has been focused on the cultivar CA (Pickersgill 1997). In this study, we observed distinct responses to LTLI, including photosynthesis, active substances, and gene expression. This indicates that different pepper species possess different resistance capacities to LTLI stress. In recent years, more than 100 new pepper species have been developed in China, and the main species in the pepper-producing areas have been updated for 4–5 times, improving pepper production and quality. It also demonstrates the apparent heterosis of pepper. However, CA genetic basis is relatively narrow, which limits further improvement in pepper yield and quality (Prince *et al*. 1995, Ou *et al*. 2012). Therefore, more species are needed in the research of pepper breeding to solve the bottleneck pepper breeding by significantly expanding pepper parental sources to effectively exploit pepper germplasm resources. Pepper is cultivated mainly in late spring/early summer, when dramatic weather changes may occur, such as sudden drops in temperature. Therefore, choosing parental varieties with strong resistance to LTLI for breeding of highly resistant species is an effective method to deal with such adverse conditions in greenhouse cultivation. In this study, we found that CA exhibited low resistance to LTLI, while CP and CB showed significantly stronger resistance. Therefore, we recommend the introduction of the stress-resistant genes in CP and CB to the breeding process of CA to improve the resistance capacity and heterosis by expanded gene exchanges.

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